Expanding the pathophysiological mechanisms of oligodendrocyte-lineage development in RNA polymerase III-related leukodystrophy: an *in vitro* approach

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Abstract

Hypomyelinating leukodystrophies are a heterogeneous group of rare, genetically determined white matter disorders characterized by a deficit of myelination during development. RNA polymerase III-related leukodystrophy is one of the most prevalent hypomyelinating leukodystrophies and arises from biallelic pathogenic variants in genes encoding subunits of the RNA polymerase III enzymatic complex. RNA polymerase III is an essential enzyme responsible for transcribing the 5S ribosomal RNA, all tRNA genes, and numerous other non-coding RNAs involved in various aspects of protein synthesis. Despite the ubiquitous nature of these RNAs, individuals with POLR3-related leukodystrophy present with involvement of specific organs, leading to neurological deficits arising from the hypomyelination and characteristic non-neurological features involving the pituitary gland, teeth, and eyes. Since the neurological manifestations are the most debilitating aspect of the disease, it is particularly intriguing how myelin is impacted. Myelin is produced by oligodendrocytes in the central nervous system, cells which undergo a complex development, beginning as oligodendrocyte precursor cells that migrate and proliferate extensively before differentiating into mature, myelinating oligodendrocytes.

The first section of this thesis focuses on the optimization of a technique to isolate oligodendrocyte precursor cells from mouse brain tissue. The isolation of oligodendrocytes in their precursor stage has traditionally been considered a challenging technique. Here, we describe an efficient and reliable immunopanning approach that enables the isolation of murine oligodendrocyte precursor cells with high yield and high purity. This isolation of cells in an early

stage of the lineage is critical for comprehensive *in vitro* studies of the fundamental aspects of oligodendrocyte biology.

In the subsequent part of this thesis, we use this optimized immunopanning method as the basis for developing a cellular model of RNA polymerase III-related leukodystrophy. We show that decreasing the endogenous transcript levels of leukodystrophy-associated RNA polymerase III subunits (e.g. *Polr3a, Polr3b,* and *Polr1c*) in oligodendrocyte precursor cells leads to defects in oligodendrocyte development. Specifically, we found that decreasing Pol III expression increased the proliferation rate of precursor oligodendrocytes and impaired their differentiation into mature oligodendrocytes, accordingly, hindering their ability to myelinate. By comparison, we noticed no defect in the migration or survival of these cells.

In summary, this thesis demonstrates that *in vitro* studies of oligodendrocyte development are an invaluable tool for researchers of white matter diseases. Additionally, we identify a role for RNA polymerase III in oligodendrocyte development, at the level of their proliferation, differentiation, and myelination. Overall, our findings contribute to a better understanding of the pathophysiological mechanisms underlying the hypomyelination seen in RNA polymerase III-related leukodystrophy and provide foundational knowledge for the development of potential therapeutics.

Resumé

Les leucodystrophies hypomyélinisantes sont un groupe hétérogène de maladies rares de la substance blanche génétiquement déterminées, caractérisées par un déficit de myélinisation au cours du développement. La leucodystrophie liée à l'ARN polymérase III (POLR3-HLD) est l'une des leucodystrophie hypomyélinisante les plus communes et est causée par des variants pathogènes biallélіques dans des gènes codant pour des sous-unités du complexe enzymatique ARN polymérase III. L'ARN polymérase III est une enzyme essentielle responsable de la transcription de l'ARN ribosomal 5S, de tous les gènes d'ARNt et de nombreux autres ARN non codants impliqués dans divers aspects de la synthèse des protéines. Malgré la nature omniprésente de ces ARN, les personnes atteintes de POLR3-HLD présentent une atteinte d'organes spécifiques, entraînant des déficits neurologiques résultant de l'hypomyélinisation et des caractéristiques non neurologiques caractéristiques impliquant l'hypophyse, les dents et les yeux. Étant donné que les manifestations neurologiques sont l'aspect le plus débilitant de la maladie, il est particulièrement intrigant de savoir comment la myéline est affectée. La myéline est produite par les oligodendrocytes du système nerveux central, des cellules qui subissent un développement complexe, commençant par des cellules précurseurs d'oligodendrocytes qui migrent et prolifèrent abondamment avant de se différencier en oligodendrocytes myélinisants matures.

La première partie de ce mémoire porte sur l'optimisation d'une technique pour isoler des cellules précurseurs d'oligodendrocytes à partir de tissu cérébral de souris. L'isolement des oligodendrocytes à leur stade précurseur a traditionnellement été considéré comme une technique difficile. Ici, nous décrivons une approche d'immunopanning efficace et fiable qui

permet l'isolement des cellules précurseurs d'oligodendrocytes avec un rendement élevé et une grande pureté. Cet isolement des cellules à un stade précoce de la lignée est essentiel pour des études *in vitro* complètes des aspects fondamentaux de la biologie des oligodendrocytes.

Dans la suite de ce mémoire, nous utilisons cette méthode d'immunopanning optimisée comme base pour développer un modèle cellulaire de leucodystrophie liée à l'ARN polymérase III. Nous montrons que la diminution des niveaux de transcription endogène des sous-unités de l'ARN polymérase III associées à la leucodystrophie (par exemple, *Polr3a, Polr3b* et *Polr1c*) dans les cellules précurseurs des oligodendrocytes entraîne des défauts dans le développement des oligodendrocytes. Plus précisément, nous avons constaté que la diminution de l'expression de l'ARN polymérase III augmentait le taux de prolifération des oligodendrocytes précurseurs et altérait leur différenciation en oligodendrocytes matures, entravant ainsi leur capacité à myéliniser. Par comparaison, nous n'avons remarqué aucun défaut dans la migration ou la survie de ces cellules.

En résumé, ce mémoire démontre que les études *in vitro* du développement des oligodendrocytes sont un outil précieux pour les chercheurs qui travaillent sur les maladies de la substance blanche. De plus, nous identifions un rôle pour l'ARN polymérase III dans le développement des oligodendrocytes, au niveau de leur prolifération, différenciation et myélinisation. Dans l'ensemble, nos découvertes contribuent à une meilleure compréhension des mécanismes physiopathologiques sous-jacents à l'hypomyélinisation observée dans la leucodystrophie liée à l'ARN polymérase III et fournissent des connaissances fondamentales pour le développement de thérapies potentielles.

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for never doubting me and for everything you have done to get me here: my mother Brenda Callahan, my sisters Jessica and Breanna and my grandmother Elizabeth Callahan.

I would like to dedicate this thesis to my grandfather, Gerald Callahan.

Manuscripts Contained in this Thesis
Chapter 1

Watt, K. E.,^ **Macintosh, J.,^** Bernard, G.,* & Trainor, P. A.* (2022). RNA Polymerases I and III in development and disease. *Seminars in cell & developmental biology*, S1084-9521(22)00100-8. https://doi.org/10.1016/j.semcdb.2022.03.027.

^Co-first authors. All excerpts from this manuscript are identified by quotation marks and marked by a [1] in the header. All excerpts written as published without modification, though with font/size changes for cohesiveness of thesis. Likewise, references are identical but are numbered according to references used in thesis entirety and may differ from numbering used in published manuscript. Permission to include in thesis by co-first author is provided by Dr. Kristin Watt. Permission for reprinting of manuscript is permitted by the author rights of Elsevier as stated by the Copyright Clearance Center.

Chapter 3

Macintosh, J., Michell-Robinson, M., Chen, X., Darbelli, L., Chitsaz, D., Kennedy, T., Bernard, G. An optimized and validated protocol for the purification of PDGFRα+ oligodendrocyte precursor cells from mouse brain tissue via immunopanning. Intended for submission to *MethodsX*. Written agreement from all co-authors has been received pertaining to the inclusion of this manuscript in my thesis.

Chapter 4

Macintosh, J., Michell-Robinson, M., Chen, X., Bernard, G. Decreased RNA polymerase III expression leads to defects in oligodendrocyte development. Intended for submission to *Frontiers in Cellular Neuroscience* upon completion of all experiments. Written agreement from all co-authors has been received pertaining to the inclusion of this manuscript in my thesis.

Contribution of Authors

Chapter 1: Literature Review
JM wrote this section in collaboration with GB.

Contains excerpts from a published manuscript (Watt, K. & Macintosh, J., *et al. Seminars in Cell and Developmental Biology*, 2022). JM, KW, PT and GB all contributed to manuscript writing and revisions.

Chapter 2: Rationale and Hypothesis

JM wrote this section in collaboration with GB.

Chapter 3: Manuscript – An optimized and validated protocol for the purification of PDGFRα+ oligodendrocyte precursor cells from mouse brain tissue via immunopanning.

JM designed and performed experiments, collected and analyzed data, and wrote the first draft of the manuscript. MMR contributed to design and performed experiments, revised manuscript. XC, LD, DC and TK helped in experiment design and revised manuscript. GB designed experiments, drafted and revised manuscript, and provided study supervision.

Chapter 4: Manuscript – Decreased RNA polymerase III expression leads to defects in oligodendrocyte development.

JM designed and performed experiments, collected and analyzed data, and wrote the first draft of the manuscript. MMR and XC helped in the design of experiments and revised the manuscript. GB conceptualized the study and designed experiments, drafted and revised manuscript, and provided study supervision.

Chapter 5: Discussion, Conclusion and Future Directions

JM wrote this section in collaboration with GB.

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List of Abbreviations

4H	Hypomyelination, Hypodontia and Hypogonadotropic Hypogonadism
5S rRNA	5S ribosomal RNA
7SK RNA	7SK small nuclear RNA
7SL RNA	RNA component of signal recognition particle 7SL1
ABCD1	ATP binding cassette subfamily D member 1 gene
AD	Autosomal dominant
ADAR	Adenosine deaminase, RNA-specific gene
AGS	Aicardi-Goutières syndrome
AIMP1	Aminoacyl tRNA synthetase complex-interacting multifunctional protein 1 gene
ALD	Adrenoleukodystrophy
AR	Autosomal recessive
ARSA	Arylsulfatase A gene
ATE1	Arginyl-tRNA-protein transferase 1
BC200/Bc1	Brain cytoplasmic 200 RNA 1
BDP1	B double prime 1
BRF1	BRF1 RNA polymerase III transcription initiation factor subunit
BRF2	BRF2 RNA polymerase III transcription initiation factor subunit
C57BL/6	C57 black 6, mouse
CC3	Cleaved caspase 3
ChIP-Seq	Chromatin immunoprecipitation sequencing
CNP	2',3'-cyclic nucleotide 3'-phosphodisesterase
CNS	Central nervous system
CSPG4	Chondroitin sulfate proteoglycan 4 gene
DAPI	4',6'-diamidino-2-phenylindole
DARS1	Aspartyl-tRNA synthetase 1 gene
DARS2	Mitochondrial aspartyl-tRNA synthetase gene
DIV	Days *in vitro*
DNA	Deoxyribonucleic acid
EARS2	Glutamyl-tRNA synthetase 2 mitochondrial gene
EdU	5'-ethyl-2'-deoxyuridine
EIF2B1	Eukaryotic translation initiation factor 2B subunit alpha gene
EIF2B2	Eukaryotic translation initiation factor 2B subunit beta gene
EIF2B3	Eukaryotic translation initiation factor 2B subunit gamma gene
EIF2B4	Eukaryotic translation initiation factor 2B subunit delta gene
EIF2B5	Eukaryotic translation initiation factor 2B subunit epsilon gene
EPRS1	Glutamyl-Prolyl-tRNA synthetase 1 gene
ERCC6	Excision repair 6, chromatin remodelling factor gene
ERCC8	Excision repair 8, CSA ubiquitin ligase complex subunit gene
FACS	Fluorescence-activated cell sorting
FAM126A	Family with a sequence similarity 126 member A gene
FUCA1	Alpha-L-Fucosidase 1 gene
FYN	Proto-oncogene tyrosine-protein kinase Fyn
GALC	Galactosylceramidase gene

GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
GJC2	Gap junction protein gamma 2 gene
gLEs	Genetic leukoencephalopathies
HEK293	Human embryonic kidney 293 cells
HEPACAM	Hepatic and glial cell adhesion molecule gene
HLD	Hypomyelinating Leukodystrophy
IFIH1	Interferon induced with helicase C domain 1 gene
KIF19-A	Kinesin family member 19
LBSL	Leukoencephalopathy with brainstem and spinal cord involvement and lactate elevation
LTBL	Leukoencephalopathy with thalamus and brainstem involvement and high lactate
MACS	Magnetic-activated cell sorting
MAF1	MAF1, negative regulator of RNA polymerase III
MBP	Myelin basic protein
mg	milligram
miRNA	microRNA
mL	millilitre
MLC	Megalencephalic leukoencephalopathy with subcortical cysts
MLC1	Modulator of VRAC current 1 gene
MLD	Metachromatic leukodystrophy
MOG	Myelin oligodendrocyte glycoprotein
MRI	Magnetic resonance imaging
mRNA	messenger RNA
miRNA	microRNA
µg	microgram
µL	microliter
µm	micrometer
NFASC	Neurofascin gene
NG2	Neural/glial antigen 2
NKX2.2	NK2 homeobox 2
nM	nanomolar
OLIG2	Oligodendrocyte transcription factor 2
OLs	Oligodendrocytes
OMIM	Online mendelian inheritance in man number
OPCs	Oligodendrocyte precursor cells
p53	Tumour protein p53
PDGFRa	Platelet derived growth factor alpha
PLP1	Proteolipid protein 1
pM	picomolar
PMD	Pelizaeus-Merzbacher Disease
PMIM	Phenotype mendelian inheritance in man number
PNS	Peripheral nervous system

Pol I	RNA polymerase I
Pol II	RNA polymerase II
Pol III	RNA polymerase III
POLR1C	RNA polymerase I and III subunit C gene
POLR3-HLD	RNA polymerase III-related hypomyelinating leukodystrophy
POLR3A	RNA polymerase III subunit A gene
POLR3B	RNA polymerase III subunit B gene
POLR3G/GL	RNA polymerase III subunit G/GL gene
POLR3K	RNA polymerase III subunit K gene
PSAP	Prosaposin gene
RARS1	Arginyl-tRNA synthetase 1 gene
RB1	RB transcriptional corepressor 1
rDNA	ribosomal DNA
RMRP	RNA component of mitochondrial RNA processing endoribonuclease
RNASEH2A	Ribonuclease H2 subunit A gene
RNASEH2B	Ribonuclease H2 subunit B gene
RNASEH2C	Ribonuclease H2 subunit C gene
RNase P	Ribonuclease P
RNA-Seq	RNA-sequencing
RNA	Ribonucleic acid
rRNA	ribosomal RNA
RNU7-1	RNA, U7 small nuclear 1 gene
RT	Room temperature
RT-qPCR	Reverse transcriptase quantitative polymerase chain reaction
SAMHD1	SAM and HD domain containing deoxynucleoside trisphosphate triphosphohydrolase 1 gene
SEM	Standard error of the mean
SINEs	Short interspersed nuclear elements
siRNA	small interfering RNA
SLC17A5	Solute carrier family 17 member 5 gene
SNAPc	SnRNA-activating protein complex subunit 1
snRNA	Small nuclear RNA
SOX10	SRY-box transcription factor 10
TBP	TATA-box-binding protein
TFIIIB	Transcription factor for RNA polymerase III B
TFIIIC	Transcription factor for RNA polymerase III C
TREX1	Three prime repair exonuclease 1 gene
tRNA	transfer RNA
TUBB4A	Tubulin beta-4A chain gene
U6	U6 spliceosomal RNA
UFM1	Ubiquitin-fold modifier 1 gene
VWM	Vanishing white matter (disease)
WRS	Wiedemann-Rautenstrauch syndrome

Introduction

Leukodystrophies are a subset of rare, genetically determined white matter disorders in which there is either insufficient myelin deposition in development (i.e. hypomyelinating leukodystrophies) or a defect in myelin homeostasis (i.e. non-hypomyelinating leukodystrophies).[1] While unified by abnormal white matter on brain imaging, they are a heterogeneous group of disorders with various genetic etiologies, phenotypic characteristics, and pathophysiological mechanisms.[2-4] These disorders typically present in previously healthy children and lead to progressive neurodegeneration, with one-third of affected individuals succumbing to the disease by the age of eight.[5] While individually rare, these disorders are now thought to collectively effect as many as 1 in 4733 live births.[6]

RNA polymerase III-related hypomyelinating leukodystrophy is one of the most prevailing types of hypomyelinating leukodystrophies and arises from hypomorphic biallelic pathogenic variants in subunits of RNA polymerase III (Pol III; e.g., *POLR3A, POLR3B, POLR1C*, *POLR3K*).[6-10] Pol III synthesizes short, non-coding RNAs that play constitutive roles, most evidently in protein synthesis.[11] As the ubiquitous nature of Pol III would suggest a multisystemic disease arises from its dysfunction, the particular vulnerability of myelin to reduced Pol III activity remains enigmatic. Myelin, produced by oligodendrocytes in the central nervous system (CNS), serves as an insulator critical to axonal integrity and which enables efficient propagation of action potentials.[12]

In the opening chapter of this thesis, we provide an overview of genetic leukoencephalopathies and leukodystrophies, later focusing on POLR3-related leukodystrophy. We also discuss RNA polymerase III function and more broadly, how defects in transcription and translation machinery are associated with tissue-specific diseases. We end this chapter with an overview of oligodendrocyte biology, from their precursor stage to myelination, and how

techniques have evolved to study their development *in vitro*. This chapter contains excerpts from a review paper (Watt, K. & Macintosh, J. *et al*., 2022) which provide insight into the global importance of the transcriptional machinery in development and how their dysfunctions lead to tissue-specific diseases with overlapping and non-overlapping features.[13]

This body of this thesis is divided into two main parts. The first, Chapter 3, details a method optimized for the isolation of oligodendrocyte precursor cells from mouse brain tissue. The isolation of oligodendrocytes at an early stage of their lineage enables a comprehensive *in vitro* study of oligodendrocyte development. This chapter is written as a manuscript which is intended for submission to *MethodsX*, titled "An optimized and validated protocol for the purification of PDGFRα+ oligodendrocyte precursor cells from mouse brain tissue via immunopanning".

The subsequent section of this thesis, Chapter 4, applies the methodology optimized in the previous chapter to enable a comprehensive *in vitro* study of oligodendrocyte development in the context of reduced Pol III function, to shed light on the pathophysiological mechanisms of POLR3-related leukodystrophy. This chapter is written as a manuscript, tilted "Decreased RNA polymerase III expression leads to defects in oligodendrocyte development", intended for submission to *Frontiers in Cellular Neuroscience* upon completion of all experiments. Concluding this thesis is an overarching discussion of the results included within and future directions for the study of the pathophysiology of POLR3-related leukodystrophy.

Chapter 1: Literature Review

1.1 Classifying genetic leukoencephalopathies and leukodystrophies

1.1.1 Genetic leukoencephalopathies and leukodystrophies

Leukodystrophies are genetically determined white matter disorders, often presenting in childhood and resulting in progressive neurodegeneration and premature death.[1] Leukodystrophies fall into a subset of genetic leukoencephalopathies (gLEs), hereditary disorders that affect white matter, with leukodystrophies considered to be primarily glial in nature and gLEs encompassing white matter disorders of both primarily glial and non-glial (e.g. neuronal, vascular, etc.) origin.[1] gLEs also encompass disorders in which white matter abnormalities are present but are overshadowed by prominent systemic features such as liver or muscle involvement, as commonly seen in inborn errors of metabolism.[1] A combination of major genetic advances and recognition of magnetic resonance imaging (MRI) patterns has substantially improved the ability to diagnose leukodystrophies.[3,14] In fact, there are now at least 30 disorders recognized as leukodystrophies, associated with 55 known genes.[1,15] While individually rare, collectively these disorders are fairly common, with an estimated incidence rate of 1 in 4733 live births.[6] The term leukodystrophy derives from the Greek origin, “dys” meaning wasting and “leuko” referring to white, as in white matter.[1] The genetic causes, clinical manifestations and pathophysiological mechanisms of leukodystrophy are broad. For one, leukodystrophies can be inherited in an autosomal dominant, autosomal recessive, X-linked or mitochondrial manner.[2] Additionally, while unified by myelin abnormalities, the clinical features of leukodystrophies are vast and certain have tissue-specific non-neuronal involvement, such as in POLR3-related leukodystrophy where neurological disorders stemming from hypomyelination are prominent

[1]Section contains excerpt(s) from Watt, K. & Macintosh, J., *et al.* (*Seminars in Cell and Developmental Biology*, 2022)

but ocular, dental and endocrine abnormalities are also seen.[14] Diagnosis is therefore based on clinical manifestations along with neuroradiological findings, commonly pattern recognition on MRI, and genetic testing to confirm a molecular diagnosis.[16] In many cases, leukodystrophies have no curative therapies and treatment options are focused on supportive care.[17]

Leukodystrophies are distinct from acquired white matter pathologies such as immunologic disorders (e.g. multiple sclerosis) or white matter damage caused by infectious or toxic insults.[1] Magnetic resonance imaging patterns are of use in distinguishing acquired from inherited myelin disorders, a valuable MRI discriminator being confluency of the white matter abnormalities.[3] Genetic disorders are noted to commonly present with bilateral/symmetric white matter involvement, which may be selective or predominant in certain cerebral lobes, subcortical or periventricular regions.[3] Opposingly, acquired myelin disorders present more often with multifocal/asymmetric white matter involvement on neuroimaging, though exceptions exist and importantly, late-stage acquired myelin disorders often result in widespread white matter damage.[3]

As a heterogeneous group of disorders, leukodystrophies can be sub-divided into hypomyelinating leukodystrophies (HLD), in which there is a failure of myelin deposition during development, and non-hypomyelinating in which myelin deposition is unaffected but myelin homeostasis is later disrupted.[1] MRIs also have high diagnostic value here. HLDs show mild hyperintensity of white matter relative to grey matter on T2-weighted MRIs and hyperintense, isointense or mildly hypointense white matter compared to grey matter on T1-weighted MRIs.[3] In comparison, non-hypomyelinating leukodystrophies show hyperintensity of white matter relative to grey matter structures on T2-weighted images and prominent hypointensity of white

[1]Section contains excerpt(s) from Watt, K. & Macintosh, J., *et al.* (*Seminars in Cell and Developmental Biology*, 2022)

matter compared to grey matter on T1-weighted images.[3] Within the categories of hypomyelinating or non-hypomyelinating, MRI patterns are further indicative of the specific disease.[3,18]

Cellular pathology is one alternative method that has been used in the classification of leukodystrophies.[4] While the glial-cell involvement of leukodystrophies was originally attributed primarily to oligodendrocytes, due to their evident role in producing myelin, advances in genetic testing has revealed a role for other glial cell types in the etiology of these disorders.[19] In line with this, it is now apparent that the cytoarchitecture of white matter is much more complex and heterogenous than previously appreciated. In a single voxel (100µm x 100µm x 100µm) of imaged white matter, there are an estimated 700 000 oligodendrocytes, 180 000 astrocytes, 76 000 microglia, 52 000 OPCs, and between 1000 to upwards of 13 000 axons.[20] In addition to cell types, white matter is vascularized and embedded in the extra-cellular matrix.[4] As such, it is now clear that various components of white matter contribute to leukodystrophies. In fact, certain leukodystrophies arise from astrocyte-specific genes and/or show predominant astrocyte involvement (i.e. astrocytopathies) while others originate from microglia-specific genes and/or show predominant microglia involvement (i.e. microgliopathies).[4] While this classification system allows for a more mechanistic view of these disorders by classifying them based on pathophysiology, and emphasizes the cellular heterogeneity of leukodystrophies, one caveat is that many of these disorders still have poorly characterized disease mechanisms.

1.1.2 Non-hypomyelinating leukodystrophies

Non-hypomyelinating leukodystrophies arise from a failure of myelin homeostasis and can be further sub-divided into demyelinating disorders, in which myelin is deposited but later

[1]Section contains excerpt(s) from Watt, K. & Macintosh, J., *et al.* (*Seminars in Cell and Developmental Biology*, 2022)

lost, dysmyelinating disorders, where myelin is deposited but is structurally or biochemically abnormal and finally, myelinolytic disorders in which vacuolation, enlarged extracellular spaces between the myelin sheath and its axon, arise and may or may not lead to myelin loss (i.e. degeneration).[4,21] This non-hypomyelinating category of leukodystrophy includes Aicardi-Goutières syndrome (AGS), Metachromatic leukodystrophy (MLD) and Vanishing White Matter disease (VWM), amongst many others (Table 1.1).[3] AGS has various genetic causes, with a common etiology of mutations in genes that encode proteins required for the metabolism of nucleic acids.[22] The consequential build-up of RNA and DNA is interpreted as foreign by the body, which mounts an immune response, culminating in the destruction of myelin.[22] By comparison, MLD is caused by biallelic pathogenic variants in *ARSA,* or less commonly *PSAP*.[23,24] Mutations in *ARSA* cause the sulfatide lipid to accumulate in cells, leading to a combination of OL toxicity and microglia activation that ultimately destroys myelin.[19] Meanwhile, VWM arises from autosomal recessive mutations in genes encoding subunits of the eukaryotic translation initiation factor 2B (EIF2B), implicated in protein synthesis.[25] Defects in these proteins induce stress on the endoplasmic reticulum of astrocytes which subsequently secrete a protein that is inhibitory to oligodendrocyte maturation.[19]

[1]Section contains excerpt(s) from Watt, K. & Macintosh, J., *et al.* (*Seminars in Cell and Developmental Biology*, 2022)

Disease Name	Inheritance pattern	Associated genes	PMIM	OMIM
Adrenoleukodystrophy (ALD)	X-linked	*ABCD1*[26]	300100	300371
Aicardi-Goutières syndrome (AGS)	AR, AD	*TREX1*[27], *RNASEH2B*[28], *RNASEH2C*[28], *RNASEH2A*[28], *SAMHD1*[29], *ADAR*[30], *IFIH1*[31], *RNU7-1*[32]	225750, 610181, 610329, 610333, 612952, 615010, 615846, 619487	606609, 610326, 610330, 606034, 606754, 146920, 606951, 617876
Alexander's disease	AD	*GFAP*[33]	203450	137780
Krabbe's disease	AR	*GALC*[34]	245200	606890
Leukoencephalopathy with brainstem and spinal cord involvement and lactate elevation (LBSL)	AR	*DARS2*[35]	611105	610956
Leukoencephalopathy with thalamus and brainstem involvement and high lactate (LTBL)	AR	*EASR2*[36]	614924	612799
Megalencephalic leukoencephalopathy with subcortical cysts (MLC)	AR	*MLC1*[37], *HEPACAM*[38]	604004, 613925	605908, 611642
Metachromatic leukodystrophy (MLD)	AR	*ARSA*[23], *PSAP*[24]	250100, 249900	607574, 176801
Vanishing white matter disorder (VWM)	AR	*EIF2B1*[25], *EIF2B2*[39], *EIF2B3*[25], *EIF2B4*[25], *EIF2B5*[39]	603896	606273, 606687, 603945, 606686, 606454

Table 1.1: Non-exhaustive list of non-hypomyelinating leukodystrophies, their inheritance patterns, associated genes and PMIM/OMIM identification (in alphabetical order of disease name). Acronyms: AD, Autosomal dominant; AR, Autosomal recessive; PMIM, Phenotype mendelian inheritance in man; OMIM, Online mendelian inheritance in man number.

[1]Section contains excerpt(s) from Watt, K. & Macintosh, J., *et al.* (*Seminars in Cell and Developmental Biology*, 2022)

1.1.3 Hypomyelinating leukodystrophies

Hypomyelinating leukodystrophies (HLDs) arise from a deficit of myelin deposition during development (Table 1.2).[1,18] Myelination normally begins *in utero* at 25 weeks of gestation and nears completion around two years of age.[3,40] The progression of myelination, as seen by MRI, follows a well-defined process.[3] As such, hypomyelination can be characterized on MRI after two years of age or, in those under the age of two years, with two MRIs taken six months apart to distinguish permanent hypomyelination from myelination delay, the latter being a non-specific feature seen in children with neurodevelopmental disorders.[3] The prototypical HLD is Pelizaeus-Merzbacher disease (PMD), an X-linked disease arising from mutations in *PLP1*, which encodes proteolipid protein, the most highly expressed protein in myelin.[41,42] PMD is one of the first documented descriptions of a white matter disorder, dating back to the 19th century.[43] HLDs also include Pelizaeus-Merzbacher-like disease (PMLD), caused by mutations in the *GJC2* gene, which encodes a connexin involved in forming gap junctions between oligodendrocytes or between oligodendrocytes and astrocytes.[44,45] Finally, hypomyelination with atrophy of the basal ganglia and cerebellum (H-ABC) is caused by *de novo* pathogenic variants in *TUBB4A,* encoding Beta-tubulin 4, that are predicted to alter tubulin polymerization and microtubule stability.[46] Notably, TUBB4A is expressed selectively in oligodendrocytes and is thought to be critical for establishing the microtubule network in oligodendrocytes which later serves as a scaffold for transporting *Mbp* mRNA, along with other myelin proteins and lipids, to process terminals.[47] While the three leukodystrophies described here arise from structural myelin proteins or proteins related to oligodendrocyte function, in recent years it has become evident that a subset of HLDs arise from

[1]Section contains excerpt(s) from Watt, K. & Macintosh, J., *et al.* (*Seminars in Cell and Developmental Biology*, 2022)

defects in ubiquitous proteins required for housekeeping processes, the most common HLD in this category being POLR3-related leukodystrophy (discussed in more detail below).[15]

Disease Name	Inheritance pattern	Associated genes	PMIM number	OMIM number
Cockayne Syndrome	AR	*ERCC6*[48], *ERCC8*[49]	133450, 216400	609413, 609412
EPRS1-related leukodystrophy	AR	*EPRS1*[50]	617951	138295
Fucosidosis	AR	*FUCA1*[51]	230000	612280
Hypomyelination with atrophy of the basal ganglia and cerebellum (H-ABC)	AD	*TUBB4A*[46], *UFM1*[52]	612438	602662, 610553
Hypomyelination with congenital cataracts (HCC)	AR	*FAM126A*[53]	610532	610531
Hypomyelination of early myelinating structures (HEMS)	X-linked	*PLP1*[54]	N.A.	N.A.
Hypomyelinating leukodystrophy with brainstem and spinal cord involvement and leg spasticity (HBSL)	AR	*DARS1*[55]	615281	603084
Pelizaeus-Merzbacher disease (PMD)	X-linked	*PLP1*[41]	312080	300401
Pelizaeus-Merzbacher-like disease (PMLD)	AR	*GJC2*[44]	608804	608803
POLR3-related leukodystrophy/4H	AR	*POLR3A*[7], *POLR3B*[8], *POLR1C*[10], *POLR3K*[9]	607694, 614381, 616494, 619310	614258, 614366, 610060, 606007
RARS1-related hypomyelinating leukodystrophy	AR	*RARS1*[56]	616140	107820
Sialic acid storage disease	AR	*SLC17A5*[57]	604369	604322

Table 1.2: Non-exhaustive list of non-hypomyelinating leukodystrophies, their inheritance patterns, associated genes and PMIM/OMIM identification (in alphabetical order of disease name). Acronyms: AD, Autosomal dominant; AR, Autosomal recessive; PMIM, Phenotype mendelian inheritance in man; OMIM, Online mendelian inheritance in man number.

[1]Section contains excerpt(s) from Watt, K. & Macintosh, J., *et al.* (*Seminars in Cell and Developmental Biology*, 2022)

1.2 RNA polymerase III

1.2.1 Eukaryotic transcription and the role of RNA polymerase III

Unlike in prokaryotes, where the task of transcription is accomplished by one RNA polymerase, eukaryotic transcription is accomplished by a division of labour amongst three distinct DNA-dependent RNA polymerases (Figure 1.1).[58] RNA polymerase I (Pol I) transcribes at ribosomal DNA (rDNA) repeats to produce the 47S precursor ribosomal RNA (rRNA) which gets processed into the 18S, 5.8S and 28S rRNAs and as such is a rate-limiting step of ribosome biogenesis.[59] Meanwhile, RNA polymerase II (Pol II) is well known for its role transcribing protein-coding genes to produce messenger RNAs (mRNAs), though is now also known to produce various non-coding RNAs (ncRNAs) including micro-RNAs (miRNAs) and small nuclear RNAs (snRNAs).[60] Finally, RNA polymerase III (Pol III) transcribes short ncRNAs with housekeeping roles, including all transfer RNAs (tRNAs) and 5S rRNA.[60] While often overshadowed by RNA polymerase II for its direct role in protein synthesis, transcription by RNA polymerase I and III accounts for an estimated 80% of all transcription in a rapidly growing cell and upwards of 95% of a cells total RNA content.[58] In particular, the 5S rRNA and tRNAs produced by Pol III account for an estimated 20% of all cellular RNA and serve a critical role in any given cell.[61]

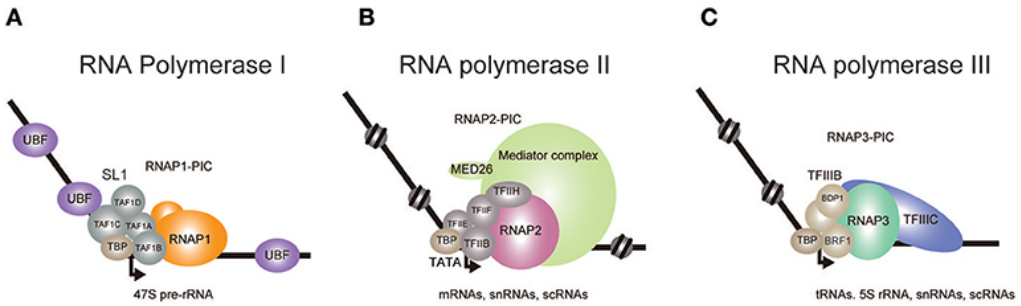

Figure 1.1: The three eukaryotic RNA polymerases and an overview of the transcripts types they produce.[62] Permission to reprint is granted by Frontiers Open Access under the terms of the Creative Commons CC-BY licence.

[1]Section contains excerpt(s) from Watt, K. & Macintosh, J., *et al.* (*Seminars in Cell and Developmental Biology*, 2022)

1.2.2 RNA polymerase III: Structure, promoter types and transcriptional machinery[1]

RNA polymerase III is an enzymatic complex composed of 17 subunits, distinguishing it as the most structurally complex RNA polymerase (Table 1.3).[63] "POLR3A/RPC1 and POLR3B/RPC2 form the active site of the enzyme and are part of the 10-subunit core which also contains two subunits shared with Pol I (POLR1C, POLR1D), and five subunits shared with Pol I and II (POLR2E, POLR2F, POLR2H, POLR2L, POLR2K), and POLR3K/RPC11. The remaining seven subunits of Pol III form subcomplexes important for transcription initiation and termination. POLR3H and CRCP form the stalk complex, important for Pol III initiation, while POLR3C, POLR3F, and POLR3G form a heterotrimer and POLR3D and POLR3E form a heterodimer, both of which are important for transcription initiation and termination.[63,64] Furthermore, Pol III can exist in two distinct isoforms defined by inclusion of subunit POLR3G or POLR3GL.[65]" As discussed in more detail below, pathogenic variants in subunits of RNA polymerase III cause tissue-specific diseases, most prevalently neurodegenerative disorders and progeria.[13] Additionally, pathogenic variants in subunits shared between polymerases cause distinct disorders based on the polymerase impacted. For example, while common to Pol I and Pol III, *POLR1C* variants disrupting Pol I are associated with Treacher Collins syndrome (TCS), while *POLR1C* variants disrupting Pol III cause a POLR3-related leukodystrophy (Figure 1.2).[13]

[1]Section contains excerpt(s) from Watt, K. & Macintosh, J., *et al.* (*Seminars in Cell and Developmental Biology*, 2022)

Gene name	Protein name	RNA polymerase associated with	Region of Pol III complex
POLR3A	RPC1	Pol III	Core
POLR3B	RPC2	Pol III	Core
POLR1C	RPAC1	Pol I, Pol III	Core
POLR1D	RPAC2	Pol I, Pol III	Core
POLR2E	RPABC1	Pol I, II, III	Core
POLR2F	RPABC2	Pol I, II, III	Core
POLR2H	RPABC3	Pol I, II, III	Core
POLR2K	RPABC4	Pol I, II, III	Core
POLR2L	RPABC5	Pol I, II, III	Core
POLR3K	RPC10	Pol III	Core/TFIIS-like
POLR3H	RPC8	Pol III	Stalk
CRCP	RPC9	Pol III	Stalk
POLR3C	RPC3	Pol III	TFIIE/F-like
POLR3D	RPC4	Pol III	TFIIF-like
POLR3E	RPC5	Pol III	TFIIF-like
POLR3F	RPC6	Pol III	TFIIE/F-like
POLR3G/GL	RPC7	Pol III	TFIIE/F-like

Table 1.3: RNA polymerase III subunits: gene names, protein names, polymerase(s) the subunit is associated with and the region of the Pol III complex it contributes to.

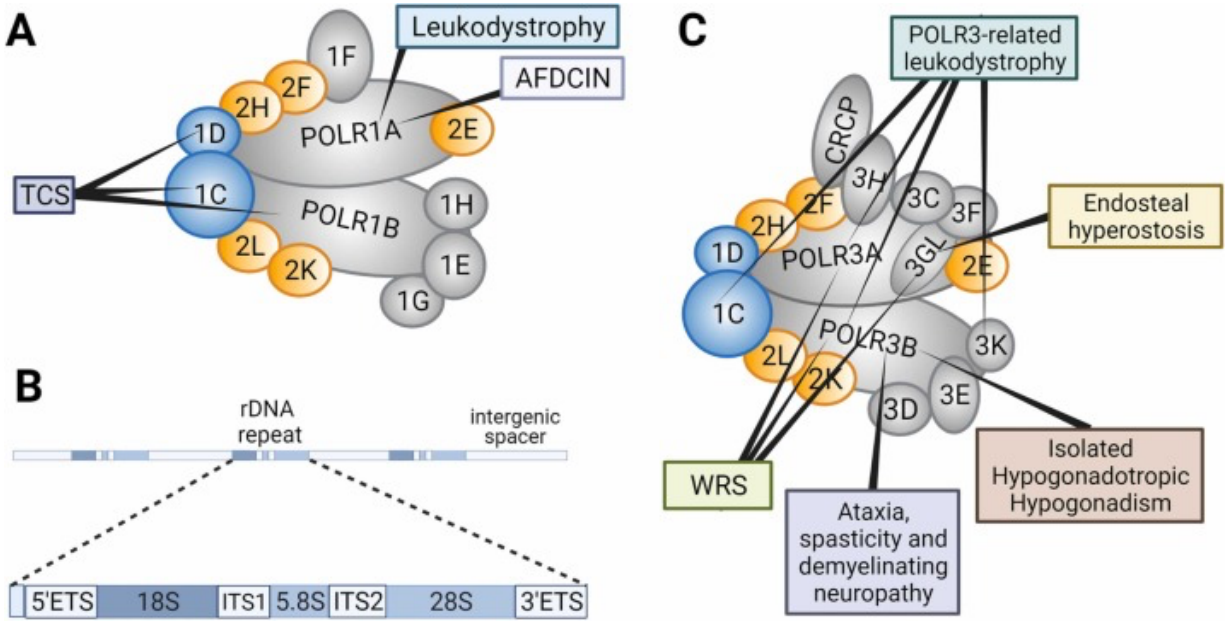

Figure 1.2: RNA polymerase I subunits and associated disorders, structure of the ribosomal DNA (rDNA) repeat and RNA polymerase III subunits and associated disorders.[13] Permission to reprint provided by the journal author rights of Elsevier under the Copyright Clearance Center confirming that authors retain the right to include published work in a thesis.

[1]Section contains excerpt(s) from Watt, K. & Macintosh, J., *et al.* (*Seminars in Cell and Developmental Biology*, 2022)

At the level of its transcription, "Pol III transcribes from three distinct promoter types, known as Type 1, Type 2, and Type 3, which vary based on the structure of the promoter elements and variable use of transcription initiation factors.[66,67] Type 1 promoters utilize transcription initiation factors TFIIIA, TFIIIB, and TFIIIC and exclusively transcribe 5S rRNA. tRNAs and some small ncRNAs are transcribed by Type 2 promoters which utilize TFIIIB and TFIIIC, while other ncRNA transcripts are transcribed by Type 3 promoters. Type 3 promoters are unique among Pol III promoters for their use of upstream promoter elements and their transcriptional machinery, specifically the use of a different form of TFIIIB, which contains a BRF2 subunit instead of BRF1, and a small nuclear RNA activating protein complex (SNAPc) instead of TFIIIC.[67] As the only factor common to all Pol III promoter types, the three subunit TFIIIB complex is an important regulator of Pol III and consists of a TATA-binding protein (TBP), BDP1, and BRF1 or BRF2.[68]" (Figure 1.3)

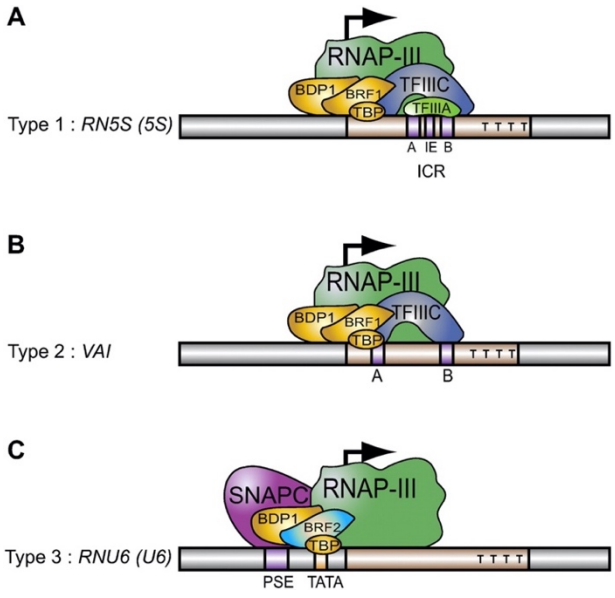

Figure 1.3: RNA polymerase III and its basal transcriptional machinery at the three promoter types.[66] Permission to reprint is granted by Cold Spring Harbor Laboratory Press Open Access under the terms of the Creative Commons CC-BY licence.

[1]Section contains excerpt(s) from Watt, K. & Macintosh, J., *et al.* (*Seminars in Cell and Developmental Biology*, 2022)

1.2.3 RNA polymerase III transcripts[1]

Following the isolation of the three eukaryotic RNA polymerases by biochemical fractionation, Pol III transcripts were originally identified via the sensitivity of their expression to the alpha-amanitin toxin.[60] Today, more systemic analysis of RNA polymerase III transcripts are enabled by genome-wide technologies such as ChIP-Seq, which have revealed previously unrecognized Pol III loci.[69]

"The function of Pol III can be defined by the roles of its transcripts, perhaps the most well-known of which are the 5S rRNA and transfer RNAs (tRNAs).[11,60] Both of these play crucial roles in protein synthesis, highlighting the critical housekeeping role for Pol III in all cells. As the sole rRNA not transcribed by Pol I, 5S rRNA is not only an integral component of the ribosome but thought to play an additional role in ribosome biogenesis through forming the peptidyl transferase center functional domain.[70] Meanwhile, tRNAs, all of which are transcribed by Pol III, are crucial for decoding mRNA transcripts to mediate translation.[71]" In line with their critical role, tRNA transcription places a large demand on RNA polymerase III as, aside from SINEs, it is predicted that 80% of loci bound by RNA polymerase III are tRNAs genes.[60,69]

"In addition, Pol III transcribes multiple small ncRNA whose function can be broadly grouped into one or more of the following categories: 1) transcription; 2) RNA processing and/or localization; and 3) translation." (Figure 1.4) "In terms of transcription, Pol III transcribes 7SK RNA, an indirect inhibitor of Pol II transcription, which illustrates a role for Pol III transcripts in the expression of protein-coding genes.[72] Pol III transcripts involved in RNA processing include U6 RNA, RNAse P RNA, and RMRP RNA. U6 RNA forms the active site of the spliceosome and works with Pol II-transcribed spliceosome RNAs to catalyze the removal of introns from pre-mRNA.[73]

[1]Section contains excerpt(s) from Watt, K. & Macintosh, J., *et al.* (*Seminars in Cell and Developmental Biology*, 2022)

RNAse P RNA regulates tRNA transcription and facilitates the removal of the 5' leader sequence of pre-tRNA, creating a link between tRNA transcription and processing.[74-76] Meanwhile, RMRP RNA functions in pre-rRNA processing through its role in cleaving the common rRNA precursor produced by Pol I, highlighting an additional role for Pol III in ribosome biosynthesis.[76] Localization of RNA in a cell is assisted by the Pol III transcripts vault RNA and 7SL.[11] Notably, vault RNAs are involved in various cellular pathways including regulation of mRNA, proliferation, differentiation, apoptosis, and autophagy.[77] However, the contribution of vault RNA to the nuclear pore complex implicates it in nuclear-cytoplasmic transport and, in turn, RNA localization.[77] 7SL is involved in targeting mRNA for secretion by serving as a scaffold for the signal recognition particle, a ribonucleoprotein that will recognize protein-coding transcripts destined for secretion and target them to the endoplasmic reticulum.[11,78] "

"Beyond these functions, certain Pol III ncRNAs play less canonical roles. Y RNAs are an emerging class of highly conserved, small ncRNAs which bind Ro60, a ring-shaped protein that associates with misfolded non-coding RNAs and pre-5S rRNA. Y RNAs play a perhaps counterintuitive role by inhibiting Ro60 from binding aberrantly folded RNA, thereby inhibiting an RNA quality control mechanism.[79] Y RNA is also required for DNA replication initiation during the cell cycle.[80] Short Interspersed Element (SINE) retrotransposons are DNA repeat elements found abundantly throughout the human genome, the physiological impact of which are beginning to be elucidated. For example, SINEs such as Alu, most commonly thought of as "genetic parasites", have been implicated in RNA editing and translation regulation.[81] Finally, the primate specific BC200 RNA, and its rodent counterpart Bc1, are expressed almost exclusively in neurons where they function in regulating local protein translation in dendrites.[82] Overall, the

[1]Section contains excerpt(s) from Watt, K. & Macintosh, J., *et al.* (*Seminars in Cell and Developmental Biology*, 2022)

functions of the various small ncRNAs transcribed by Pol III illustrate the crucial and extensive roles for Pol III in all cells."

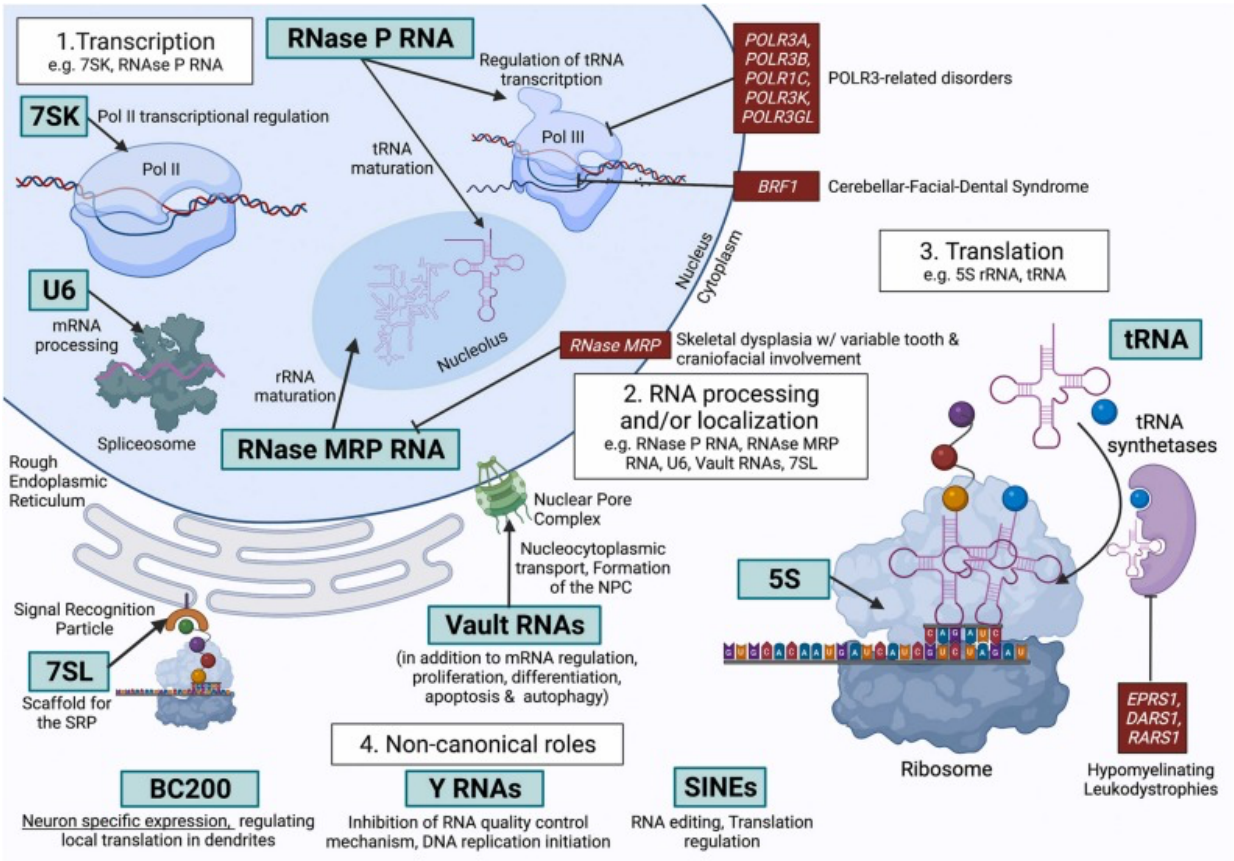

Figure 1.4: Broad groupings of RNA polymerase III transcripts based on their role in transcription, RNA processing and/or localization and translation.[13] Permission to reprint provided by the journal author rights of Elsevier under the Copyright Clearance Center confirming that authors retain the right to include published work in a thesis.

1.2.4 RNA polymerase III regulation[1]

"Pol III activity in a cell is under control of the master regulator MAF1, a mechanism conserved from *Saccharomyces cerevisiae* to mammalian cells, as well as tumor suppressor and oncogene pathways including p53 and RB1.[83-86] MAF1 inhibits Pol III activity in response to

[1]Section contains excerpt(s) from Watt, K. & Macintosh, J., *et al.* (*Seminars in Cell and Developmental Biology*, 2022)

nutrient deprived conditions, as Pol III transcription places a large metabolic demand on a cell.[61,87] In response to environmental and genetic stressors, Maf1 becomes dephosphorylated, triggering its import and accumulation in the nucleus. This enables Maf1 to bind to and allosterically inhibit Pol III, such that it cannot interact with the basal transcription machinery Brf1-Tbp promoter complex, thereby preventing Pol III transcription at the initiation step.[88-91] Maf1 also binds elongating Pol III, playing an additional repressive role by preventing re-initiation and local recycling of the complex.[89] MAF1 in humans is thought to be more evolved and play a more complex role than in yeast. While Maf1 in yeast can function under normal growth conditions, it is predominantly active during stress, such as nutrient deprivation. In contrast, MAF1 in humans has been shown to have a significant role in mediating Pol III-repression in unstressed conditions.[86] Furthermore, in humans, MAF1 represses Pol III function through an additional indirect function, physically binding to and regulating BRF1 as well as the gene that encodes TBP, thus acting through both direct and indirect mechanisms. Pol III transcription is further regulated by the tumor suppressor proteins p53 and RB1.[83,84,86] Similar to MAF1, p53 inhibits Pol III transcription at the initiation step, in this case by binding the TBP subunit of TFIIIB and preventing TFIIIB interaction with other transcription factors at the promoter site.[84,92] Thus, p53 blocks the formation of the basal transcription machinery complex and in turn, halts the recruitment of Pol III to its DNA target sites.[92] Similarly, the RB1 tumor suppressor protein also blocks the formation of the basal transcription machinery complex and prevents Pol III recruitment by binding to BRF1 in TFIIIB.[83,87] MAF1, p53, and RB1 therefore all function, at least in part, by hindering the activity of TFIIIB, the transcription initiation factor ultimately responsible for Pol III recruitment and thereby blocking transcription initiation.[83,87,92,93] ”

[1]Section contains excerpt(s) from Watt, K. & Macintosh, J., *et al.* (*Seminars in Cell and Developmental Biology*, 2022)

1.3 RNA polymerase III-related leukodystrophy

1.3.1 Genetic etiology of POLR3-related leukodystrophy[1]

"A subset of leukodystrophies, inherited white matter disorders of the brain, were the first diseases found to arise from abnormal Pol III function. Biallelic pathogenic variants in *POLR3A* (OMIM 607694) and *POLR3B* (OMIM 614381), encoding the two largest subunits of Pol III and forming its catalytic core, were found to cause five hypomyelinating leukodystrophies: leukodystrophy with oligodontia; ataxia delayed dentition with hypomyelination; 4H syndrome; hypomyelination with cerebellar atrophy and hypoplasia of the corpus callosum; and tremor ataxia with central hypomyelination.[7,8,94-99] Because of the overlapping clinical and radiological features, together with the identification of *POLR1C* (OMIM 616494) and *POLR3K* (OMIM 619310) as causative genes, these disorders are now recognized as POLR3-related hypomyelinating leukodystrophy (POLR3-HLD) or 4H (Hypomyelination, Hypodontia and Hypogonadotropic Hypogonadism) leukodystrophy.[9,10,100] Variants in *POLR3A*, *POLR3B*, *POLR3K* and *POLR1C* genes include missense, nonsense, small insertions or deletions, exonic or intronic splice site variants and large exonic deletions.[7,8,100-106]" The fact that no patient presents with two null alleles suggests that some residual function of the Pol III complex is a requirement for life.[7]

1.3.2 Clinical features of POLR3-related leukodystrophy[1]

"Individuals with POLR3-HLD display diffuse hypomyelination with sparing of early-myelinating structures (i.e. dentate nucleus, optic radiation, anterolateral nucleus of the thalamus, globus pallidus, and, in some patients, corticospinal tracts at the level of the posterior limb of the internal capsule), with or without thinning of the corpus callosum and cerebellar

[1]Section contains excerpt(s) from Watt, K. & Macintosh, J., *et al.* (*Seminars in Cell and Developmental Biology*, 2022)

atrophy.[18,102,107] Phenotypically, these individuals present with varying degrees of neurological and non-neurological manifestations. The former consists predominantly of motor signs, including cerebellar (e.g., ataxia, dysmetria, dysarthria), pyramidal (e.g., spasticity) and extrapyramidal (e.g., dystonia) signs with variable cognitive involvement. The later includes abnormal dentition (e.g., hypodontia, delayed dentition, natal teeth), endocrine abnormalities (e.g., hypogonadotropic hypogonadism leading to absent/delayed/arrested puberty, short stature with/without growth hormone deficiency) and ocular abnormalities (commonly myopia).[102,106,108-114]"

"Most patients present in early childhood with motor anomalies and exhibit disease progression over several years. A minority of patients present late in childhood with intellectual disability or cognitive plateauing and then subsequently develop motor anomalies. There is however a wide phenotypic spectrum, including a very severe form presenting in the first few months of life with failure to thrive, prominent hyperkinetic movement disorder, rapid developmental regression and premature death, to an extremely mild form of the disease diagnosed incidentally in adult patients via brain MRI.[102,115,116] Interestingly, the severely affected patients all carry a nonsense variant in a compound heterozygous state with a specific *POLR3A* splice site variant that has been previously implicated in a striatal phenotype involving the putamen, caudate nucleus and red nucleus, but in which affected individuals have normal myelination.[117-120] Biallelic *POLR3B* variants have also been identified in individuals with isolated Hypogonadotropic Hypogonadism.[121] Some patients with variants in *POLR3B* also present with endosteal sclerosis.[122]"

[1]Section contains excerpt(s) from Watt, K. & Macintosh, J., *et al.* (*Seminars in Cell and Developmental Biology*, 2022)

1.3.3 Pathophysiology of POLR3-related leukodystrophy

The pathophysiology of POLR3-related leukodystrophy remains enigmatic and it is currently unclear why white matter shows a particular vulnerability in POLR3-related leukodystrophy. RNA polymerase III function is thought to be hypomorphic in the disease state. Indeed, functional work further supports a hypomorphic outcome of pathogenic variants, with reduced protein expression of the affected subunits in fibroblast and brain lysates from individuals with POLR3-HLD.[7,115] One attractive idea is that the metabolically taxing process of CNS myelination by oligodendrocytes poses a large burden on RNA polymerase III, a demand that cannot be met in a hypofunctional state. There are two main hypotheses as to how that might be. The first, that a reduced tRNA pool could impair protein synthesis during a critical demand in myelination and the latter, that certain ncRNAs may be a requirement for oligodendrocyte development and myelination.[10,93,123] Research is underway to deepen our understanding the pathophysiology of this disorder, as the lack of insight into mechanism poses a challenge for the development of therapeutics.[124] While many questions remain, there are various previous lines of work that have shed light on disease mechanisms. These studies have focused on three main methods: 1) Assessing the impact of mutations on the Pol III complex 2) deciphering the impact of pathogenic variants on the Pol III transcriptome and 3) modelling the disease in mice for a more thorough study of pathophysiological mechanisms.

To act as crucial transcriptional machinery in the nucleus, RNA polymerase III subunits must be assembled, imported into the nucleus and bound to DNA for transcription.[125] Initial work on understanding the impact of Pol III pathogenic variants on the enzyme function began by extrapolating variant sites onto a highly conserved yeast structure.[7,8,10] This structural modelling

[1]Section contains excerpt(s) from Watt, K. & Macintosh, J., *et al.* (*Seminars in Cell and Developmental Biology*, 2022)

revealed that numerous variants in *POLR3A, POLR3B* and *POLR1C* map to regions that interact with adjacent Pol III subunits, predicted to affect complex biogenesis, while a minority of variants in *POLR3A* and *POLR3B* localize to the DNA binding domain.[7,8,10] These predictions were further supported in 2020, when the human structure of the RNA polymerase III complex was resolved.[89,126] Various mutations in *POLR3A, POLR3B* and *POLR1C* were found to cluster in hotspots at subunit interfaces, with predicted outcome on complex biogenesis or interface stability.[63,126] In line with these findings, complex assembly studies have shown certain pathogenic Pol III variants impair assembly of the affected subunit with its interacting partners, with a consequential decrease in the resulting Pol III complex formed.[10,127] Nuclear import of mutated Pol III has also been shown to be affected by certain variants.[10] Finally, studies using ChIP-Seq have revealed impaired chromatin association of Pol III in a diseased state.[10] Importantly, these findings suggest a common hypomorphic outcome amongst leukodystrophy-causing mutations in Pol III.

The function of RNA polymerase III is to transcribe housekeeping non-coding RNAs.[11] As such, one avenue of studying the pathophysiological mechanisms of POLR3-HLD has focused on deciphering the impact of hypomorphic Pol III on the expression of its genes. In an analysis done by Thiffault *et al.* in 2015, HeLa cells expressing wild-type or mutated *POLR1C* were analyzed by ChIP-Seq to assess Pol III occupancy at over 659 Pol III transcribed genes.[10] When stratified according to promoter type, Pol III occupancy was decreased at all three promoters in cells harboring mutations in *POLR1C*.[10] Additional RT-qPCR and RNA-Seq analyses have revealed a variable impact on the Pol III transcriptome. While the type 1 transcript *5S* rRNA was decreased in fibroblasts from two individuals with biallelic pathogenic *POLR3K* variants, it was shown to be

[1]Section contains excerpt(s) from Watt, K. & Macintosh, J., *et al.* (*Seminars in Cell and Developmental Biology*, 2022)

increased in blood samples from individuals with striatal *POLR3A* variants, associated with a severe form of the disease.[9,117] The type 2 transcript *7SL* has often shown to be decreased, as has been seen in blood samples from individuals with striatal *POLR3A* variants and fibroblasts from individuals with *POLR3K* variants.[9,117] However, this is not consistent, as expression was decreased in fibroblasts from an individual with *POLR3A* variants associated with a more typical disease phenotype.[128] Meanwhile, the type 3 transcript *7SK* was shown to be unaffected in fibroblasts from the individuals with *POLR3K* variants but increased in blood from individuals with striatal *POLR3A* variants.[9,117] While increased expression of certain Pol III transcripts seems paradoxical to the hypomorphic nature of POLR3-HLD variants, this finding for type 3 transcripts can be rationalized by Pol II occupancy at these promoters, which is believed to provide some compensation.[123] Interestingly, tRNAs have consistently shown to be decreased in numerous studies. Indeed, northern blots of fibroblasts from individuals with *POLR3K* variants showed a significant decrease in $tRNA^{Met}$, while RNA-Seq data on blood from individuals with striatal variants in *POLR3A* showed a decrease in a few tRNAs and a HEK293 model of *POLR3A* variants associated with a typical disease course showed a global reductions of tRNAs levels.[9,117,123]

As the generation of a representative POLR3-HLD animal model would provide a valuable tool for a detailed study of pathophysiological mechanisms, various attempts at generating a mouse model have been made, with variable success. Initial attempts at generating a transgenic mouse model of POLR3-HLD included a $Polr3a^{G672/G672E}$ and $Polr3a^{G672E/-}$, both of which displayed normal myelination and accordingly, no neurological abnormalities.[129] Following this, a transgenic $Polr3b^{R103H/R103H}$ mouse was generated but proved to be embryonically lethal in the homozygous state.[127] A double mutant $Polr3a^{G672E/G672E}$;$Polr3b^{+/R103H}$ was then generated, which

[1]Section contains excerpt(s) from Watt, K. & Macintosh, J., *et al.* (*Seminars in Cell and Developmental Biology*, 2022)

showed complete and normal myelination and no motor phenotype.[127] Finally, a more recent attempt, an *Olig2*-cre conditional knock-in of two adjacent point mutations in *Polr3a* (W671R and G672E) was successful in generating a mouse model (*Polr3a*$^{cKI/cKI}$) with a myelin phenotype, though no motor abnormalities were present.[130] There are various possibilities why generating a transgenic mouse that models a POLR3-HLD disease state has proven challenging. It is possible that Pol III vulnerability varies between species, or that the altered pace of oligodendrogenesis in mice or their lower percentage of white matter compared to humans could make them less susceptible to myelin defects.[131,132] Indeed, attempts at modelling other leukodystrophies in mice have proven to be difficult, in some instances with more severe mutations having to be used, such as the case for mouse models of Pelizaeus-Merzbacher-like disease, adrenoleukodystrophy, vanishing white matter disease and hypomyelination with atrophy of the basal ganglia and cerebellum.[133-136] As a result of the limited availability of PORL3-HLD models, large gaps remain in understanding disease mechanism.

1.4 Implications of defective Pol III beyond leukodystrophy; defects of Pol III transcriptional machinery and transcripts in CNS development and myelination

1.4.1 Ataxia, spasticity and demyelinating neuropathy[1]

"POLR3-HLD is not the only POLR3-related disorder. Recently, it was shown that *de novo* pathogenic variants in *POLR3B* cause a completely different phenotype characterized by ataxia, spasticity, and demyelinating sensorimotor peripheral neuropathy, with variable intellectual disability, motor delay and epilepsy, but normal brain myelination.[137] Further, these individuals do not display endocrine dysfunction, growth defects, dental or ocular abnormalities. A recent report of one patient with a previously published *de novo POLR3B* variant displaying isolated

[1]Section contains excerpt(s) from Watt, K. & Macintosh, J., *et al.* (*Seminars in Cell and Developmental Biology*, 2022)

demyelinating peripheral neuropathy suggests that this novel POLR3-related disorder is also associated with a spectrum of severity.[137,138] Protein modelling and affinity purification coupled to mass spectrometry has shown these *de novo POLR3B* variants either cluster in a region of POLR3B that interacts with DNA and/or at a subunit interface, impacting the interaction between POLR3B and either POLR3A, POLR3C, POLR3F, POLR2H, POLR2K, or CRCP. This is distinct from POLR3-HLD where mutations at subunit interfaces are thought to disrupt the entire POL III complex.[126] Moreover, in fibroblasts cultured from an individual with a *de novo* mutation in *POLR3B,* there was no reduction of POLR3B expression, indicating that the variants have the potential to function in a dominant-negative manner.[137]"

1.4.2 Wiedemann-Rautenstrauch syndrome[1]

"Beyond the critical role Pol III seems to play in the nervous system, mutations in *POLR3A* (OMIM 264090)*, POLR3GL* and most recently, *POLR3B,* have been shown to underlie some cases of Wiedemann-Rautenstrauch syndrome (WRS).[139-144] WRS is a rare genetic disorder with heterogeneous clinical features including intrauterine growth restriction, poor postnatal growth, facial dysmorphia, and lipodystrophy.[145,146] Similar to POLR3D-HLD, dental abnormalities including natal teeth and hypodontia are common in WRS, while myopia and hyperopia are found in a subgroup of patients.[141] Typically, no hypomyelination is observed in WRS, and most individuals display normal motor function, cognition and speech.[141] However, a subset of patients exhibits developmental delay and/or hypotonia, and one individual has been reported with cerebellar signs, muscle weakness and unintelligible speech.[139-141] Short stature is almost universal in WRS, whereas only 61% of individuals with POLR3-HLD are similarly affected.[102,108,141]

[1]Section contains excerpt(s) from Watt, K. & Macintosh, J., *et al.* (*Seminars in Cell and Developmental Biology*, 2022)

Additionally, while not present in POLR3-HLD, WRS individuals have characteristic facial features including mandibular hypoplasia, triangular face, widened fontanelles and pseudohydrocephalus.[141] WRS-associated variants in genes encoding Pol III subunits are thought to cause partial loss of Pol III function, with *POLR3A* mutations disrupting biogenesis or stability of the Pol III complex, while at least one *POLR3B* missense mutations lies in the catalytic site and disrupts Pol III-DNA interactions.[63,126,141,142] The reported homozygous *POLR3GL* mutation is a nonsense mutation thought to be degraded via nonsense-mediated mRNA decay and shown to cause an 84% reduction in overall *POLR3GL* mRNA, indicative of a severely hypomorphic state.[139]"

1.4.3 Endosteal Hyperostosis[1]

"Recently, exome sequencing identified *POLR3GL* mutations in three individuals with endosteal hyperostosis, oligodontia, growth impairment, dysmorphic facial features and in two of those individuals, delayed puberty (OMIM 619234).[147] Other than delays in motor development which were seen in all individuals, neurological impairment was minimal, and no ocular abnormalities were present. One individual exhibited a thin corpus callosum, without hypomyelination. The variants in *POLR3GL* reported to date are all splice site variants, found in either a homozygous or compound heterozygous state, and cause skipping of exon 2, which is involved in translation initiation, or exon 5, that is part of the domain thought to mediate interactions between POLR3GL and POLR3C.[148] RNA-sequencing of blood revealed an absence of full length *POLR3GL* RNA.[147] Since POLR3GL is the sole POLR3 subunit with an isoform, and POLR3G has been shown to compensate for POLR3GL *in vivo,* this perhaps explains how the lack of wild-type POLR3GL does not affect viability.[149]"

[1]Section contains excerpt(s) from Watt, K. & Macintosh, J., *et al.* (*Seminars in Cell and Developmental Biology*, 2022)

Overview of common clinical findings in diseases arising from variants in genes encoding Pol III subunits.

	POLR3-related leukodystrophy	Ataxia, spasticity and demyelinating neuropathy	Wiedemann-Rautenstrauch syndrome	Endosteal hyperostosis	Isolated Hypogonadotropic Hypogonadism
Pol III subunit affected	*POLR3A, POLR3B, POLR1C, POLR3K*	*POLR3B*	*POLR3A, POLR3B, POLR3GL*	*POLR3GL*	*POLR3B*
Inheritance	Autosomal recessive	*De novo*	Autosomal recessive	Autosomal recessive	Autosomal recessive
Neurological features	Cerebellar (ataxia, dysmetria, dysarthria), Pyramidal (spasticity), Extrapyramidal (dystonia) signs, Cognitive involvement	Pyramidal (spasticity), Cerebellar signs (ataxia, dysmetria, dysarthria) Sensorimotor demyelinating peripheral neuropathy Developmental delay, Epilepsy	Not common – Hypotonia and/or Developmental delay in some individuals	Motor delay, Hypotonia, Mild intellectual disability	–
Myelin	Diffuse brain hypomyelination	Peripheral nerve demyelination	–	–	–
Teeth	Hypodontia, Oligodontia, Delayed dentition, Natal teeth, etc.	–	Oligodontia, Natal teeth	Oligodontia	–
Endocrine	Hypogonadotropic hypogonadism (absent, delayed or arrested puberty), Growth hormone deficiency, others	–	–	Hypogonadotropic Hypogonadism	Hypogonadotropic Hypogonadism
Growth	Short stature	–	Short stature	Short stature	–
Eyes	Myopia	–	Myopia, Hyperopia or normal vision	Hyperopia	–
Bone	Osteosclerosis (rare), Endosteal Sclerosis in some patients with biallelic *POLR3B* variants	–	Congenital fractures	Axial endosteal hyperostosis	–
Adipose tissue	–	–	Lipodystrophy, Abnormal distribution of fat tissue	–	–
Craniofacial bones	Not common - reported in some patients with biallelic *POLR1C* variants	–	Characteristic facial features ex. triangular face, widened fontanelles, mandibular hypoplasia	Dysmorphic facial features	–

Table 1.4: Common clinical features of POLR3-related disorders.[13] Permission to reprint provided by the journal author rights of Elsevier under the Copyright Clearance Center confirming that authors retain the right to include published work in a thesis.

1.4.4 Disease associations of RNA polymerase III transcription machinery and transcripts[1]

The CNS, and myelin in particular, shows a particular vulnerability to defects of transcription and translational machinery. Beyond disorders directly related to RNA polymerase III, various disorders arise from defects in Pol III co-factors and transcripts.

"Biallelic variants in genes encoding proteins that interact with Pol III have been shown to cause a phenotype reminiscent of POLR3-related disorders. These include variants in *BRF1*, a subunit of the transcription factor TFIIIB, which is involved in recruiting Pol III to its DNA targets.[150] Hypomorphic biallelic pathogenic variants in *BRF1* alter Pol III recruitment and transcription, and affected individuals present with cerebellar hypoplasia and thin corpus

[1]Section contains excerpt(s) from Watt, K. & Macintosh, J., *et al.* (*Seminars in Cell and Developmental Biology*, 2022)

callosum, as well as non-neurological features such as dysmorphic facial features, dental abnormalities, and short stature.[150] Conditional deletion of *Brf1* in mice results in perturbed 5S rRNA and tRNA transcription, diminished 80S ribosome production and loss of translation.[151] These studies suggest that *Brf1* may be required in a dynamic spatiotemporal manner. Overall, this cerebellar-facial-dental syndrome further implicates Pol III hypofunction in this specific subset of tissues."

"Mutations in certain Pol III transcripts cause disorders with partially overlapping phenotypic features. For example, hypomorphic mutations in *RMRP* RNA, the Pol III transcript involved in processing the common rRNA precursor produced by Pol I, is known to underlie various forms of skeletal dysplasia, including cartilage-hair hypoplasia (CHH), anauxetic dysplasia (AD) and kyphomelic dysplasia (KD).[152,153] These three disorders share abnormalities in connective tissues, which manifest as short stature, while individuals with AD also exhibit mild intellectual disability and abnormal dentition, and patients with KD display mild facial dysmorphia.[153] Each disorder demonstrates a critical role for Pol III transcription in connective tissues, dentition, and neurodevelopment."

Finally, disorders related to tRNAs represent another connection between Pol III transcripts and neurological disease. Defects in aminoacyl-tRNA synthetases (e.g., *EPRS1, DARS1, RARS1),* each responsible for catalyzing the aminoacylation reaction between amino acids and their cognate tRNA, are known to be associated with hypomyelination.[50,55,56] In turn, both POLR3-HLD and tRNA synthetase disorders share a potential common mechanism whereby deficient tRNA availability or lack of aminoacylated tRNAs could inhibit protein synthesis.[93] Further supporting a critical role for tRNAs in the CNS, mutations in the *CLP1* and *TSEN* genes, which

[1]Section contains excerpt(s) from Watt, K. & Macintosh, J., *et al.* (*Seminars in Cell and Developmental Biology*, 2022)

encode proteins important for tRNA processing and maturation, are associated with pontocerebellar hypoplasia and neurodegeneration.[71]

1.5 Oligodendrocyte lineage and myelination

1.5.1 Oligodendrocyte precursor cells

OPCs can be characterized both by their intrinsic migratory behaviour and their extensive proliferative ability (Figure 1.5).[154,155] Rodent studies have demonstrated that three populations of OPCs arise in precisely-timed temporal waves, originating in the ventral forebrain (Embryonic day 12.5; E12.5), ventricular zone (E14.5), and cortex (postnatally), a pattern which is thought to be conserved in humans.[155-157] These waves of OPCs have experimentally shown to be functionally redundant, as ablation of one wave in mice via targeted, temporally-controlled expression of a Diphtheria toxin in OPCs, revealed that another wave of OPCs will compensate for this loss, allowing for complete myelination.[158] In line with this functional redundancy, if not subjected to ablation, OPCs will normally compete for access to axons, and in turn survival.[156] To reach their final myelination site, OPCs must therefore travel significant distances from their sites of origin, a process whereby OPCs associate with endothelial cells and travel along vasculature, driven by a combination of chemoattractant and chemorepellent, short-range and long-range cues.[154,159] Once at their final destination, OPCs will proliferate extensively, making them the largest population of dividing cells in the nervous system.[160] This process of proliferation is thought to be homeostatically regulated, as the OPC pool will expand until a critical balance is met and further able to proliferate in the face of cell death or migration failure.[155] While the putative role of OPCs is to differentiate into OLs for myelination, it is interesting to point out that

[1]Section contains excerpt(s) from Watt, K. & Macintosh, J., *et al.* (*Seminars in Cell and Developmental Biology*, 2022)

large numbers of OPCs persist in the adult brain, comprising ~3-8% of all brain cells, depending on the region.[160] While this adult OPC pool is for critical myelination repair in the face of myelin injury, it is thought that these OPCs have additional roles in modulating neural function via their ability to receive GABAergic and glutaminergic input and their ability to contact both pre- and post-synaptic terminals.[160]

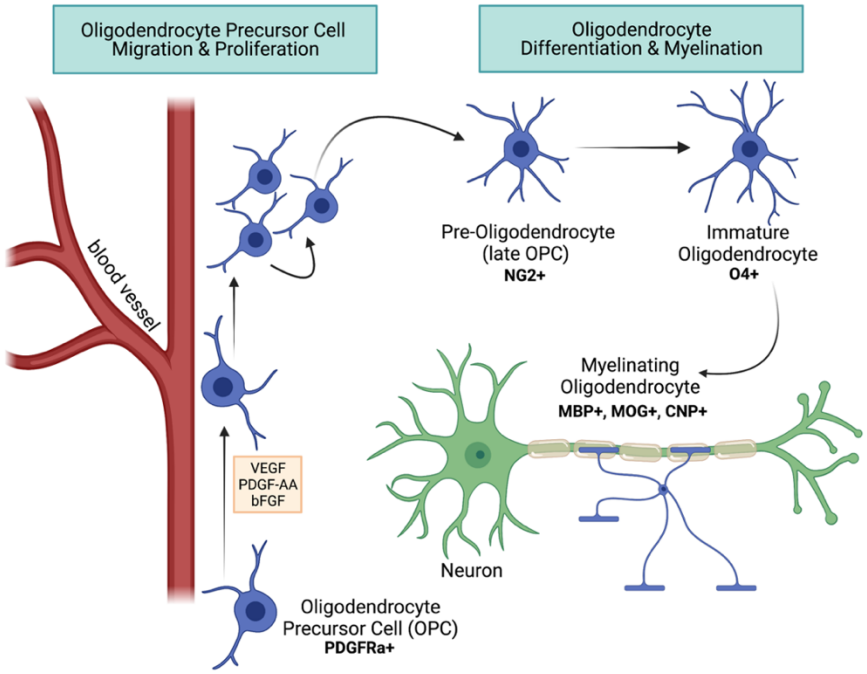

Figure 1.5: A simplified overview of oligodendrocyte development. Figure made using BioRender.

1.5.2 Oligodendrocyte maturation

Differentiation of OLs is known to be controlled by a variety of both extracellular signalling cues and OL-intrinsic transcription factors, epigenetic mechanisms, post-translational modifications, and miRNAs.[161] At an OL-intrinsic level, the transcription factors OLIG2 and NKX2.2 must be co-expressed as a pre-requisite for the initiation of differentiation, while transcription factors such as SOX10 are critical for differentiation to proceed and for the expression of myelin-

[1]Section contains excerpt(s) from Watt, K. & Macintosh, J., *et al.* (*Seminars in Cell and Developmental Biology*, 2022)

related proteins.[161] Additional epigenetic regulation including DNA methylation and histone acetylation controls access to genes involved in proliferation or differentiation, while post-translational modifications to transcription factors control gene expression and signalling pathways.[161] Moreover, miRNAs have been elucidated as having a critical role in oligodendrocyte differentiation, with certain miRNAs (e.g. miR-219) having oligodendrocyte-specific expression and roles in inhibiting repressors of OL differentiation.[162,163] In the post-mitotic stage, cells lose their proliferative and migratory capacity, alter their expression of lineage-restricted stage-specific cell markers and begin to undergo drastic morphological changes.[164] While OPCs express markers such as platelet-derived growth factor receptor alpha (i.e. PDGFRa) and neural/glial antigen 2 (i.e. NG2), immature OLs express markers such as O4 and mature OLs can be distinguished by the expression of myelin proteins such as myelin basic protein (i.e. MBP) or myelin oligodendrocyte glycoprotein (i.e. MOG).[165] The stage of a cell in the OL-lineage can also be distinguished based on its morphological complexity.[164] Beginning as a bipolar or tripolar cell in the precursor stage, an OL extends numerous processes to enable axon contact for the beginning of myelination.[156]

1.5.3 Myelination

Previous estimates have suggested that an individual OL at the peak of myelination produces up to 50 000 μm^2 of myelin, or as much as 5000 um^2 per day, and three times the weight of its soma in proteins daily.[12,166,167] In fact, estimates on the quantity of myelin have suggested that if the myelin fibers from a single human brain were laid out from end to end, the length would be sufficient to wrap around the Earth three times.[20] The demand of myelination by

[1]Section contains excerpt(s) from Watt, K. & Macintosh, J., *et al.* (*Seminars in Cell and Developmental Biology*, 2022)

oligodendrocytes is brought on by several factors, including the quantity of proteins that an OL must produce to make myelin, many of which are myelin-specific proteins, such as proteolipid protein (PLP1; 38% of CNS myelin proteome), myelin basic protein (MBP; 30%), 2'3'-cyclic nucleotide 3'-phosphodiesterase (CNP; 5%) and myelin oligodendrocyte glycoprotein (MOG; 1%).[42] An additional demand on protein synthesis is brought on by the production of enzymes involved in lipid synthesis.[21,42] Lipids constitute the bulk of myelin, or 70% of its dry weight, and while these lipids are not myelin-specific, this high lipid content enables the insulation role of myelin.[21] Importantly, oligodendrocytes also face greater demands than their peripheral nervous system (PNS) counterparts.[12,42] While an individual Schwann cell myelinates one axon, an individual OL must myelinate an estimated fifty to eighty axons, an event which is thought to occur within a time span of only twelve to eighteen hours.[168,169] These taxing demands on oligodendrocytes could help explain why decreased Pol III function, presumably leading to a protein synthesis deficit, may be detrimental to CNS myelination while sparing PNS myelination.

The process of axon contact during myelination is highly regulated, with *in vivo* timelapse studies on zebrafish myelination revealing that oligodendrocytes will extend and retract their processes numerous times before settling on a site for myelination.[165] These processes will likewise sense nearby cells, ensuring proper distance between oligodendrocytes to allow for myelination with uniform spacing of nodes.[170] Once axon contact is stabilized, a complex process is ensued involving OLs wrapping axons and cytoplasmic exclusion for myelin compaction.[12] As such, the myelin sheath is an extension of oligodendrocytes.[12] Functionally, myelin will serve as a low-capacitance insulator, enabling action potentials to travel between nodes of Ranvier with minimal energy required to transmit the action potential along the myelinated segment, a

[1]Section contains excerpt(s) from Watt, K. & Macintosh, J., *et al.* (*Seminars in Cell and Developmental Biology*, 2022)

process known as saltatory conduction.[165] This myelin is thought to accelerate nerve conduction by 20-100 fold compared to non-myelinated axons.[12] Beyond the role of insulating axons, a post-myelinating OL continues the long-term task of providing metabolic and trophic support to neurons, such as by supplying lactate as a source of energy and providing neuroprotective cues via exosomes.[171,172] Finally, while the vast majority of myelination is completed by two years of age, myelination is not thought to be static. Rather, a process of adaptive myelination has been suggested in which axons that are more active gain more myelin, reinforcing active pathways.[173]

1.5.4 Isolating oligodendrocyte-lineage cells from mice

Originally described by McCarthy & de Vellis in 1980, the shake-off method was one of the first methods proposed to isolate oligodendrocytes and astrocytes from rodent brains, enabling the characterization of cell types by purifying them from the cellularly heterogeneous CNS.[174] Relying on the ability of a mixed-glial culture to stratify, this technique separated OPCs from their underlying astrocyte layer by exposure to shear force.[174] While this method proved to be efficient, and is still used for the isolation of OL-lineage cells from rat, mouse oligodendrocytes have shown to be more difficult to detach from astrocytes.[175] As such, various alternative techniques have been proposed for the isolation of OL-lineage cells from rodents, including fluorescence-activated cell sorting (i.e. FACS) and magnetic-activated cell sorting (i.e. MACS), which rely on the expression of cell surface antigens to isolate cells via fluorescent labelling or antibody-coated beads.[176,177] While perhaps more reliable than the shake-off method, these alternatives have their own challenges, in part because mice have reduced expression of the antigens traditionally used by these techniques for the isolation of rat cells.[178] The gold standard

[1]Section contains excerpt(s) from Watt, K. & Macintosh, J., *et al.* (*Seminars in Cell and Developmental Biology*, 2022)

for the isolation of oligodendrocyte-lineage cells from mice is immunopanning in which a cell suspension derived from digested brain tissue is sequentially incubated on cell culture dishes which have absorbed reagents (e.g. glycoproteins, antibodies). The cell suspension is first incubated on cell culture plates that non-OL cell types bind to, for depletion, and finally, the cell suspension is plated on a positive selection plate that binds oligodendrocytes at a specific stage of the lineage (Figure 1.6).[179] The isolation of OL-lineage cells for *in vitro* studies enables a comprehensive assessment of the fundamental aspects of OL-lineage development, while eliminating the spatiotemporal complexity of oligodendrocyte development *in vivo*.

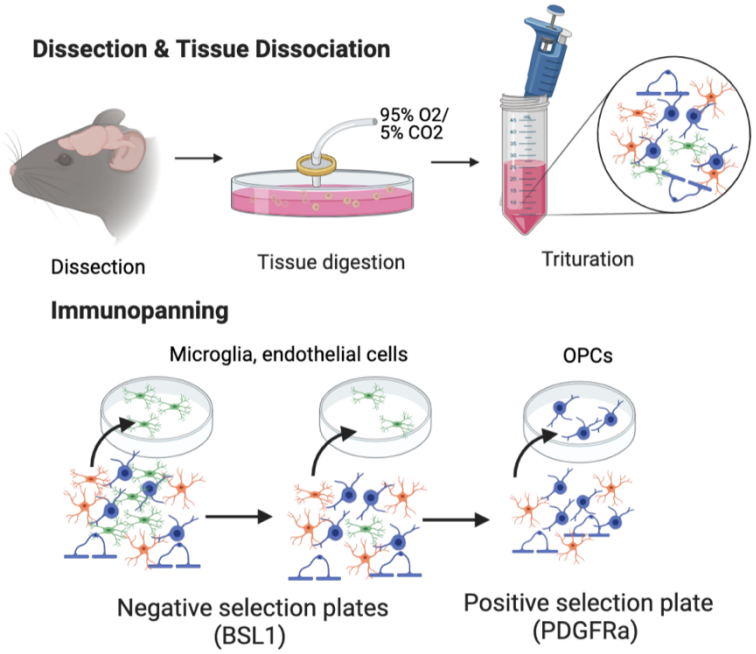

Figure 1.6: A schematic overview of the immunopanning technique. Figure made using BioRender, modified version of a similar figure in Emery & Dugas.[179]

[1]Section contains excerpt(s) from Watt, K. & Macintosh, J., *et al.* (*Seminars in Cell and Developmental Biology*, 2022)

Chapter 2: Rationale and Hypothesis

Pol III activity is ubiquitous and critical for life, as evidenced by no individual having been described with two null mutations and as mice harboring two null mutations are embryonic lethal.[7,129] Thus, how altered Pol III activity causes tissue-specific involvement is unclear. Indeed, how OLs are particularly impacted in the disease, leading to hypomyelination and consequently neurodegeneration, is unknown. In this thesis, we characterized how reducing Pol III expression in primary mouse OPCs impacts their development, to shed light on the pathophysiological mechanisms underlying hypomyelination in POLR3-related leukodystrophy.

In the first part of this thesis, we set out to optimize a method for the isolation of oligodendrocyte precursor cells from mouse brain tissue. An efficient method for the isolation of oligodendrocyte-lineage cells enables the study of a cell type that is physiologically relevant to disease and without the confounders of being derived from cancerous cells and immortalized, as is the case with cell lines. Additionally, the isolation of cells in an early stage of the lineage allows for a comprehensive study of oligodendrocyte development, from the precursor stage to myelination, which enabled the subsequent part of this thesis.

To address the gaps that remain in understanding the cellular pathophysiology of hypomyelination in POLR3-related leukodystrophy, we developed a cellular model that utilized a knockdown approach in primary murine OPCs. We hypothesized that reducing Pol III activity would lead to defects in OL-lineage development, either in the migration, proliferation, differentiation and/or myelination of these cells. To address this hypothesis, we had the following research aims:

AIM 1: Characterize the impact of reduced RNA polymerase III in oligodendrocyte precursor cells at the level of their migration and proliferation.

AIM 2: Investigate how reducing RNA polymerase III activity affects differentiation of oligodendrocyte precursor cells into mature oligodendrocytes.

AIM 3: Assess how reduced Pol III activity affects myelination.

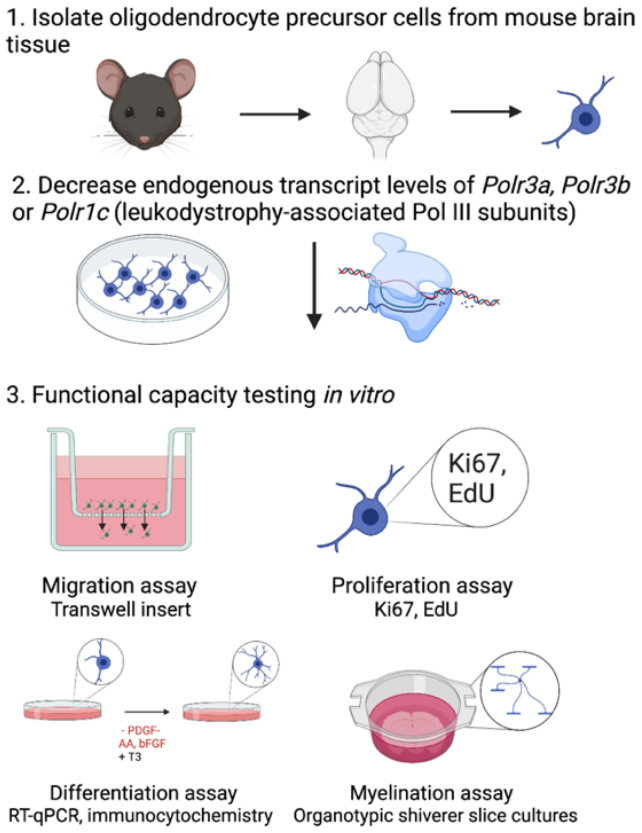

Figure 2.1: Graphical overview of thesis project. Step 1 relates to the method optimized in **Chapter 3,** for the isolation of oligodendrocyte precursor cells from mouse brain tissue, whereas Steps 2 and 3 provides a workflow for the research described in **Chapter 4**.

Chapter 3: Optimizing an immunopanning protocol for the isolation of mouse oligodendrocyte precursor cells

3.1 Preface

This chapter contains a manuscript which describes an immunopanning protocol optimized for the isolation of OPCs from mouse brain tissue, a stage of the lineage that has traditionally been considered difficult to isolate. This method was imperative for the subsequent chapter of this thesis, which utilizes mouse primary OPCs as the basis of an RNA polymerase III knockdown approach to identify a role for Pol III in OL-development and to shed light on the cellular pathophysiology of hypomyelination in POLR3-related leukodystrophy. Macintosh, J. et al, intended for submission to *MethodsX*.

3.2 Title Page

An optimized and validated protocol for the purification of PDGFRα+ oligodendrocyte precursor cells from mouse brain tissue via immunopanning

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Keywords: Immunopanning, Oligodendrocyte Precursor Cells, mouse primary cells, oligodendrocyte purification, *in vitro*

Declaration of competing interests

The authors declare no competing interests.

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Additional information

Our protocol is a modified version of the immunopanning oligodendrocyte lineage cells paper by Dugas & Emery published in Cold Spring Harbour Protocols.[1]

3.3 Abstract

Immunopanning is an efficient and reliable method for isolating primary cells from rodent brain tissue, making it a valuable tool for researchers interested in *in vitro* glial models. Here, we present an immunopanning protocol optimized for the isolation of Platelet-Derived Growth Factor Receptor Alpha positive (PDGFRα+) oligodendrocyte precursor cells (OPCs) from mouse brain tissue that results in a high yield of pure OPCs from minimal quantities of starting tissue.

- The protocol presented here is optimized for a PDGFRα-dependent selection of mouse OPCs using a commercial antibody, accounting for the relatively weaker cell-plate interaction as compared to other oligodendrocyte lineage markers (e.g. MOG).
- A modified papain digestion with humidified carbogen perfusion step significantly enhances the yield of dissociated cells and final yield of OPCs.
- Culturing PDGFRα+ OPCs permits the expansion of cells in culture, facilitating studies using transgenic mice.
- Isolating OPCs at the PDGFRα+ stage enables studies on the development of the oligodendrocyte lineage without the spatial and temporal complexity of *in vivo* studies.

3.4 Introduction

Various techniques have been developed for the isolation of oligodendrocyte precursor cells (OPCs) and oligodendrocytes (OLs) from rodent brain tissue, such as magnetic-activated cell sorting (i.e. MACS), fluorescence-activated cell sorting (i.e. FACS), and physical separation by shear force from mixed glial cultures (i.e. shake-off method).[2-6] While many of these techniques have proven successful for the culture of OL-lineage cells from rat brains, culturing mouse OPCs remains a challenge. While rat OPCs and astrocytes stratify in mixed glial cultures and can be separated when exposed to shear force, mouse OPCs are difficult to detach and isolate from

astrocytes, resulting in low yield or impure cultures.[7] Furthermore, MACS and FACS have traditionally relied on cell-surface antigens that are highly expressed in rat OPCs but show relatively lower or less specific expression in mouse cells, leaving these techniques flawed for OPC isolation from mouse brain tissue.[7,8] Consequently, many *in vitro* studies of the oligodendrocyte lineage have relied on rat cells.

Given the preponderance of transgenic models in mice among rodents and their widespread use in studies of brain development, function, and disease, a reliable method to isolate primary OPCs from the mouse brain is of great significance. Equally important, such a method must yield adequate numbers of healthy cells in order to pursue detailed *in vitro* studies from minimal starting material. Transgenic breeding programs often yield a genotype of interest in ≤25% of pups. It is often the case that a standard litter (~6 pups) from two heterozygous breeders yields just one pup having a target genotype. Therefore, the challenge of efficiently isolating healthy mouse OPCs in sufficient numbers is of equal or even greater importance than the challenge of isolating the cells themselves.

Immunopanning is a reliable and sensitive method for the isolation of mouse OL-lineage cells, whereby a single cell suspension derived from dissociated brain tissue is sequentially added onto cell culture dishes containing absorbed materials (e.g., lectin, antibody). The cell suspension is first added to negative selection plates, depleting unwanted cell types from the mixture, and finally onto a positive selection plate, to retain the desired cell type.[1] The original published protocol, Emery & Dugas (2013), summarizes immunopanning for the isolation of oligodendrocyte-lineage cells at various stages of development (e.g. PDGFRα selection for OPCs, O4 selection for immature oligodendrocytes, MOG selection for mature oligodendrocytes). Here,

we present an improved immunopanning protocol for the isolation of PDGFRα+ OPCs from mouse brain, which accounts for both the poor recovery of these cells following tissue dissociation and the relatively weak binding of OPCs to anti-PDGFRα plates during the PDGFRα-dependent selection, resulting in a high-yield and pure cultures.

3.5 Materials and Reagents

Dissection and Papain Dissociation Reagents

- Earle's Balanced Salt Solution (EBSS; Wisent, 311210CL)
- Papain (Worthington, LS003126)
- Papain buffer
    - $MgSO_4$ (100 mM)
    - EGTA (1 M)
    - Glucose (30%)
    - $NaHCO_3$ (1 M)
    - EBSS (10X) stock
- L-cysteine (Sigma, C7477)
- 10X papain inhibitor, 6X papain inhibitor
    - Bovine Serum Albumin (BSA; Sigma, A3912)
    - Ovomucoid (Worthington, LS003086)
    - D-PBS with Ca^{2+}/Mg^{2+} (ThermoFisher, 14287080)
    - NaOH
- DNase I
    - DNase I (CedarLane, LS002007)
    - EBSS (Wisent, 311210CL)

Immunopanning Reagents

- BSL 1 (VECTL1100, BioLynx)
    - Hepes Buffered Saline
        - NaCl
        - Na_2HPO_4
        - HEPES
        - NaOH
        - $CaCl_2$
        - NaN_3
- Goat anti-rat IgG (Jackson ImmunoResearch, 115-005-167)
- Tris-HCl (50 mM) stock solution
- Phosphate buffered saline (PBS; ThermoFisher, 10010023)
- Anti-PDGFRα antibody (BD Pharmigen, 558774)
- Immunopanning buffer

    - Bovine Serum Albumin (Sigma, A3912)
    - Insulin from bovine pancreas (Sigma, I6634)
    - D-PBS with Ca^{2+}/Mg^{2+} (ThermoFisher, 14287080)

Trypsinization Reagents

- Trypsin from bovine pancreas (Sigma, T9935)
- EBSS (Wisent, 311210CL)
- EBSS (10X) stock
    - NaCl
    - KCl
    - $NaH_2PO_4 \bullet H_2O$
    - Glucose
    - Phenol red (0.5%) (Sigma, P0290)
- Fetal Bovine Serum (FBS; Wisent, 089-150)

Cell culture reagents

- Poly-D-Lysine (PDL) hydrobromide (Sigma, P8099)
    - Borate buffer
        - H_3BO_3
        - $Na_2B_4O_7$
- Cell culture media
    - Dulbecco's Modified Eagle's Medium (DMEM; Wisent, 319-005-CL)
    - SATO supplement
        - BSA (Sigma, A3912)
        - Transferrin (Sigma, T1147)
        - Putrescine (Sigma, 221732)
        - Progesterone (Sigma, P8783)
        - Sodium selenite (Sigma, S5261)
    - Insulin (0.5 mg/mL) stock solution
    - Penicillin-Streptomycin (Wisent, 450-201-EL)
    - Sodium pyruvate (Gibco, 11360070)
    - GlutaMAX (Gibco, 35050061)
    - N-Acetyl-L-Cysteine (Sigma, A7250)
    - Forskolin (Sigma, F3917)
        - Dimethyl sulfoxide (DMSO; Fisher, BP231-100)
    - D-Biotin (Sigma, B4639)
    - Trace Elements B (Fisher Scientific, MT99175Cl)
    - B-27 without vitamin A (ThermoFisher, 12587010)
    - Recombinant human PDGF-AA (Preprotech, 100-13A)
    - Recombinant human bFGF (Preprotech, 100-18B)
    - Triiodothyronine (T3, 4 µg/mL) (Sigma, T6397-100mg)
- Trypsin/EDTA (optional, for passaging) (Wisent, 325.043-cl)

Materials and equipment

- Ready Warm Hot plate (LW scientific, FWL-04PL-BTD3)
- 500 mL vacuum flask
- Tubing (~0.5 cm wide)
- Connectors for tubing
- 500 mL flask stopper with one-hole
- 10 cm petri dishes (Fisher, FB0875713)
- Petri lid
- 95% O_2/5% CO_2 carbogen tank (MEGS Air liquide, A0490943)
- 40 µM cell strainer, DNase/RNase free, non-pyrogenic, sterile (VWR, 76327-098)
- Syringe filter (0.22 µM) (Milipore, SLGV033RS)
- 50 mL Syringe with Luer-Lok tip (BD, 309653)
- 15 mL, 50 mL conical tubes (Sarstedt, 62.554.205 and 62.547.205)
- 2 mL, 5 mL, 10 mL, 25 mL serological pipettes (Sarstedt, 86.1252.001, 86.1253.001, 86.1254.001 and 86.1685.001)
- 20 µL, 200 µL, 1000 µL pipette tips
- 10 cm cell culture dishes (Sarstedt, 83.3902)
- 6-well (Sarstedt, 83.392O) or 12-well cell culture dishes (Sarstedt, 83.392) (optional, for immunocytochemistry)
- Coverslips (Pre-Treated German Glass Coverslips 15 mm #1; Electron Microscopy Sciences) (optional, for immunocytochemistry)
- Dissection kit: large scissors for decapitation, small scissors, forceps
- Razor Blade (VWR, 55411-055) or scalpel (FisherScientific, 08-927-5F)
- Tabletop centrifuge with tube adaptors for 15 mL and 50 mL tubes
- Biological Safety Cabinet
- Cell culture CO_2 incubator set to 10% CO_2
- Water bath, set to 37˚C

3.6 Method Workflow

A note on planning experiments: The protocol described here is sufficient for processing three P6 mouse brains which yields an average of $6x10^6$ PDGFRα+ OPCs in the final step. These are either collected directly or further expanded *in vitro* for downstream testing. To increase throughput, simply coat additional plates and prepare additional reagents.

Preparation the day before immunopanning

1. Coat positive selection plate with secondary antibody.
    a. Dilute 30 µL of anti-rat IgG (1.3 mg/mL) (Jackson ImmunoResearch) in 10 mL of Tris-HCl (50 mM, pH 9.5).
    b. Add 10 mL antibody solution to a 10 cm petri dish and swirl to evenly coat plate.
    c. Incubate the panning plate at 4˚C overnight.
2. Coat cell culture plates with PDL.

a. For 10 cm dishes: dilute 50 µL of PDL (10 mg/mL) in 10 mL of sterile water (per dish). Add 10 mL of PDL solution to a 10 cm tissue culture plate and swirl to evenly coat plastic.
b. For coverslips: Plate 1 coverslip in each well of a 6- or 12-well plate. Dilute 120 µL PDL solution in 12 mL of sterile water (per plate). Add 2 mL of PDL solution to each well of a 6-well plate or 1 mL to each well of a 12-well plate and swirl to evenly coat plastic.
c. Incubate overnight at room temperature, keep sterile.
d. Note: A higher concentration of PDL is used for glass relative to plastic as OPCs are more readily adherent to plastic cell culture dishes. It is not necessary to wash the coverslips listed above with ethanol before or after coating.

3. Prepare 0.2% BSA solution.
    a. Dilute 2 mL of 4% BSA in 38 mL of D-PBS.
4. Prepare 0.5 mg/mL insulin solution.
5. Prepare proliferation media.
    a. Note: As the biological activity of growth factors decreases with time, and the concentration of PDGF-AA is critical for OPC cultures and amplification of cells, media should be prepared fresh when possible. Once growth factors are added, store at 4˚C for up to 7 days.

<u>Preparation the morning of the immunopanning experiment</u>

6. Rinse and dry PDL-coated culture dishes.
    a. Rinse each plate three time with sterile water.
    b. Leave plates open in hood for a minimum of 2 hours to air dry.
    c. Note: make sure plates are dried completely as this may impact OPC attachment (Figure 3.4A).
7. Coat two 10 cm petri dishes with BSL 1 solution for negative selection.
    a. Dilute 20 µL BSL 1 in 20 mL D-PBS.
    b. Add 10 mL BSL 1 solution to each plate and swirl to evenly coat.
    c. Note: Emery & Dugas (2013) suggest two 15 cm dishes.[1] While this is more or less equivalent to four 10 cm dishes in terms of surface area, in our experience two 10 cm dishes limits reagent use and is sufficient as microglia cell contamination has not been a problem with our protocol.
8. Coat positive selection dish with primary antibody.
    a. Rinse plate coated with secondary antibody (prepared in step 1) three times with PBS. Do not let plate cell culture surface dry out after coating.
    b. Prepare 40 µL rat anti-PDGFRα antibody in 10 mL of 0.2% BSA D-PBS solution.
    c. Coat plate with primary antibody solution and let sit at room temperature until use, or for a minimum of two hours.
9. Prepare solutions for papain digestion, immunopanning and trypsinization.
    a. For 10X ovomucoid solution, combine:
        a. 1 mL of 10X ovomucoid stock with 9 mL D-PBS.
    b. For 6X ovomucoid solution, combine:
        a. 1 mL of 6X ovomucoid stock with 5 mL D-PBS.

c. For immunopanning buffer, combine:
    a. 13.5 mL D-BPS, 1.5 mL 0.2% BSA D-BPS, and 150 µL of 0.5 mg/mL insulin.
d. For 30% FBS, combine:
    a. 3 mL heat-inactivated FBS and 7 mL D-BPS.

10. Equilibrate EBSS in 37˚C incubator.
    a. Dilute 800 µL 10X EBSS in 7.2 mL sterile water and incubate in 10% CO_2 incubator until use.
11. Equilibrate papain buffer.
    a. Set-up hot plate in BSC.
        i. The hot plate should be set so that papain digestion can occur at 34˚C.
        ii. Note: The hot plate will likely have to be set at a higher temperature to maintain the buffer at 34˚C during dissociation, for reference ours is set to 44˚C. Before carrying out the protocol, test your hot plate with some papain buffer and a thermometer to determine an appropriate temperature.
    d. Add 10 mL papain buffer to petri dish, place on hot plate and cover with petri lid.
    e. Set-up the humidified-carbogen perfusion system (see Figure 3.1D,E).
        i. Add around 100 mL of sterile water to a 500 mL vacuum flask.
        ii. Add a stopper to the top of the flask with a 2 mL pipette through it.
        iii. Connect carbogen tank to 2 mL pipette with tubing.
        iv. Connect outlet of flask to petri lid with tubing.
    f. Add 200 U of papain to papain buffer.
    g. Add 200 µL of DNase I solution (2500 U) to papain buffer.
    h. Add 200 µL of 10 mg/mL L-cysteine solution to papain buffer.
        i. Emery & Dugas (2013) recommend adding 2 mg of L-cysteine.[1] As weighing out 2 mg can be tedious and inaccurate, we opt to aliquot 10 mg and when ready to use, resuspend in 1 mL of sterile water. Discard any extra.
    i. Leave the papain mixture on the hot plate with humidified gas flow for 5 to 10 minutes before adding brain tissue to activate the papain.
        i. Note: This optimized papain tissue digestion with humidified carbogen perfusion is crucial to the final OPC yield (Figure 3.1B).

<u>Dissection of the mouse brain (we typically use three P6 mice per experiment)</u>

12. Add EBSS to a petri dish and place on ice.
13. Cryo-anesthetize and decapitate post-natal day 6 (P6) mice.
14. Starting at the vertex, cut the skin along the midline, removing it to expose the skull.
15. Starting at lambda, cut along the sagittal suture of the skull towards the nasal bone.
16. Using forceps, peel back the skull to reveal the brain.
17. Gently use forceps to cut any underlying nerves and remove the brain.
18. Dissect desired brain regions (*e.g.*cortices)
    a. Note: For standard cultures, yield is best when both telencephalon and mesencephalon are included in the dissociation (i.e. remove olfactory bulbs, cerebellum, pons, and any residual spinal cord)

19. Place the dissected brain in a 15 mL conical tube with EBSS while dissecting the remaining mice.
    a. Note: Work carefully but quickly during the dissection as this improves cell viability.

Papain digestion

20. Add dissected brains to papain buffer and chop, with a razor blade or scalpel, into small chunks.
21. Incubate the tissue in papain solution for 90 minutes, gently agitating the plate every 30 minutes.
22. Just before the 90 minute mark, add 100 µL DNase 1 (1250 U) to the 10X ovomucoid solution (prepared in step 9).
23. At the end of the 90 minutes, when the digestion is complete, transfer the papain buffer with digested brain tissue into a 15 mL conical tube and add 1 mL of 10X ovomucoid solution (with DNase 1).
24. Pellet the solution for 5 minutes at 220 xg at room temperature.
    a. This modification adds negligible time but increases yield and minimizes the risk of aspirating tissue, compared to leaving the tissue to settle.
25. Carefully aspirate the supernatant.
26. Add 1.5 mL of 10X ovomucoid solution to digested tissue and triturate slowly (set pipette gun to low speed) with a 5 mL pipette by pipetting up and down, seven times.
27. Let tissue settle and transfer supernatant to a new 15 mL conical tube.
28. Repeat steps 26-27 once.
29. Add 1 mL of 10X ovomucoid solution to digested tissue and triturate with a 1000 µL pipette tip, by pipetting up and down, seven times.
30. Let tissue settle and transfer supernatant to a new conical tube.
31. Repeat steps 29-30 until all 10X ovomucoid solution is used.
32. Pellet cells by centrifugation for 15 minutes at 220 xg (room temperature).
33. Aspirate supernatant and resuspend pellet in 6 mL of 6X ovomucoid solution (prepared in step 9) by gently pipetting up and down once.
34. Pellet cells by spinning for 15 minutes at 220 xg (room temperature).
35. Aspirate the supernatant and resuspend pellet in 6 mL immunopanning buffer (prepared in step 9).
36. Prewet 40 µM cell strainer with 2 mL immunopanning buffer.
37. Filter the cell solution in panning buffer and rinse filter with the remaining 7 mL of immunopanning buffer.
    a. At this point, collect 10 µL of the cell suspension for a post-papain cell count. The expected yield is 30.25×10^6 cells/brain (Figure 3.1A).
38. Immediately before use, rinse one BSL 1 coated plate three times with D-PBS.
    a. Note: Ensure panning plates are never allowed to dry.
39. Add filtered cell suspension to the rinsed BSL 1 dish.
40. Incubate plate at room temperature for 15 minutes, gently agitating the plate every 5 minutes.
41. Immediately before using, rinse the second BSL 1 dish three times with D-PBS.

42. Gently shake the plate to loosen non-adherent cells and transfer non-adherent cells to the second BSL 1 plate.
43. Repeat step 40.
44. Immediately before using, rinse PDGFRα antibody-coated plate three times with D-PBS.
45. Gently shake the plate to loosen non-adherent cells and transfer non-adherent cells to a PDGFRα antibody-coated plate.
46. Incubate the plate at room temperature for 45 minutes, with gentle agitation every 15 minutes.
47. At the end of the 45 minutes, rinse the plate three times with D-PBS.
    a. While seemingly trivial, the modification to reduce washes from the Emery & Dugas (2013) protocol is key for the isolation of PDGFRα+ cells.[1] The interaction between OPCs and the anti-PDGFRα plate is relatively weak – if washing steps are not done gently, cells will detach and can be completely lost at this step.
    b. When washing, set pipette to gravity and pipette onto the same area of petri dish with every wash, swirl once very gently, pipette off. Do *not* pour D-PBS on and off the plate.
    c. It is more beneficial to minimize washes than to be too stringent about non-OPC cell contamination. The media used to culture the cells is pro-oligodendrocyte lineage and contamination of other glial cell types, such as residual astrocytes, is of minimal concern.

<u>Trypsinization and plating</u>

48. Add 400 µL of trypsin to the equilibrated 8 mL 1X EBSS (prepared in step 10).
49. Add the trypsin solution to cells on the PDGFRα coated plate.
50. Incubate the plate at 37˚C for 6 to 8 minutes.
51. Stop the trypsin reaction by adding 30% FBS solution.
52. Dislodge and collect the cells in a conical tube.
53. Spin for 15 minutes at 220 xg, room temperature.
54. Resuspend the cells in 1 mL proliferation media and count.
    a. The expected yield is 2.17×10^6 cells/brain (Figure 3.1B).
55. Plate 500 000 cells per 10 cm culture dish.
    a. Note: Cells will proliferate rapidly. It is ideal to start with a low cell density, so the cells do not become too confluent.
    b. Emery and Dugas (2013) suggests adding cells in a small suspension volume, spreading with a sterile glass spreader and incubating prior to adding media, in order to increase cell viability.[1] In our protocol, viability of cells has not proven to be problematic and therefore we have not used this. However, one may consider doing this if experiencing low viability.
56. Culture mouse OPCs in a 10 % CO_2 incubator.
57. At 2 DIV, supplement media with PDGF-AA, bringing the final concentration to 20 ng/mL PDGF-AA.
    a. Supplementing the cells with PDGF-AA will push for an oligodendrocyte-lineage culture and induce a high rate of OPC proliferation to expand cultures (Figure 3.2).

58. At DIV4, do a half media change.
59. Continue half media changes every two days.
60. Passage cells.
    a. We typically passage at DIV 7 into cell culture dishes for experiments, as the cells at this point have proliferated but are not so dense that they start to differentiate.
    b. If the experimental objective involves differentiation, passage cells into differentiation media. Passaging will help to ensure PDGF-AA and bFGF are removed from culturing conditions and is a preferable alternative to simply replacing media with T3-containing media.

3.7 Reagent Preparation

<u>Papain dissociation reagents:</u>

Papain Buffer (500 mL)

EBSS Stock (10X)	50 mL
$MgSO_4$ (100 mM)	60.19 mg
Glucose (30%)	2.3 g
EGTA (1 M)	1 mL
$NaHCO_3$ (1 M)	13 mL

Bring the volume to 500mL with ddH_2O and autoclave to sterilize. Store at 4˚C.

EBSS (250 mL,10X stock)

NaCl	17 g
KCl	1 g
$NaH_2PO_4 \bullet H_2O$	0.35 g
Glucose	2.5 g
Phenol red (0.5%)	2.5 mL

Bring the volume to 250 mL with ddH_2O and filter to sterilize.

L-Cysteine

Prepare 10 mg aliquots of L-cysteine and store at room temperature.

Do not dissolve in water until right before use.

10X Ovomucoid papain inhibitor (33.3 mL)

BSA	0.5 g
Ovomucoid	0.5 g

Combine in D-PBS. Adjust pH to 7.4 with NaOH. Bring final volume to 33.3 mL with D-PBS. Filter and sterilize with a 0.22 µm filter. Aliquot the papain inhibitor in 1mL aliquots and store at -20˚C.

6X Ovomucoid papain inhibitor (33.3 mL)

BSA	1 g
Ovomucoid	1 g

Combine in D-PBS. Adjust pH to 7.4 with NaOH. Bring final volume to 33.3 mL with D-PBS. Filter and sterilize with a 0.22 µm filter. Aliquot the papain inhibitor in 1mL aliquots and store at -20˚C.

DNase I
Combine 12 500 U of DNase I per 1 mL EBSS, on ice. Filter and sterilize. Aliquot in 300 µL aliquots and store at -80˚C.

Immunopanning reagents:
BSL 1
Resuspend 5 mg BSL 1 in 1mL of 1X HEPES buffered saline. Aliquot in 20 µL aliquots and store at 4˚C.

HEPES buffered saline (HBS) (100 mL, 2X stock)

NaCl	0.8 g
Na_2HPO_4	21 mg
HEPES	0.283 g

Combine in ddH_2O and bring pH to 8.5 with NaOH.
Then add in:

$CaCl_2$	1.12 mg
NaN_3	40 mg

Bring final volume to 100 mL and filter-sterilize.

Tris-HCl (1L, 50 mM)
Combine 7.88 g of Tris-HCl with ddH_2O. Bring pH to 9.5 with NaOH. Autoclave to sterilize and store at 4˚C.

BSA (50 mL, 4%)
Dissolve 2 g of BSA in 50 mL D-PBS. Adjust pH to 7.4 with NaOH. Filter through a 0.22 µm filter. Aliquot in 1 mL aliquots and store at -20 ˚C.

Trypsinization reagents:
Trypsin
Dissolve 50 000 U/mL in EBSS.
Trypsin should be aliquoted (400 µL) and stored at -80˚C to prevent self-cleavage.[9]

FBS
Heat shock FBS in a water bath set to 56˚C for 1 hour. Aliquot and store at -20˚C.

Cell culture reagents:
Poly-D-Lysine (PDL) hydrobromide (10 mg/mL)
Dissolve 100 mg of PDL in 10 mL of 1 X borate buffer.
Aliquot in 250 µL aliquots and store at -20˚C.

Borate buffer (400 mL)

H_3BO_3	1.24 g
$Na_2B_4O_7$	1.9 g

Dissolve in ddH_2O, bring the pH to 8.5 and the volume to 400 mL with ddH_2O.

SATO Supplement (20 mL)

BSA	200 mg
Transferrin	200 mg
Putrescine	32 mg
Progesterone stock	5 µL
Sodium selenite stock	200 µL

Bring to a total volume of 20 mL with DMEM. Filter with a 0.22 µM filter to sterilize. Aliquot in 500 µL aliquots and store in -20˚C.

Progesterone stock solution (make fresh when preparing SATO reagent)
Combine 5 mg of progesterone in 200 µL ethanol to make the progesterone stock solution.

Sodium selenite stock solution (make fresh when preparing SATO reagent)
Combine 4 mg of sodium selenite, 10 µL of 1N NaOH solution and 10 mL DMEM to make the sodium selenite stock solution.

Insulin (0.5 mg/mL)
Dissolve 5 mg of insulin with 10 mL of sterile water and 50 µL of 1N HCl. Mix and filter through a 0.22 µM filter. Store at 4˚C. Remake stock every 4 weeks.

N-acetyl-L-cysteine
Dissolve 25 mg of N-acetyl-L-cysteine in 5 mL DMEM for a concentration of 5 mg/mL. Aliquot in 50 µL aliquots and store at -20˚C.

Forskolin (4.2 mg/mL)
Dissolve 50 mg forskolin in 1 mL of sterile DMSO. Add another 11 mL DMSO to bring the volume to 12 mL for a final concentration of 4.2 mg/mL. Aliquot in 50 µL aliquots and store at -20˚C.

Biotin (50 µg/mL)
To prepare a 10 µM stock solution, dissolve 100 mg of biotin in 40 mL of DMEM.
To prepare the final solution, dilute 20 µL of the 10 µM stock solution in 980 µL DMEM.
Aliquot in 10 µL aliquots and store at -80˚C.

PDGF-AA (10 µg/mL)
Dissolve 50 µg PDGF-AA in 5 mL 0.2% BSA D-PBS. Aliquot in 50 µL aliquots and store at -80˚C.

bFGF (10 µg/mL)
Dissolve 50 µg bFGF in 5 mL of 0.2% BSA D-PBS. Aliquot in 50 µL aliquots and store at -80˚C.

T3 (4 µg/mL)
To prepare a 20 mg/mL stock solution, dissolve 100 mg of T3 in 5 mL DMSO.

To prepare a 400 µg/mL solution, dilute 100 µL of the above 20 mg/mL solution in 4.9 mL DMEM.
To prepare the final solution, dilute 50 µL of the 400 µg/mL solution in 4.95 mL DMEM.
Aliquot in 500 µL aliquots and store at -80˚C.

For 50mL of Proliferation media, combine:

Dulbecco's Modified Eagle's Medium (DMEM)	49 mL
B-27 (- vitamin A)	1 mL
Penicillin-Streptomycin	500 µL
GlutaMAX	500 µL
Sodium Pyruvate	500 µL
Insulin (0.5 mg/mL)	500 µL
SATO supplement	500 µL
Trace Elements B	50 µL
N-acetyl-L-cysteine	50 µL
Forskolin	50 µL
Biotin	10 µL
PDGF-AA	50 µL
bFGF	50 µL

Filter through a 0.22 µm filter.

Note: Emery & Dugas (2013) add CNTF and NT-3 growth factors to the media.[1] CNTF has been shown to direct OPCs toward astrocyte differentiation and we therefore omit it from the media.[10] Additionally, NT-3 has been suggested to induce differentiation of OPCs cultured on poly-lysine plates into OLs, hence we do not use NT-3 as a growth factor in our cultures.[11]

<u>For 50 mL differentiation media:</u>
Omit PDGF-AA and bFGF and rather, include 500 µL of 4 µg/mL T3.

3.8 Method Validation

To validate our protocol, we compared the total cell yields following papain digestion and the OPC yield after immunopanning, in the absence or presence of humidified carbogen perfusion during the enzymatic tissue digestion, to the expected yields of the Emery & Dugas (2013) paper (Figure 3.1A, B).[1] Omitting carbogen resulted in a drop in yield at the post-papain count (*p=0.0309) but to our surprise, provided comparable OPC yields (n.s., p=0.0657). In comparison, our perfusion method led to a significant improvement in yield at both the post-papain step (****P<0.0001) as well as a the final OPC yield (****P<0.0001). This finding suggests

an optimized papain digestion impacts the yield after the papain step and has a significant influence on the final OPC yield.

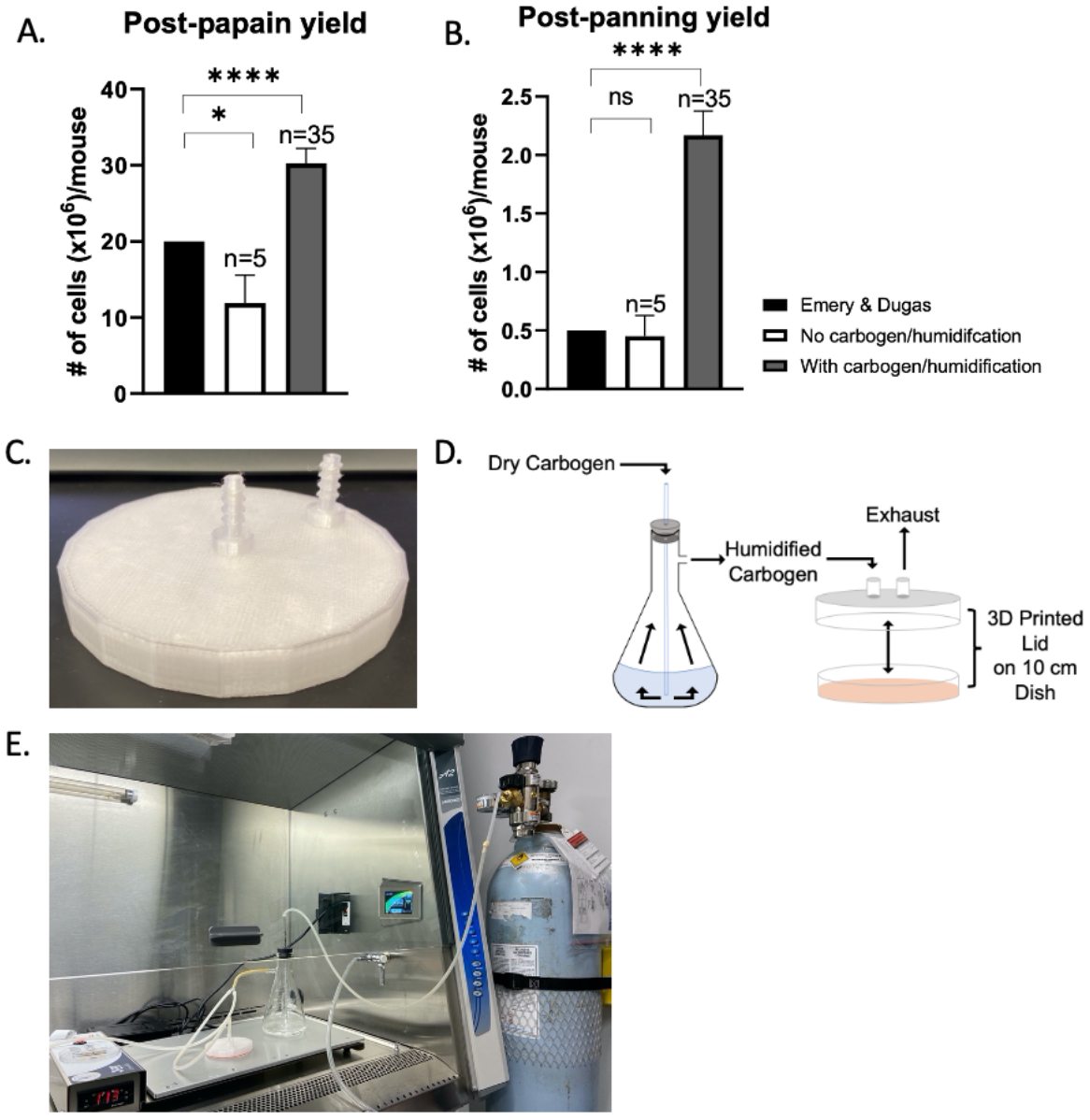

Figure 3.1: Improved cell yields and final OPC yields with optimized papain digestion**.** A. Quantification of total cell yields (expressed as x 10^6/mouse brain) following papain digestion in the Emery & Dugas (2013) protocol[1], without carbogen/humidification (n=5) and with carbogen/humidification (n=35). B. Quantification of OPC yields (expressed as x 10^6/mouse brain) following papain digestion in the Emery & Dugas (2013) protocol[1], without carbogen/humidification (n=5) and with carbogen/humidification (n=35). Single sample t-test. Data represented as mean ± SEM. C. Image of the petri lid used for perfusion. D. Schematic of the carbogen perfusion set-up . E. Image of the carbogen perfusion set-up.

Moreover, isolation at the PDGFRα+ precursor stage means the cells are highly proliferative when maintained in culture, overcoming the often-inherent challenge of limited cell yield for primary cell culture work (Figure 3.2A). In line with this, high percentages of cells were positive for EdU in an EdU uptake experiments using standard proliferation media (avg. 77.5% EdU+, following 20 hours of 10 µM EdU exposure) and this proportion was significantly enhanced when the cells were maintained using media supplemented with PDGF-AA (*p=0.011; final PDGF-AA concentration 20 ng/mL) (Figure 3.2B,C). Due to the high proliferative rate (avg. cell count $60.4x10^6$ cells/mouse after 7DIV), we found that one panning dish for all immunopanning experiments, or the equivalent of up to three mouse brains, was sufficient to generate large quantities of cells, while limiting the use of reagents. The extensive proliferative capacity of early PDGFRα+ OPCs is relevant to the amplification of OPCs from transgenic mice, where the availability of mice with the desired genotype limits source tissue quantities.

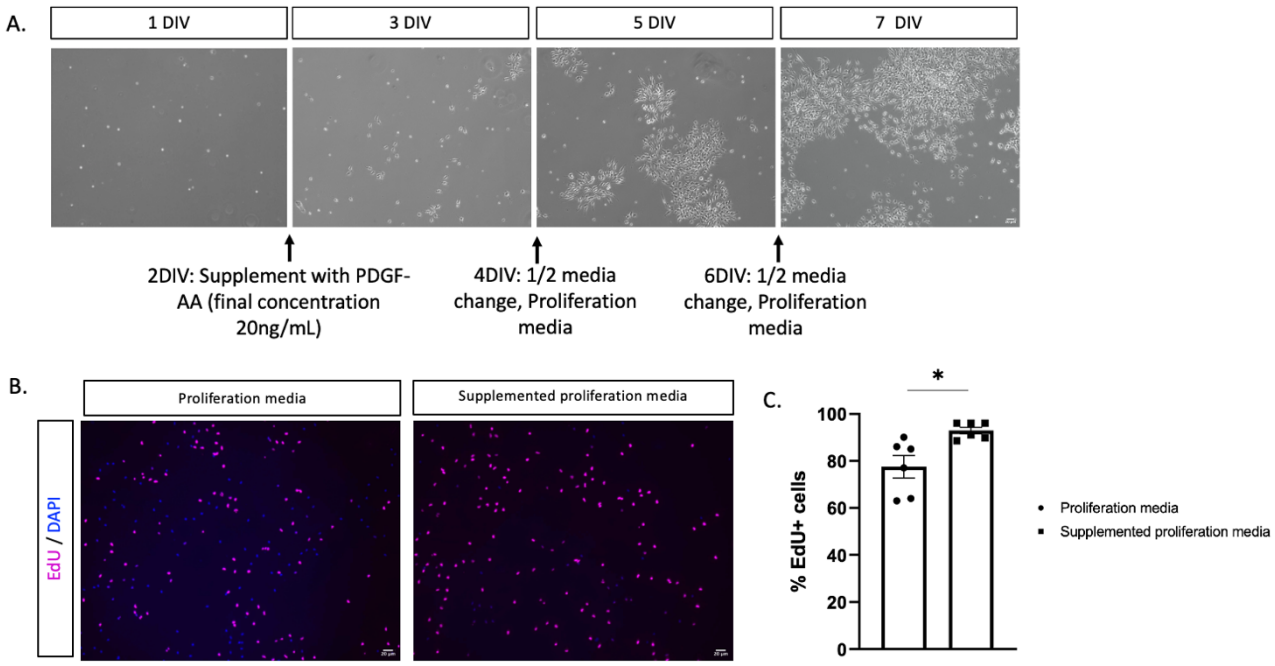

Figure 3.2: PDGFRα+ OPCs are highly proliferative**.** A. Brightfield images of OPCs in culture at 1, 3, 5 and 7 days *in vitro* (DIV) and maintenance protocol (written below the images). Scale bar, 20 µm. B. Representative immunofluorescence images (from three independent experiments) of OPCs in standard proliferation media or media supplemented with PDGF-AA (final concentration: 20 ng/mL) labelled with EdU (magenta). Nuclei stained with DAPI. Images taken on an EVOS light microscope, Cy5 light cube. Scale bar, 20 µm. C. Quantification of EdU+ cells as a percentage of DAPI in standard or supplemented proliferation conditions. Data represents mean $\pm$ SEM from three independent experiments, *p < 0.05. Unpaired, two-sided Student's t test.

Next, to assess that the modifications to the protocol, namely decreasing the surface area used during the BSL 1 negative selection steps and reducing the number of washes between the PDGFRα+ coated panning dish and trypsinization step, did not affect the purity of cultures, we characterized the cultures using immunocytochemistry. All cells were immunolabelled for their respective lineage markers at their corresponding stage of development, including all cultured cells under proliferative conditions labelling for the OPC markers PDGFRα+ and/or NG2+, which have different but overlapping temporal expression (Figure 3A).[12] Additionally, when induced to differentiate by passaging into T3-containing media, cells labelled for immature oligodendrocyte markers O4 (Figure 3B; 2 DIV in differentiation media) or mature oligodendrocyte markers MBP/MOG (Figure 3C,D; 3 DIV in differentiation media). Additionally, when isolating OPCs from constitutive Olig2-Cre YFP+ mice, all cells in the cultures were YFP+, consistent with our immunocytochemical findings indicating the purity of the oligodendrocyte-lineage culture, although of note, distinct populations of Olig2+ astrocytes do exist in the mouse brain (Figure 3E-G).[13]

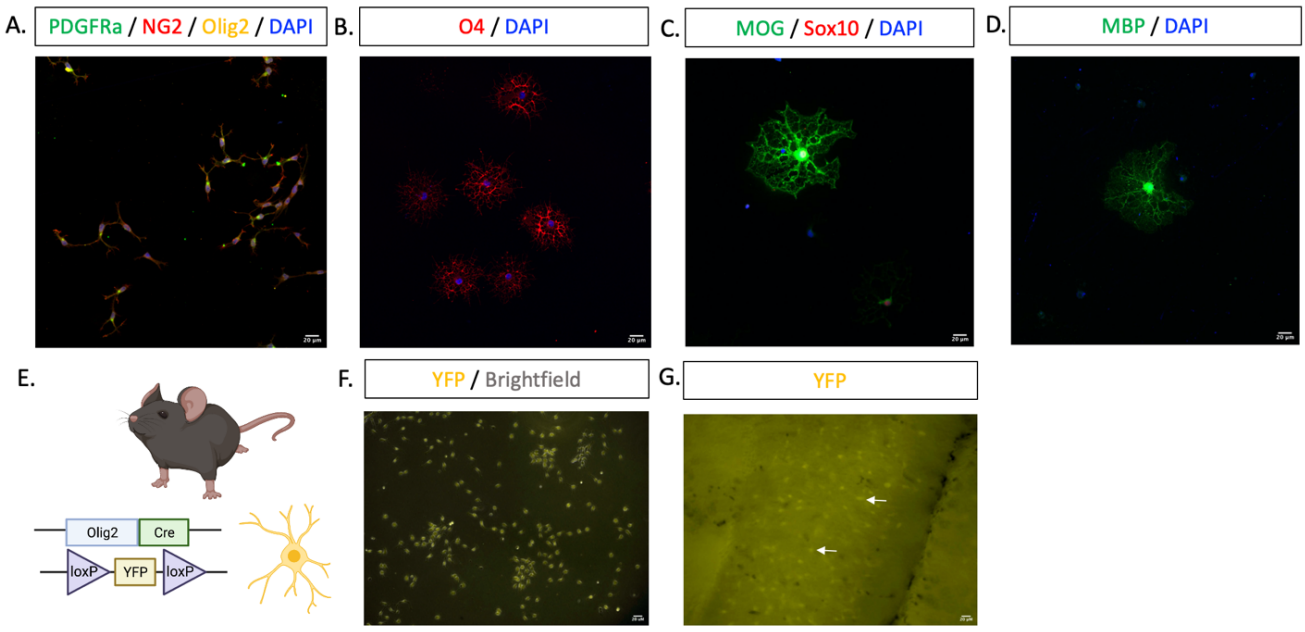

Figure 3.3: Immunocytochemistry characterization of cultures and purification from Olig2-Cre YFP+ mice. A. OPCs at 7 DIV under proliferative conditions positively labelled for PDGFRα, NG2 and Olig2, B. Immature oligodendrocytes after 2 DIV in differentiation/T3-containing media stained for O4. C. Oligodendrocytes after 3 DIV in differentiation/T3-containing media stained for MOG and Sox10 or D. MBP. E. Olig2-Cre YFP+ transgenic mouse. F. OPCs from Olig2-Cre YFP+ transgenic mice in culture (2 DIV, EVOS light microscope, YFP light cube) G. Distinct YFP+ cells (white arrows) visible in cerebellar tissue of Olig2-YFP mice. Antibodies: rat anti-PDGFRα (BD Pharmigen, 1:100), rabbit anti-NG2 (Milipore, 1:200), goat anti-Olig2 (R&D, 1:200), mouse anti-O4 (R&D, 1:200), goat anti-Sox10 (R&D, 1:250) rat anti-MOG (R&D, 1:300), rat anti-MBP (Novus, 1:500), AlexaFluor 488 donkey anti-rat (ThermoFisher, 1:1000), AlexaFluor 647 donkey anti-rabbit (Jackson ImmunoResearch, 1:500), AlexaFluor 594 anti-mouse secondary antibody (Jackson ImmunoResearch, 1:500), AlexaFluor 555 donkey anti-goat (Invitrogen, 1:500). Nuclei stained with DAPI. Labelling imaged using Zeiss LSM780 confocal microscope, YFP+ cells imaged with an EVOS light microscope. Scale bar, 20 µm.

Once isolated, OPC maintenance in culture is straightforward. Nonetheless, certain considerations should be kept in mind, for one, ensuring a proper PDL coating of the cell culture dish prior to plating. Poor PDL coating or failure to dry the plates sufficiently at this step can limit cell adherence to the cell culture substrate (Figure 3.4A). Moreover, while immunopanning is advantageous compared to other isolation techniques at yielding a relatively pure OPC population, contamination by non-oligodendrocyte lineage cell types may arise in culture and are

most often attributable to a high cell density, poor maintenance in pro-OPC lineage media conditions or inclusion of factors that promote the differentiation of astrocytes in the media (Figure 3.4B,C,D).

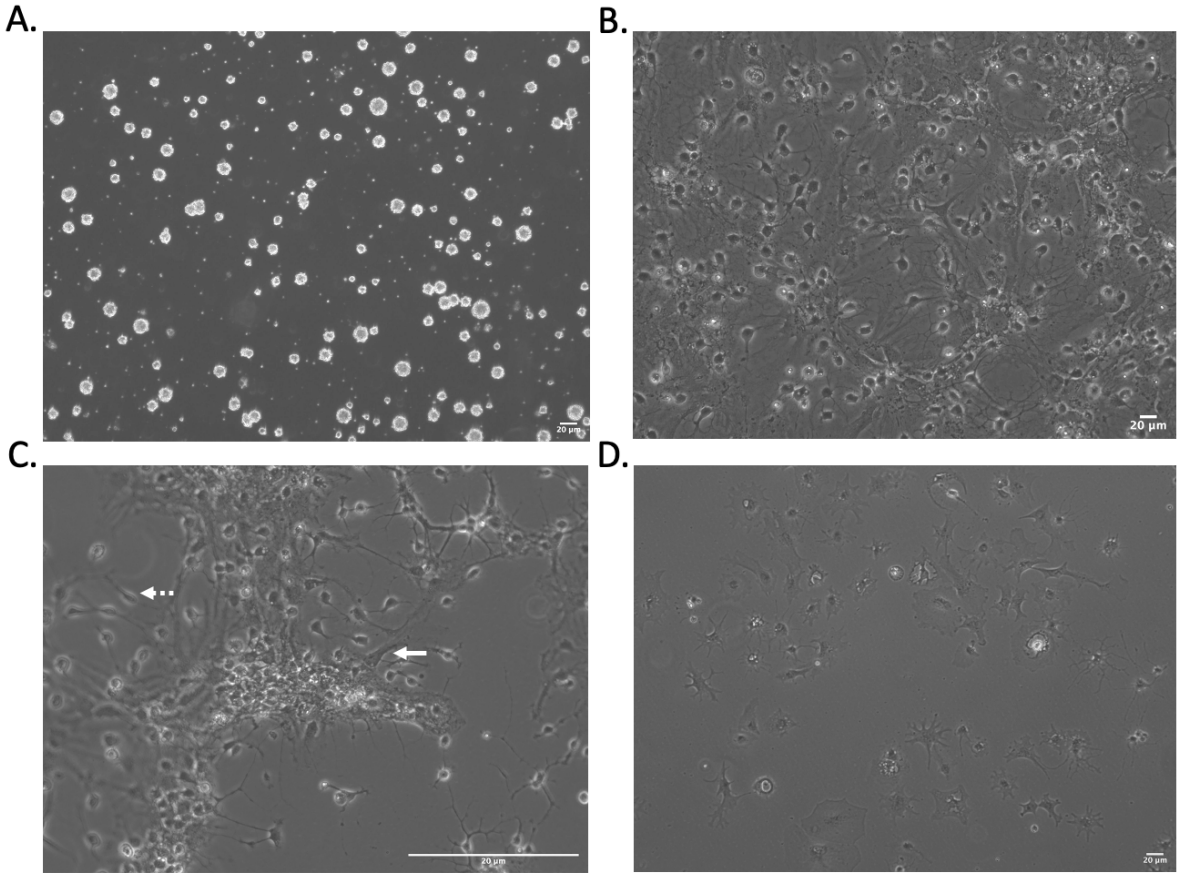

Figure 3.4: Maintenance of OPCs in culture and recognizing contaminating cell types A. PDL coating of cell culture dishes is critical for the adherence of OPCs. If the PDL coating is suboptimal, or cell culture substrates are not dried at this step, OPCs may fail to attach to the plates and subsequently form spheres, as seen here. B. In certain cases, contaminating cell types may be present in these cultures. In the case illustrated here, cells were maintained for 13 DIV under proliferative conditions but without passaging, and as a result became over-confluent at which point cells may differentiate into astrocyte-like cells. C. Here as well, contaminating cell types (white arrow) are seen amongst the OPCs (white dashed arrow), which are themselves easily recognizable as small, rounded cells. D. The media used to culture OPCs is serum-free. Inclusion of serums like Fetal Bovine Serum (FBS), such as was done in this example, directs cell fate towards the astrocyte lineage resulting in a mixed cell culture population. Scale bar, 20 µm.

Finally, as OPCs in culture can be subjected to various assays that recapitulate their development *in vivo*, an efficient method for isolating oligodendrocytes at the precursor stage provides a powerful tool for studying fundamental aspects of oligodendrocyte development. For example, OPCs in culture can be subjected to assays that assess their migration (e.g. Transwell insert migration assay; Figure 3.5A) and proliferation (e.g. Ki67 staining, EdU uptake experiment; Figure 3.5B). Moreover, upon differentiation, these cells provide a valuable tool to study myelination using organotypic slice culture techniques (Figure 3.5C) and nanofiber cell culture systems (Figure 3.5D).[14,15]

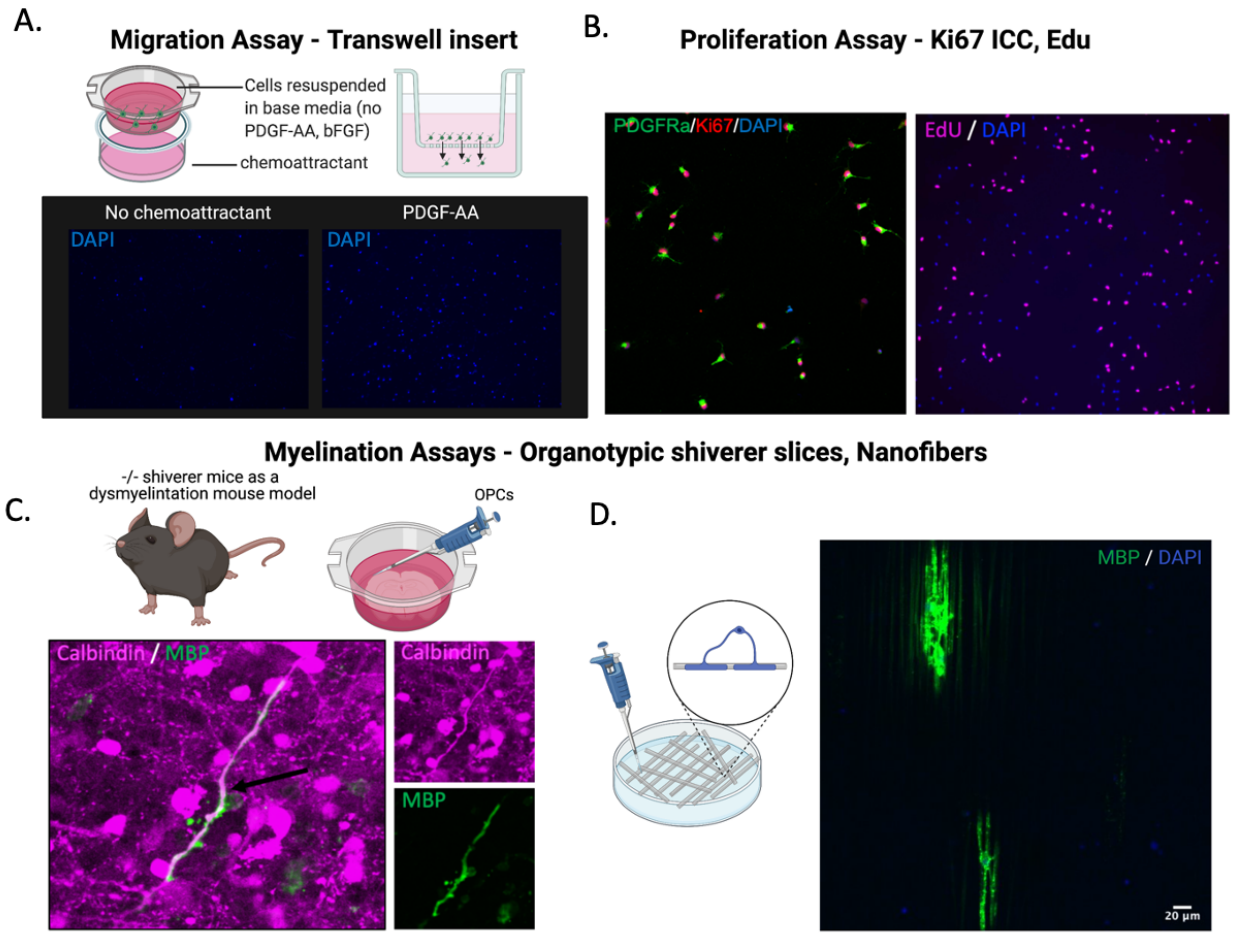

Figure 3.5: Isolation of PDGFRa+ OPCs enables the study of oligodendrocyte development. A. Transwell insert migration assay schematic and assessment of OPC migration in a control (no chemoattractant, intrinsic migration) versus chemoattractant (PDGF-AA, 20 ng/mL) driven

migration. B. Proliferation assays that can be utilized to assess OPC proliferation include Ki67 staining and EdU uptake experiments. Once differentiated, oligodendrocytes *in vitro* can be used to assess myelination. C. Schematic of transplantation of OPCs into organotypic shiverer slice and immunofluorescence image demonstrating co-localization of MBP and calbindin, suggestive of axon ensheathment by transplanted OPCs, in shiverer slices collected at three weeks. D. Immunofluorescence image of positive MBP labelling in a nanofiber 3D cell-culture myelination assay, collected at two weeks. Antibodies: Antibodies: sheep anti-KI67 (Novus, 1:200), rat anti-PDGFRα (BD, 1:100), rat anti-MBP (Novus, 1:500), rabbit anti-calbindin (Swant, 1:5000), AlexaFluor 488 donkey anti-rat (ThermoFisher, 1:1000), AlexaFluor 594 donkey anti-sheep (Jackson ImmunoResearch, 1:500), AlexaFluor 647 donkey anti-rabbit (Jackson ImmunoResearch, 1:500).

3.9 Discussion

In this study, we present an optimized immunopanning protocol for the isolation of PDGFRα+ oligodendrocyte precursor cells from mouse brain tissue. Despite the advent of numerous techniques to isolate oligodendrocyte-lineage cells from rat, for various reasons, many of these techniques have proven to be inefficient for isolation from mouse brain tissue. While the immunopanning technique first described by Emery & Dugas has been of great value, the relatively weak binding of OPCs to the anti-PDGFRα plate requires special considerations when isolating PDGFRα+ mouse OPCs.[1] As the isolation of OPCs in the early, PDGFRα+ stage allows for the expansion of these cells in culture and for the study of oligodendrocyte lineage development *in vitro*, here we present an immunopanning protocol optimized specifically for PDGFRα-dependent selection.

Our data emphasizes the importance of the papain digestion step as critical to obtain increased cell yields. We found that enhancing the carbogen perfusion via implementation of 3D-printed lids custom-designed for perfusion via gas inlets and outlets and humidifying the system to limit evaporation of the papain buffer on the hot plate improved the OPC yield dramatically. Moreover, the various other additions to the protocol have optimized a PDGFRα positive cell selection, without adding significant time to the protocol. Specifically, we noted that adding a

short spin following the papain digestion, in the presence of a low concentration of ovomucoid solution, minimized cell loss following enzymatic digestion. Additionally, two BSL 1 coated 10 cm dishes (10 mL of solution/plate) was sufficient for the negative selection panning step and halved the amount of reagents required at this step. Moreover, limiting the number of washes of the anti-PDGFRα coated positive selection plate prior to trypsinization was crucial to enhance the yield derived from PDGFRα cell selection. In early attempts, we found that using the full six washes resulted in far fewer cells on the panning dish at the trypsinization step, while attempting a "pour-off/pour-on" wash method led all cells to detach and zero yield. Importantly, the reduced number of washes did not decrease purity as our immunocytochemistry characterization supports the purity of our cultures. Still, maintaining cell density and growth factors within desired ranges is required to avoid contamination by undesired cell types which occasionally arise through differentiation. Once in culture, OPC maintenance is quite straightforward though various considerations are merited, discussed herein to facilitate reproducibility.

In summary, our modified immunopanning protocol for the isolation and culturing of PDGFRα+ oligodendrocytes from mouse brain tissue presented here enables a high yield of a pure population of OPCs, enabling the study of various stages of oligodendrocyte development *in vitro* and with potential applications to studies of transgenic mouse models and white matter diseases.

Statistical Analysis

All data analysis was performed using GraphPad Prism 9. Statistical analysis was performed using a Single sample t-test to compare our value (minimum of five independent experiments) to the

expected baseline value provided in the Emery & Dugas (2013) protocol.[1] Statistical significance was set at *p<0.05. Data shown as mean ± SEM.

EdU uptake experiment

As a means to assess proliferation, transfected OPCs were treated with Click-IT™ EdU Cell Proliferation Kit (ThermoFisher) for imaging as per manufacturer's protocol. Briefly, OPCs were seeded onto 15mm coverslips (EMS) in proliferation media with (20ng/mL) or without (10ng/mL) extra PDGF-AA supplementation. Forty-eight hours later, OPCs were treated with 10μM EdU solution and incubated for another 20 hours. Following the EdU incubation, cells were fixed (4% PFA in PBS, 10 mins, RT), washed (3% BSA in PBS, 2x 10 mins, RT), permeabilized (0.5% TX-100 in PBS, 20 mins, RT) and incubated with Click-IT™ reaction cocktail to detect EdU labelling (30 mins, RT). After washing, cells were stained with DAPI and mounted with Immu-Mount (ThermoFisher). Each coverslip was imaged in two randomly chosen 20x objective fields using an EVOS epifluorescent microscope. EdU and DAPI signal were counted using the analyze particles function on ImageJ software and the amount of EdU+ cells (as a percentage of DAPI) were averaged for each individual coverslip. Three independent experiments were performed for all conditions.

Animal work

C57BL/6 were obtained from Charles River Laboratory Canada. *Olig2-cre* mice were obtained from Jackson laboratories (#025567) and backcrossed onto a C57BL/6 background. R26-stop-YFP mice were a generous gift from Dr. William Richardson (University College London).[16] All procedures with animals were approved by the Animal Resource Division of the RI-MUHC

(protocol # 2018-8055) and performed in accordance with the Canadian Council for Animal Care Guidelines for animal use in research.

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Chapter 4: Insight into the pathophysiological mechanisms of hypomyelination in POLR3-related leukodystrophy

4.1 Preface

The cellular mechanisms underlying the hypomyelination seen in individuals with POLR3-related leukodystrophy are poorly characterized. Specifically, it is still unclear whether the hypomyelination seen arises from a defect in oligodendrocyte development at the level of their migration, proliferation, differentiation and/or myelination. This chapter utilizes the method optimized in Chapter 3 as the basis of a cellular model of POLR3-related leukodystrophy, to better delineate the role of Pol III in oligodendrocyte development. Included within is a manuscript that details results from the functional experiments used to comprehensively characterize oligodendrocyte development in the face of reduced Pol III activity. Macintosh, J. et al, intended for submission to *Frontiers in Cellular Neuroscience* upon completion of experiments.

4.2 Title Page

Decreased RNA polymerase III expression leads to defects in oligodendrocyte development

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Declaration of competing interests

The authors declare no competing interests.

4.3 Abstract

RNA polymerase III (Pol III) is a critical enzymatic complex tasked with the transcription of ubiquitous non-coding RNAs including 5S rRNA and all tRNA genes. Despite the constitutive nature of this enzyme, hypomorphic biallelic pathogenic variants in genes encoding subunits of Pol III lead to tissue-specific features and cause a hypomyelinating leukodystrophy, characterized by a severe and permanent deficit in myelin. The pathophysiological mechanisms in POLR3-related leukodystrophy and specifically, how reduced Pol III function impacts oligodendrocyte development to account for the devastating hypomyelination seen in disease, remain poorly

understood. In this study, we characterize how reducing endogenous transcript levels of leukodystrophy-associated Pol III subunits effects oligodendrocyte maturation at the level of their migration, proliferation, differentiation and myelination. Our results show that decreasing Pol III expression accelerated the proliferation of oligodendrocytes precursor cells but had no impact on migration. On the other hand, reducing Pol III activity impaired differentiation of these precursor cells into mature oligodendrocytes and concomitantly hindered their ability to myelinate. In turn, our findings provide insight into the role of Pol III in oligodendrocyte development and shed light on the pathophysiological mechanisms of hypomyelination in POLR3-related leukodystrophy.

4.4 Introduction

Hypomyelinating leukodystrophies encompass a group of genetically-determined white matter disorders in which there is a failure of central nervous system (CNS) myelination in development.[1,2] They present in previously healthy children and lead to progressive neurodegeneration and premature death.[1] While hypomyelinating disorders were historically thought to be due to defects in genes that encode structural myelin proteins, the canonical example being Pelizaeus-Merzbacher disease resulting from pathogenic variants in *PLP1,* the genetic etiology of these heterogenous disorders has since broadened. Indeed, more recent advances have identified a subset of hypomyelinating leukodystrophies that arise from defects of transcription and translation machinery, as in the case of RNA polymerase III-related hypomyelinating leukodystrophy (POLR3-related leukodystrophy).[2] Now known to be one of the most common hypomyelinating leukodystrophies, POLR3-related leukodystrophy arises from

biallelic pathogenic variants in genes that encode subunits of RNA polymerase III (Pol III), namely *POLR3A*, *POLR3B*, *POLR1C* and *POLR3K*.[3-7]

Pol III is a 17-subunit enzymatic complex responsible for the transcription of ubiquitous and essential RNAs, including 5S rRNA, all tRNA genes and a variety of functionally distinct non-coding RNAs (ncRNAs) such as 7SL, which serves as a scaffold for the signal recognition particle and U6, a component of the spliceosome.[8] Pathogenic variants in genes associated with POLR3-related leukodystrophy are diverse and include missense, nonsense, small insertions or deletions, exonic or intronic splice site variants and large exonic deletions.[3-5,9-14] These variants are believed to share a common hypomorphic outcome. Indeed, mapping pathogenic variants to the human Pol III structure has revealed clustering of variants at subunit interfaces, predicted to affect biogenesis and/or stability of the polymerase.[15,16] Moreover, pathogenic variants have also been shown to impair nuclear import of the complex or impede its ability to bind DNA for transcription, while functional work has shown reduced protein expression of the affected Pol III subunit in fibroblasts and brain lysates in affected individuals, further supporting a hypomorphic nature of Pol III in a diseased state.[3,5] Notably, the absence of any patients with two null alleles suggests that some residual function of the polymerase is required for life.

Despite advances in the genetic etiology of these disorders, the pathophysiological mechanisms remain enigmatic. POLR3-related leukodystrophy is known to have a spectrum of disease severity, ranging from a very severe form that presents in the first few months of life and lead to progressive decline and premature death, to a mild form diagnosed incidentally on magnetic resonance imaging (MRI).[11,17,18] While the constitutive housekeeping role of Pol III would suggest a multi-system disease arises from its dysfunction, POLR3-related leukodystrophy

presents with characteristic neurological and non-neurological manifestations. Neurological features, stemming from hypomyelination, often present as motor delays or regression and can include cerebellar (e.g. ataxia, dysphagia, dysmetria), pyramidal (e.g. spasticity) and extrapyramidal (e.g. dystonia) features.[11,14,19,20] Variable cognitive involvement is also seen and may involve learning difficulties, intellectual disabilities and/or cognitive regression.[11,14] Meanwhile, non-neurological features include abnormal dentition, myopia and endocrine abnormalities, such as hypogonadotropic hypogonadism with absent, delayed or arrested puberty.[11,14,21] Diagnosis of POLR3-related leukodystrophy is therefore based on the presentation of these clinical findings, hypomyelination on MRI with a characteristic pattern, and genetic testing to confirm the molecular basis.[22]

Associated with the most debilitating aspect of disease, the hypomyelination seen in patients remains especially intriguing. Myelin, produced by oligodendrocytes (OLs) in the CNS, serves a crucial function in protecting neurons and enabling saltatory conduction for efficient propagation of action potentials.[23] Previous work on the histopathology of POLR3-related leukodystrophy support a primary involvement of OLs in the disease pathogenesis.[11,24] OLs undergo a complex developmental process to become myelinating cells, beginning with an oligodendrocyte precursor cell (OPC) migrating significant distances to reach its final site of myelination.[25] Having reached its destination, an OPC will proliferate until reaching a homeostatically regulated critical threshold.[26] Eventually becoming post-mitotic, an immature oligodendrocyte will then undergo drastic increases in morphological complexity as it differentiates to enable contact with numerous axons for subsequent myelination.[25]

Research into the underlying mechanism of hypomyelination in POLR3-related leukodystrophy is ongoing, with various prior attempts at modelling disease having proven to be challenging.[27,28] Recently, progress has been made in modelling POLR3-related leukodystrophy in mice using a conditional knock-in of two *Polr3a* hypomorphic variants present in patients and which were elucidated as pathogenic in combination via yeast studies.[29] This mouse was successful in exhibiting a myelin phenotype and recapitulating certain aspects of disease.[29] Intriguingly, flow cytometry analysis of these mice revealed a reduction in mature, MOG+ oligodendrocytes which the authors proposed could be due to a defect in oligodendrogenesis.[29] As a detailed study of oligodendrocyte maturation in model organisms is complicated by the spatiotemporal complexity of OL-lineage development *in vivo*, we sought to characterize how reduced Pol III impacts oligodendrocyte development *in vitro*.

In this study, we downregulated endogenous transcript levels of leukodystrophy-associated Pol III genes, namely *Polr3a, Polr3b* and *Polr1c*, using small interfering RNAs (siRNA) in primary mouse OL-lineage cells in order to model the hypomorphic effect of variants in a cell-type predicted to be dysfunctional in disease. We then subjected these cells to developmental processes that recapitulate their development *in vivo* (e.g. migration, proliferation, differentiation and myelination) in order to shed light on a role for Pol III in oligodendrocyte maturation and on POLR3-related pathophysiology.

4.5 Methods

Animals

C57BL/6 mice were obtained from Charles River Laboratory Canada. All procedures with animals were approved by the Animal Resource Division of the RI-MUHC (protocol #2018-8055) and

performed in accordance with the Canadian Council for Animal Care Guidelines for animal use in research.

Mouse brain primary OPC cultures

OPCs were isolated from P6 wild-type (C57BL/6) mice of either sex using a modified version of the previously described immunopanning protocol.[41] Briefly, mice were cryo-anesthetized and brains dissected before dissociation in papain, L-cysteine and DNase I for 90mins at 37˚C with perfusion of 95% O_2/5% CO_2 gas. Post-dissociation, the digested brain tissue was pelleted before gently triturating in 10x ovomucoid solution to form a single-cell suspension. Cells were spun down (220g, 15min, RT) and resuspended in 6x ovomucoid, followed by resuspension in immunopanning buffer (D-PBS supplemented with 0.2% BSA and 0.5 mg/mL insulin). The resulting cell suspension was filtered and sequentially passed onto 2x10cm Petri dishes coated with bandeiraea simplicifolia lectin (15min, RT) to which microglia and endothelial cells adhere. The residual cell suspension was then passaged onto a positive selection plate coated with an anti-PDGFRα antibody (45 mins, RT). The plate was rinsed with D-PBS and the adhered cells were dissociated with trypsin to be spun down. OPC pellets were resuspended in serum-free OPC proliferation media (DMEM with 2mM glutamine, 1mM sodium pyruvate, 5ug/mL insulin, 5ug/mL N-acetyl-L-cysteine, 1X trace elements B, penicillin-streptomycin, 10ng/mL biotin, 10mg/mL BSA, 10mg/mL transferrin, 1.6mg/mL putrescine, 6ug/mL progesterone, 4ug/mL sodium selenite, 4.2ug/mL forskolin, 1X B-27, 10ng/mL PDGF-AA and 10ng/mL bFGF) and seeded onto poly-D-lysine coated 10cm plates. OPCs were maintained in a 10% CO_2 incubator and by supplementing with PDGF-AA (10ng/mL) at 2 DIV followed by half media changes every 2 days

starting at DIV 4. For experimental objectives involving differentiation, cells were passaged and plated in differentiation media (+T3, 40 ng/mL, -PDGF-AA, -bFGF).

OPC/OL siRNA transfection

Primary OPCs were passaged at 7DIV into media (+PFGF-AA/bFGF or +T3/-PDGF-AA/-bFGF, depending on experimental objectives) containing Lipofectamine® RNAiMax transfection reagent (Invitrogen) and 15 pM siRNA (Dhamarcon siRNA SMARTPool: *Polr3a, Polr3b,* or *Polr1c*) prepared following manufacturer's instructions at an siRNA to Lipofectamine ratio of 1:1. The level of knockdown was assessed through RT-qPCR using the ΔΔCt method with three reference genes (*18s, Gusb, Pgk1*). A non-targeting siRNA (siGENOME siRNA pool #2) was used as a negative control in respective experiments.

Immunofluorescence

Cells (OPCs/OLs) were fixed in 4% paraformaldehyde. After washing, cells were blocked in blocking buffer (PBS with 5% BSA, 0.2% Triton X-100) for 1 hour at RT. Samples were then washed and incubated with primary antibodies (Supplemental Table S4.1) in blocking buffer for 2 hours at RT. After washes, cells were incubated for 2hrs at RT with DAPI and corresponding secondary antibodies. For Ki67 staining, OPCs were transfected at 7 DIV and fixed 48hrs later for staining of Ki67 and respective OL-lineage markers. For staining of slice cultures, slices were fixed in 4% PFA (1 hr, ice). Following washes, slices were treated with 5% glacial acetic acid (20 mins) and washed. Slices were blocked in blocking buffer (3% horse serum, 2% BSA, 0.25% TX-100 and 0.1% sodium azide; 2 hrs, RT) before incubation with primary antibodies in primary incubation buffer (2% BSA. 0.25% TX-100, 0.1% sodium azide) for 20 hours at RT. Slices were washed and incubated with respective secondary antibodies (overnight, RT). Following washes, slices were mounted with

Immu-Mount. All stainings were imaged using a EVOS Epifluorescent microscope or Zeiss LSM780 laser scanning confocal microscope and processed using ImageJ or ZEN microscope software.

RNA extraction and reverse transcription quantitative polymerase chain reaction

Cells (OPCs/OLs) were lysed in TRIzol reagent (ThermoFisher) and total RNA extracted using a miRNeasy kit (QIAGEN), following manufacturer's instructions and with on-column DNase I treatment (Qiagen). For the first few extractions, RNA was run on a bleach gel to confirm quality and effectiveness of the DNase I treatment in removing genomic DNA. RNA concentration and quality was assessed on a ND-1000 Nanodrop spectrophotometer. For each sample, 500 ng of RNA was reverse transcribed with M-MLV reverse transcriptase (Promega) and a 1:1 mix of Oligo(d)T (ThermoFisher) and random hexamer primers (IDT). Primers were designed to span exon-exon junctions using the Primer-Blast NCBI and OligoAnalyzer IDT software (Supplemental Table S4.2). All primers were validated using cDNA derived from mouse brain tissue to meet MIQE guidelines.[64,65] cDNA was amplified using a Roche LightCycler® 96 instrument using the following parameters: pre-incubation (95˚C for 180s), 3-step amplification (95˚C for 10s, 60˚C for 30s, 72˚C for 30s; 45 cycles) and melting (95˚C for 10s, 65˚C for 60s, 97˚C for 1s) and expression assessed using SsoAdvanced Universal Sybr green supermix (Bio-Rad Laboratories). Gene expression of each biological replicate was analyzed in technical triplicates using the ΔΔCt method with a 1:40 cDNA dilution and normalized to three reference genes (*18s*, *Gusb* and *Pgk1*). Expression was presented as relative to the negative control condition.

Transwell insert migration assay

OPCs were resuspended in base media (OPC media -PDGF-AA/-bFGF) at a density of 1.25 x 10^5 cells/mL and 100µL was seeded into the upper chamber of a poly-D-lysine coated 8 µm pore

polycarbonate transwell insert (Corning™ Transwell™ Multiple Well Plate with Permeable Polycarbonate Membrane Inserts, Fisher) similarly to previously described protocols.[66,67] The insert was placed over a well of a 24-well tissue culture plate containing 600µL of base media and 20 ng/mL PDGF-AA. OPCs were left to migrate through pores in the insert for 16 hours at 37˚C. A cotton swab was used to remove non-migrating OPCs remaining in the upper chamber, before fixing cells that had migrated to the lower chamber in 4% PFA (30 minutes, RT). Following rinses with PBS, nuclei were stained with DAPI. For each transwell insert, 3-6 images were taken of a random 4X objective field on an epifluorescent microscope. For quantitative assessment, cells were counted using the analyze particles function on ImageJ software, with cell counts from each individual insert averaged. Three independent experiments were performed for all conditions.

OPC EdU uptake experiment

As a means to assess OPC proliferation, transfected OPCs were treated with Click-IT^{TM} EdU Cell Proliferation Kit (ThermoFisher) for imaging as per manufacturer's protocol. Briefly, OPCs were seeded onto 15mm coverslips (EMS) and transfected in proliferation media. Forty-eight hours post-transfection, OPCs were treated with 10µM EdU solution and incubated for another 20 hours. Following the EdU incubation, cells were fixed (4% PFA in PBS, 10 mins, RT), washed (3% BSA in PBS, 2x 10 mins, RT), permeabilized (0.5% TX-100 in PBS, 20 mins, RT) and incubated with Click-IT^{TM} reaction cocktail to detect EdU labelling (30 mins, RT). After washing, cells were stained with DAPI and mounted with Immu-Mount (ThermoFisher)**.** Each coverslip was imaged in 5 randomly chosen 20x objective fields using an epifluorescent microscope. EdU and DAPI signal were counted using the analyze particles function on ImageJ software and the amount of EdU+

cells (as a percentage of DAPI) were averaged for each individual coverslip. Five independent experiments were performed for all conditions.

Sholl Analysis

As a measure of morphological complexity, OPCs transfected with siRNA (control or Pol III targeting) and passaged into T3-containing differentiation media were fixed 3 DIV post-transfection and stained for MOG. Staining was imaged using a Zeiss LSM780 laser scanning confocal microscope and process branching complexity was quantified using the concentric ring analysis Plugin on ImageJ for Sholl analysis.[68] As an overview, a series of concentric rings were superimposed on the cell, starting at the soma (parameters: inner radius 20uM, outer radius 140uM, step size 10μM), and the number of intersections at each ring was determined. Data from four independent experiments for each condition were used, in which 5-8 cells were analyzed per coverslip.

ReadyProbe live/dead assay

To look at cell death, cells were treated with a cell-permeable NucBlue® live reagent (Hoescht 33342) and cell-impermeable NucGreen® dead reagent using the ReadyProbe assay kit (ReadyProbes™ Cell Viability Imaging Kit, Blue/Green). Fluorescence signal was imaged using a DAPI and GFP filter on the EVOS epifluorescent microscope and cell counts quantified using the analyze particles function on ImageJ. Percentage of cell death was determined by # of dead (GFP+) cells / # of dead (GFP+) + live (blue) cells. Data is presented as fold-change of the control condition.

Genotyping Shiverer mice

Tails were taken from mice prior to dissecting cerebellums and DNA extracted using the Extracta DNA prep for PCR (QuantaBio) according to manufacturer's instructions. Shiverer mice were genotyped with a multiplex PCR using the following primers: WT band flanking exon 6 (deleted in *Shi*/*Shi* mice) [5' - AGCTCTGGTCTTTCTTGCAG – 3' and 5' – CCCCGTGGTAGGAATATTACATAAC – 3'] and primers complementary to the shiverer breakpoint [5' – CAGGGGATGGGGAGTCAGAAGTGAG – 3' and 5' – ATGTATGTGTGTGTGTGCTTATCTAGTGTA – 3']. PCR was performed with Taq polymerase (Promega) and the cycling conditions were as follows: 3 mins at 94˚C, 35 cycles of 45 sec at 94˚C, 45 sec at 60˚C, 1 min at 95˚C and 10 mins at 72˚C. For genotype analysis, PCR products were separated using agarose gel electrophoresis (2% agarose gel; 120V for 30mins) and imaged using a Doc EZ System (BioRad).

Shiverer organotypic slice cultures

Cerebellar slices from shiverer mice were cultured and transplanted with OPCs, as previously described.[69] Briefly, shiverer mice at P0 were cryo-anesthetized and cerebellums dissected and sectioned into 300uM sagittal sections with a McIlwain tissue chopper. Slices were transferred onto Millicell inserts (MilliporeSigma™ Millicell™ Culture Plate Inserts, Fisher) in serum-containing media (MEM with Earle's salts, EBSS, heat-inactivated horse serum, 1X Glutamax, 1X penicillin-streptomycin, 6.25g/mL glucose, 0.5ug/mL Fungizone). Slices were maintained with media changes every 2 days that progressively replaced serum-containing media with serum-free media (DMEM/F-12, 1% GlutaMAX, 1X B-27, 1X N2, 1X penicillin-streptomycin, 0.5ug/mL Fungizone). At 21 DIV, purified siRNA-treated OPCs (Ctrl siRNA, siPolr3a, siPolr3b, siPolr1c) were injected into the slice (20 000 OPCs/slice in 2x 0.5 µL injections). Slices were maintained in serum-

free media for a further 3-6 weeks, at which time slices were fixed (4% PFA, 1hr on ice) and stained using methods described under immunofluorescence.

Statistical Analysis

All data analysis was performed using GraphPad Prism 9. Quantifications were performed from at least three independent experiments. Statistical analysis was performed using a Student's t-test to compare between two groups. Statistical significance was set at *p<0.05. Data shown as mean $\pm$ SEM.

4.6 Results

Decreasing Pol III subunit expression in OPCs accelerates their proliferation

To study the role of Pol III in a precursor stage of oligodendrocyte development, we transfected OPCs with siRNA targeting *Polr3a, Polr3b* or *Polr1c* to downregulate endogenous expression of Pol III genes (by ~60%). Migration of OPCs during development is required for the cells to reach their axon targets, a failure in which could result in a myelin deficit.[25] As such, we assessed whether downregulation of disease-associated Pol III subunits impacts migration of OPCs by subjecting the cells to a transwell insert migration assay (Figure 4.1A). We found the number of migrated cells under PDGF-AA chemoattractant-driven conditions comparable between the treatment and control conditions, suggesting migration was unaffected in the context of a Pol III downregulation (Figure 4.1B; siPolr3a*:* p = 0.808, siPolr3b: p = 0.847, siPolr1c: p = 0.992). We next assessed the role of Pol III in the precursor stage of the lineage, at the level of their proliferation, by staining for the proliferation marker Ki67, a nuclear antigen expressed in G1, S, G2 and M phases of the cell cycle.[30] Triple immunolabelling of OPCs in proliferation media for Ki67, PDGFRα and NG2 showed that Pol III siRNA-treated and control cells were

likewise positive for Ki67 (Figure 4.1C). When we quantified the proportion of PDGFRα+ or NG2+ cells (OPC markers) that were Ki67+, there was no statistically significant difference between the treatment and control conditions, with all PDGFRα+ and NG2+ cells positive for Ki67 (Figure 4.1D). As Ki67 immunofluorescence is a nonspecific marker of proliferation, we then performed an EdU uptake experiment to look directly at actively dividing cells and to further assess the possibility of altered OPC proliferation in the context of decreased Pol III expression. An EdU uptake experiment suggested there were more EdU+ cells in the Pol III siRNA-treatment conditions (Figure 4.1E). Indeed, when we quantified the proportion of EdU+ cells, we found a significantly increased proliferating cell population in the Pol III siRNA-treated conditions relative to the control (Figure 4.1F; EdU+ siPolr3a: $p = 0.01$, siPolr3b*:* $p = 0.01$, siPolr1c*:* $p = 0.008$). Overall, these findings indicate that decreasing Pol III expression during the precursor stage of the OL-lineage has no effect on migration but accelerates the proliferation of OPCs.

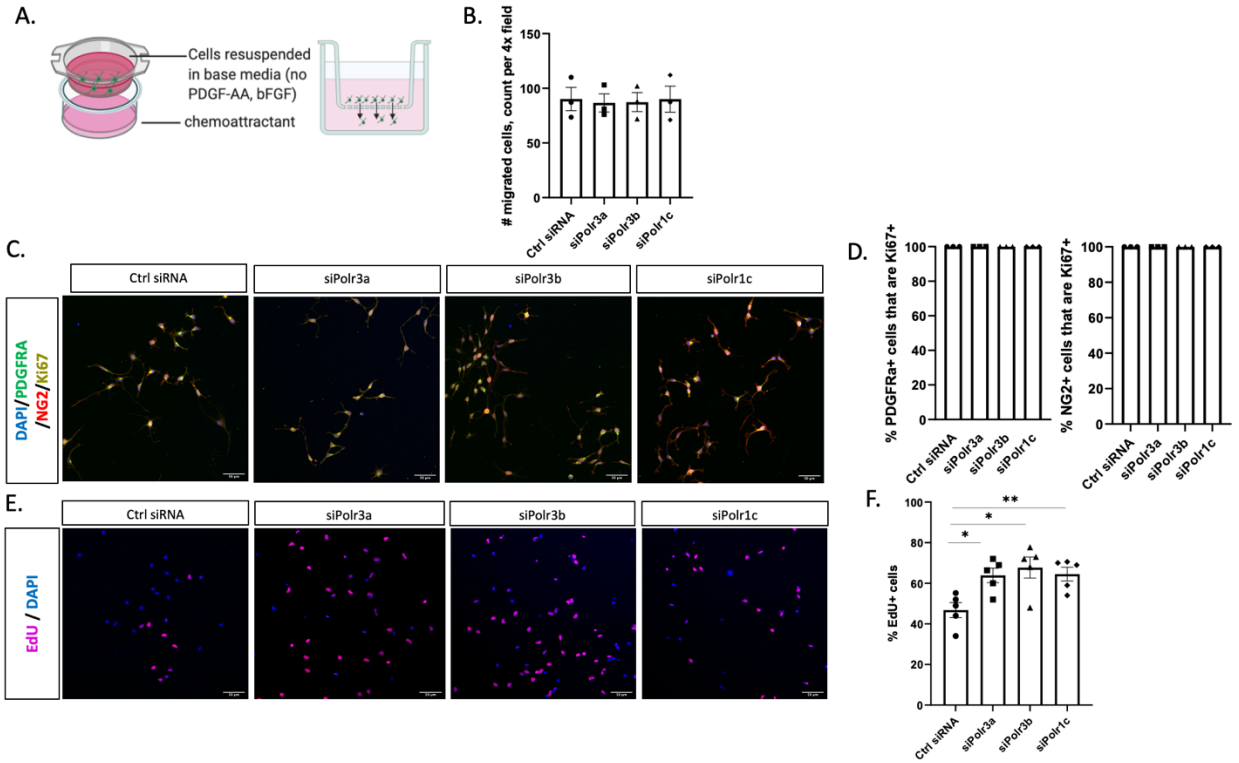

Figure 4.1: Decreased expression of leukodystrophy-associated Pol III subunits accelerates OPC proliferation but does not affect migration. A. Schematic of the transwell insert migration assay. B. Quantification of siRNA-treated OPC migration, as determined by cell count in a randomly imaged 4x field. Data represents mean ± SEM from three independent experiments (each presented as an average of 3-6 technical replicates). C. Representative immunofluorescence images (from three independent experiments with similar results) of siRNA-treated OPCs in proliferation media, showing PDGFRα (green), NG2 (red) and Ki67 (yellow). Nuclei stained with DAPI. Scale bar, 50 μm. D. Quantification of the % PDGFRα+ (left) and NG2+ (right) cells that are Ki67+ from three independent experiments. E. Representative immunofluorescence images (from five independent experiments with similar results) of siRNA-treated OPCs labelled with EdU. Nuclei stained with DAPI. Scale bar, 50 μm. F. Quantification of EdU+ cells as a percentage of DAPI following siRNA-treatment. Data represents mean ± SEM from five independent experiments (each presented as an average of 5 technical replicates), *p<0.05, **p<0.01, unpaired, two-sided Student's t-test.

Pol III is required for differentiation of OPCs into mature OLs

To characterize the role of Pol III in OL differentiation, we performed RT-qPCR analysis on RNA collected from cells after three days in differentiation media. Analysis of myelin markers demonstrated Pol III subunit downregulation (siPolr3b and siPolr1c (preliminary), siPolr3a in progress) significantly decreased the expression of these transcripts, including *Mbp, Mog* and *Cnp* (Figure 4.2A: *Mbp* - siPolr3b: p= 0.025,; *Mog* - siPolr3b: p= 0.001,; *Cnp* - siPolr3b: p= 0.007). A reduction in myelin transcripts could be due to a decrease in the production of myelin or a defect in differentiation, in which case the cells could be too immature to produce transcripts enriched in late-stage oligodendrocyte. Following the idea of a maturation block, we next looked at the expression of developmental markers enriched at the immature oligodendrocyte stage. Specifically, we performed RT-qPCR for *Fyn*, *Nfasc* and *Kif19a*, transcripts involved in cytoskeletal activity, organizing the nodes of Ranvier and microtubule binding activity, respectively.[31-34] RT-qPCR analysis demonstrated expression of immature OL transcripts were likewise decreased in the context of a *Polr3b* downregulation, though not all significantly (Figure 4.2B: *Fyn* - siPolr3b: p= 0.704,; *Nfasc* - siPolr3b: p= 0.077,; *Kif19a* - siPolr3b: p= 0.004). In comparison, there was a

slight increase in expression of early OPC markers *Pdgfrα* and *Cspg4* (which encodes NG2) in the Pol III siRNA-treated cells under differentiation conditions (Figure 4.2C: *Pdgfrα* -siPolr3b: p = 0.207; *Cspg4* - siPolr3b: p= 0.0164). While still preliminary, these findings appear consistent between the siPolr3b and siPolr1c conditions, perhaps emphasizing the role of each subunit as impactful to the function of the entire enzymatic complex. Overall, these results suggest that decreasing Pol III subunit expression in OPCs induced to differentiate impairs their expression of late-stage OL transcript, suggesting a potential impairment of OL maturation.

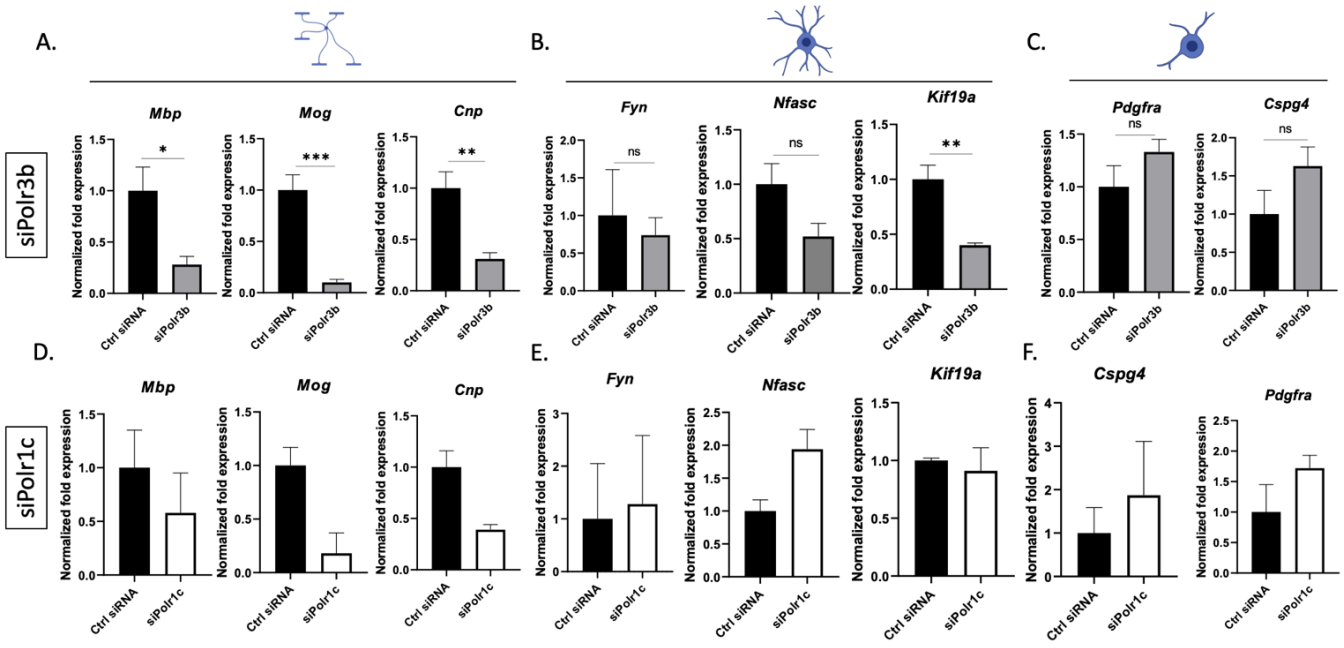

Figure 4.2: Reducing expression of Pol III subunits impairs differentiation of OPCs, as determined by expression of OL-lineage transcripts. A-C. RT-qPCR analysis of RNA derived from siPolr3b OLs passaged into T3-containing differentiation media and collected at 3 DIV, demonstrating A. decreased expression of late-stage OL transcripts *Mbp, Mog* and *Cnp,* B. decreased expression of the immature OL transcript *Kif19a* and a non-significant decrease in *Fyn* and *Nfasc* C. increased expression of early-stage OPC markers *Pdgfrα* and *Cspg4,* though not significantly. D-F. preliminary siPolr1c work shows findings similar to siPolr3b (siPolr3a work in progress). Data represents mean ± SEM normalized fold expression of siPolr3b (n=4) or siPolr1c (n=2, thus far) after normalizing to reference genes *18s, Gusb* and *Pgk1.* *p < 0.05, **p < 0.01, *** p < 0.001, ns: not significant, unpaired, two-sided Student's t-test.

Reduced Pol III expression decreases branching complexity of OLs

Higher-order branching of OLs is a requirement for the cell to function in myelinating numerous axons.[25] Given our RT-qPCR findings of reduced expression of late-stage OL transcripts, we next performed immunocytochemistry analysis to evaluate the morphological complexity of these cells and further assess the possibility of impaired differentiation. Immunofluorescence staining for MOG under differentiation conditions revealed the Pol III siRNA-treated cells retained a more immature branching network than their control-transfected counterparts (Figure 4.3A). To quantify a difference in morphological complexity, we evaluated process branching using Sholl analysis (Figure 4.3B). This revealed the Pol III siRNA-treated cells had a less elaborate network of processes than the control cells (Figure 4.3C). This difference could be seen at the level of primary processes, the processes that protrude directly from the soma, in the siPolr3a and siPolr3b conditions (Figure 4.3C; 20µm ring – siPolr3a*:* p = 0.024*,* siPolr3b*:* p = 0.04*,* siPolr1c*:* p = 0.09) but was most substantial at the level of process branching (30+ µm from soma) (Figure 4.3C). While the control cells demonstrated an intricate branching complexity, the Pol III siRNA-treated cells showed, on average, significantly fewer intersections at each concentric ring (Figure 4.3C). Additionally, at the most distant concentric ring, superimposed 140 µm from the soma, the control condition had significantly more intersections than the Pol III conditions (Figure 4.3C; 140µm ring – siPolr3a*:* p = 0.038*,* siPolr3b*:* p = 0.0009, siPolr1c*:* p = 0.0006). This suggested that in addition to having a more immature branching complexity, the Pol III siRNA-treated OLs had fewer long processes, or that the processes were on average shorter, further supporting an immature morphology. Overall, these results reinforce a defect in oligodendrocyte development when Pol III function is decreased.

Oligodendrocyte differentiation is known to be regulated via a bottleneck process, with a substantial proportion of immature OLs lost to apoptosis during development.[35] As such, we sought to determine whether impaired differentiation in the Pol III siRNA-treated cells subjected them to apoptosis. Immunofluorescence staining of Pol III siRNA-treated OLs after three days in differentiation conditions showed they did not stain for cleaved-caspase 3, a marker of apoptosis (Figure 4.3D). To further assess the possibility of cell death in these cells, we treated cells with NucBlue™ live reagent (Hoechst 33342) and NucGreen™ dead reagent as part of a ReadyProbe assay to determine the percentage of cell death. While some cell death was seen in all conditions, this data suggested cell death was comparable in the treatment and control conditions (Figure 4.3E; *Polr3b*: p = 0.5, *Polr3a*: p = 0.8, *Polr1c*: p = 0.5). Taken together, these findings support that reducing Pol III subunits expression impairs differentiation of OLs, with cells retaining a more immature morphology, but that these cells nonetheless remain alive.

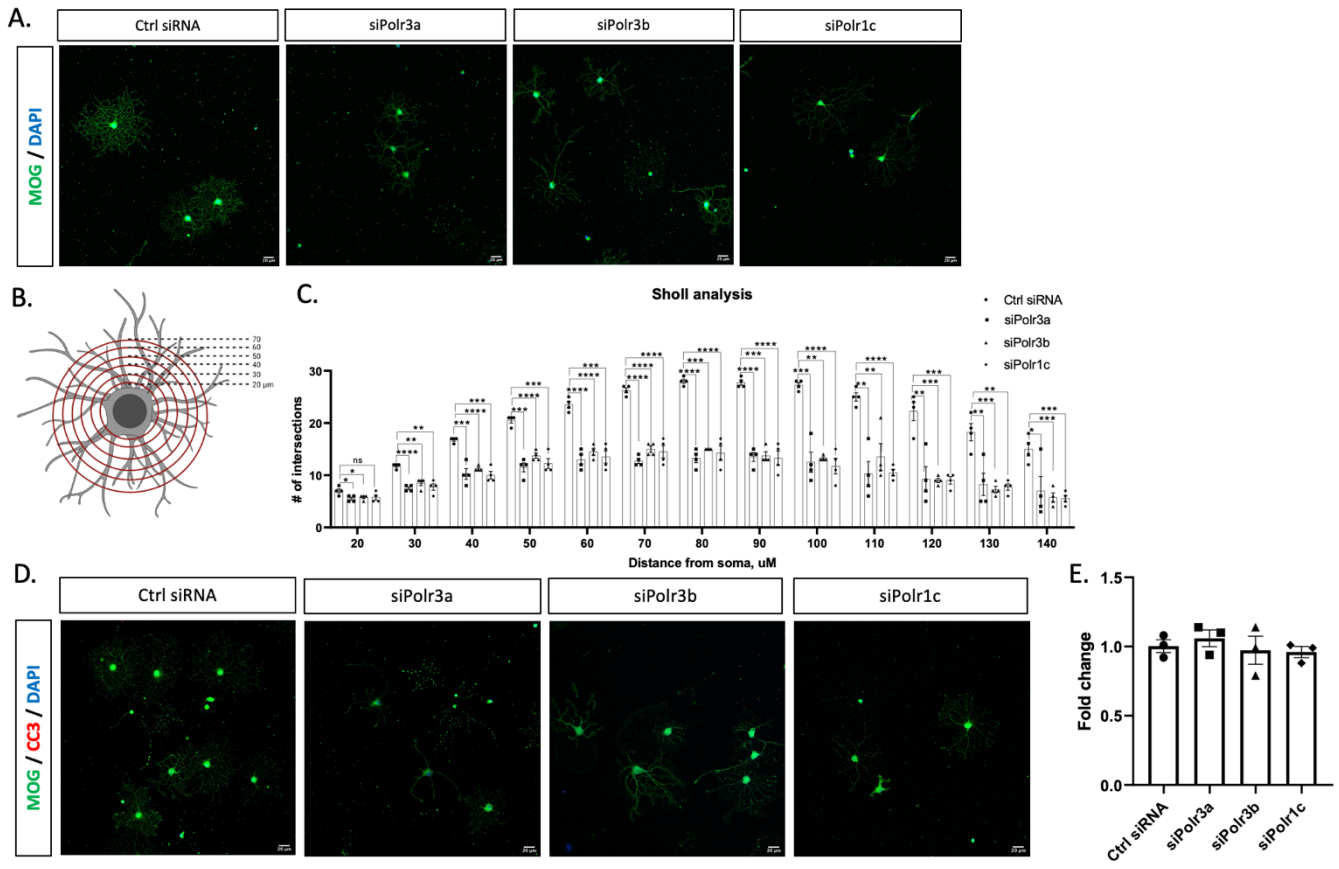

Figure 4.3: Reduced morphological complexity of cells with decreased expression of Pol III subunits. A. Representative immunofluorescence images (from four independent experiments with similar results) of siRNA-treated OLs in differentiation conditions, showing MOG+ cells (green). Nuclei stained with DAPI. Scale bar, 20 µm. B. Schematic of Sholl analysis, in which a series of concentric rings are super-imposed on the cell as a measure of process networks (parameters - inner radius 20µM, outer radius 140µM, step size 10uM). C. Quantification of Sholl analysis, depicted as mean ± SEM number of intersections at each ring from four independent experiments (each presented as an average of 5-8 technical replicates). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. D. Representative immunofluorescence images (from three independent experiments with similar results) of siRNA-treated OLs in differentiation conditions, showing MOG+ cells (green) stained for CC3 (red). Nuclei stained with DAPI. Scale bar, 20 µm. E. Percent cell death as determined by ReadyProbe assay. Data presented as fold change of control condition, graphed as mean ± SEM.

Decreased levels of Pol III subunits impairs myelination

Considering the findings of impaired differentiation, we next looked at how a Pol III deficit might impact the ability of oligodendrocytes to myelinate. To assess myelination in an *ex vivo*

micro-environment, we generated organotypic slice cultures from the homozygous Shiverer mouse, a naturally occurring *Mbp* mutant that produces no myelin.[36] We transplanted siRNA-treated OPCs into the slice cultures and fixed slices at 3- and 6-week timepoints to assess their myelination capacity, as inferred by MBP signal. Immunofluorescence staining for MBP at 3-weeks revealed fewer MBP+ regions in the slices with Pol III siRNA-treated cells, as compared to the condition with control cells (Figure 4.4). Moreover, this finding of less MBP+ regions was consistent at the 6-week timepoint, suggesting the impaired myelination state persisted.

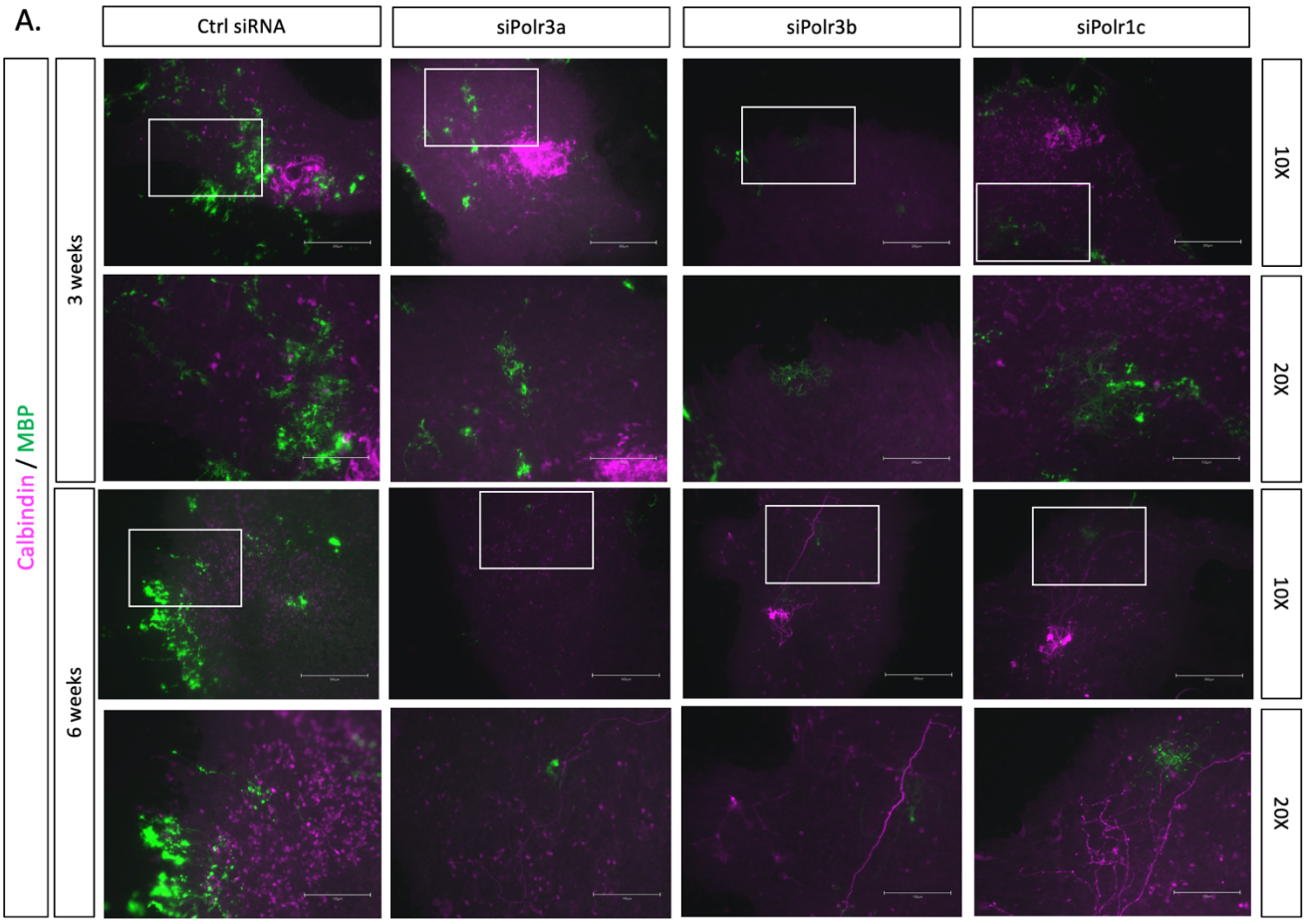

Figure 4.4: Reducing Pol III expression in OLs impairs myelination. Representative immunofluorescence images (from one independent experiment, in progress) of *Shi/Shi* organotypic slices transplanted with siRNA-treated OPCs (Ctrl siRNA, siPolr3a, siPolr3b, siPolr1c)

showing calbindin (magenta) and MBP (green) and collected at 3- or 6-weeks. Scale bar, 300µm (10X), 150 µm (20X).

Assessment of Pol III transcription in a defective oligodendrogenesis state

RNA polymerase III transcripts can be split into three categories based on their promoter type and basal transcription machinery used.[8] Type 1 transcripts include 5S RNA exclusively while type 2 transcripts include tRNA genes, with some ncRNAs like 7SL using a hybrid type 2 promoter, with upstream regulatory elements, also included in this category as well. Finally, type 3 promoters, which are unique in their use of an upstream promoter include ncRNAs such as U6, a component of the spliceosome.[8] To assess what might be occurring at the level of Pol III activity in the cells which demonstrated defective differentiation, we performed RT-qPCR analysis of Pol III transcripts. Specifically, as a proxy for each promoter type, we assessed the expression levels of *5s, 7sl* and *U6* RNA following Pol III subunit downregulation (siPolr3b and siPolr1c (preliminary), siPolr3a in progress). We observed no differences in the expression of any of these transcripts (Figure 4.5; *5s* – siPolr3b: $p = 0.567$, *7sl* – siPolr3b: $p = 0.965$, *U6* – siPolr3b: $p = 0.973$). Thus, our results suggest that the impaired differentiation seen is unlikely to be explained by altered *5s* rRNA, *7sl* or *U6* function. Rather, it is probable there are other Pol III transcripts, such as tRNAs or ncRNAs, underpinning the defects seen here.

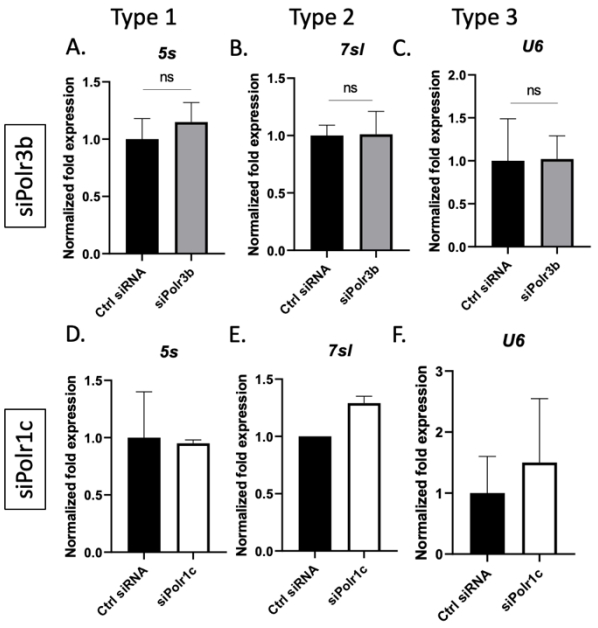

Figure 4.5: Transcriptional impact of decreasing Pol III expression in differentiated OLs. RT-qPCR analysis of RNA derived from siPolr3b-treated OLs passaged into T3-containing differentiation media and collected at 3 DIV demonstrated no change in expression of Pol III transcripts *5s* (A)*, 7sl* (B) or *U6* (C). D-F. Preliminary siPolr1c work shows findings similar to siPolr3b (siPolr3a work in progress). Data represents mean ± SEM normalized fold expression of siPolr3b (n=4) or siPolr1c (n=2) after normalizing to reference genes *18s, Gusb* and *Pgk1.* Unpaired, two-sided Student's t-test.

4.7 Discussion

RNA polymerase III-related leukodystrophy is a devastating neurodegenerative condition. A failure in myelin deposition would suggest the involvement of OLs in a diseased state. Nonetheless, where a defect might arise during the complex development of oligodendrocytes remains poorly understood. As such, it is unclear whether the hypomyelination seen in patients arises from a failure of OPCs to migrate to sites of myelination, to proliferate or whether a differentiation defect or intrinsic failure of myelination is at play. A better understanding of oligodendrocyte development in the context of reduced Pol III activity would provide insight into disease mechanism. Here, we utilized a downregulation approach to reduce expression of leukodystrophy-associated Pol III subunits in primary murine OL-lineage cells and then subjected

these cells to various *in vitro* assays that recapitulate their maturation, in order to study the role of Pol III in oligodendrocyte development and shed light on disease pathogenesis.

Our assessment of the precursor stage suggested migration was unaffected when *Polr3a, Polr3b* or *Polr1c* were downregulated, proposing that failure of OPCs to migrate to myelination sites is unlikely to explain the hypomyelination seen in POLR3-related leukodystrophy. Though, given that we did not completely downregulate Pol III subunit expression in our experiments, migration activity of OPCs may be affected in situations where Pol III expression/activity are more heavily downregulated. To our surprise, decreasing Pol III activity in the precursor stage of development accelerated the rate of OPC proliferation. Proliferation is one cellular aspect that has been previously implicated in Pol III mutants of yeast, BHK-1 cells and zebrafish gut development.[37-39] However, all of these models demonstrated a specific cell cycle stall at the G1-S phase transition. Our results suggest that OPCs might respond differently at the level of proliferation to hyomorphic Pol III than other cell types; however, the basis of this difference is unclear. Each of the cited models leverages different mutations in Pol III subunits, whereas our model specifically studies how Pol III subunit downregulation impacts OPC behaviour, which may be one reason our proliferation findings differ from prior observations. Perhaps a compensatory increase in proliferation is seen in light of the differentiation and myelination defects. Nevertheless, Ki67 staining in cells at the same timepoint and the labelling of cells in the EdU experiment as PDGFRα+ and NG2+ (Supplemental Figure 4.1) would suggest that all this is a pure, proliferating OPC population, rather than a mixed cell population where one would imagine signalling from neighbouring post-mitotic oligodendrocytes could be at play. In turn, our findings suggest that a failure of OPCs to proliferate is not likely to be the cause of hypomyelination in

POLR3-related leukodystrophy and that conversely, affected individuals may even have a higher proportion of OPCs.

OL-lineage cells with reduced expression of Pol III subunits exhibited impaired maturation when induced to differentiate, with reduced expression of late-stage OL and myelin transcripts, retained expression of OPC transcripts and impaired branching complexity of oligodendrocytes. Previous work has suggested defective oligodendrocyte development may be implicated in POLR3-related leukodystrophy pathophysiology.[29,40] Our works proposes a specific vulnerability of oligodendrocyte development to reduced Pol III activity in the immature OL stage. Comprehensive transcriptomic data has demonstrated Pol III subunit expression is dynamic during different stages of the OL-lineage.[31] Intriguingly, the expression of various Pol III subunits (e.g. *Polr3b, Polr1c)* decreases in the post-mitotic phase, with OPCs having higher expression levels than newly formed OLs.[31] This is perhaps counterintuitive as one would imagine that high protein synthesis demands during the immature OL stage, both at the level of cytoskeletal proteins for process extension and myelin proteins for myelination, would require more Pol III activity. However, this is not consistent, as *Polr3a* expression is slightly increased in newly formed OLs when compared to OPCs.[31] Expression of all three of these subunits (*Polr3a, Polr3b,* and *Polr1c)* decreases in mature OLs, though completion of myelination in these cells likely rationalizes why less Pol III would be present.[31,41] Transcription by Pol III is known to reduce with differentiation as, in general, cells that are dividing would be expected to require more protein synthesis.[42] Indeed, Pol III transcription at all three promoter sites decreases during differentiation of teratoma-derived F9 embryonic carcinoma cells and remains decreased.[42] Nevertheless, in a review paper on differentiation-dependent Pol III regulation published prior to

the identification of the first two causative genes in POLR3-related leukodystrophy, it was postulated that this general finding would not hold true for actively myelinating glial cells which require synthesis of myelin proteins post-mitotically.[42] Perhaps a limited availability of Pol III at a time point where one would imagine OLs have a high demand for this polymerase may decrease the threshold whereby hypomorphic Pol III, as in a diseased context, is acceptable.

Consistent with our differentiation results, we noticed that reducing Pol III in OLs hindered the amount of myelin present, as inferred by fewer MBP+ regions in organotypic shiverer slice cultures. Notably, diffuse hypomyelination is a cardinal feature of POLR3-related leukodystrophy.[11] Our earlier findings would suggest this hypomyelination is an outcome of a differentiation failure in the transition from precursor cells to oligodendrocytes though it is possible that, in addition to a differentiation defect, an intrinsic deficit in myelin deposition could also be contributing to this myelination finding.

We performed a RT-qPCR assessment of Pol III transcription in Pol III siRNA-treated cells under differentiation conditions, to see how Pol III transcriptional activity might relate to our impaired differentiation findings. We did not identify any changes in *5s, 7sl* or *U6* expression suggesting these transcripts are not likely to be at play in our impaired differentiation findings. Previous work has revealed variable outcomes of diseased state on Pol III transcript levels. Indeed, *5S* expression has been shown to be unaffected in HEK293 cells harboring a pathogenic *POLR3A* variant associated with a typical POLR3-related leukodystrophy phenotype, increased in blood from patients with an atypically severe form of POLR3-related leukodystrophy and yet decreased in fibroblasts from patients with *POLR3K* mutations.[6,40,43] The expression of *7SL* has often shown to be decreased, in line with the idea that the internal nature of Type II promoters

might make them more susceptible to Pol III dysfunction, although of note, *7SL* has a Type 2 promoter yet upstream promoter elements that might reduce its vulnerability relative to true Type II transcripts like tRNAs.[6,40,43] In certain cases, *7SL* has been shown to be unaffected in patient fibroblasts.[44] Meanwhile, Type III transcripts have often shown to be unaffected in a disease state, a finding which has been rationalized by an upstream promoter capable of receiving compensatory transcription by RNA polymerase II.[40] There are two main disease mechanisms pertaining to how Pol III transcription might relate to POLR3-related leukodystrophy. The first, that certain ncRNAs may be required for OL-lineage development and myelination, and the second, that tRNA homeostasis might be altered in a diseased state, leading to insufficient translation and therefore protein synthesis during critical timepoints in development.[45]

One such ncRNA that has been suggested as playing a role in the disease state is BC200 RNA, a brain-enriched transcript thought to play a role in neurons by local translation of mRNA in dendrites.[8,40] In MO3.13 BC200^{KO} cells, there is reduced expression of *MBP* mRNA upon differentiation.[40] However, glial transcriptomic profiling has revealed that BC200 is not normally expressed in human OLs, and neither is the functional analog Bc1 RNA in mouse OLs, suggesting the BC200 expression in MO3.13 cells could be due to their cancerous origin.[31,46,47] While this does not preclude that another, unidentified, Pol III transcript might be relevant for OL maturation and/or myelination, or that BC200 may contribute to neuronal phenotypes (e.g. cerebellar or striatal involvement) seen in a diseased state, it is improbable to explain the findings here. Certain miRNAs have also been identified for their importance in OL differentiation and

myelination and while once thought to be transcribed exclusively by Pol II, Pol III is now known to transcribe a subset of miRNAs.[48,49]

Various studies on the transcriptional impact of POLR3-related leukodystrophy both in patient samples and model organisms have supported involvement of tRNAs in the pathophysiology of the disease.[6,40,43,50] Further, the identification of hypomyelinating leukodystrophies that arise from biallelic variants in genes encoding cytoplasmic aminoacyl tRNA synthetases and a cerebellar-facial-dental syndrome arising from mutations in *BRF1*, transcription machinery used solely by Type 1 and Type 2 promoters, further supports this idea, as does the intricate connection between tRNAs and neurological disorders.[51-55] Importantly, changes in the tRNA pool could have tissue and even lineage-specific effects based on codon usage bias in these cell types, and further transcript-specific effects based on codon preference in mRNAs, suggesting a possible mechanism for our findings of a defect in the transition of OL-lineage stages.[56,57] Moreover, tRNA availability for protein arginylation is an additional way tRNAs may be important for OL maturation and myelination. The transfer of arginine from tRNA onto proteins, or arginylation, a process catalyzed by arginyl-protein-transferase 1 (Ate1), is a post-translational modification recently shown to play a critical role in OL maturation.[58] Various cytoskeletal regulators of OL maturation are subject to arginylation and in line with this, Ate1 expression peaks during differentiation of OPCs.[58] In primary cultures of mouse OLs with a genetic ablation in Ate1, branching complexity was significantly reduced and *in vivo,* these mice showed fewer mature (MOG+ or MBP+) OLs, which the authors suggested could be due to an impairment of OL differentiation.[58] It is interesting to note that biallelic variants of the

cytoplasmic arginine aminoacyl-tRNA synthetase (*RARS1*) are associated with *RARS1*-related hypomyelinating leukodystrophy.[52]

Oligodendrocytes bare large transcriptional and translational demands which could put them at an increased vulnerability in the face of altered tRNA homeostasis and a protein synthesis defects, the major demand of an OL being to produce canonical myelin proteins such as MBP, CNP and MOG.[59] Second to myelin-related proteins, cytoskeletal proteins constitute a major demand for OLs and are synthesized earlier in the lineage.[60] Notably, biallelic variants in genes encoding OL cytoskeletal proteins are implicated in hypomyelination, with variants in *TUBB4A* (encoding tubulin ß4, a microtubule specific to OLs) predicted to alter tubulin polymerization and microtubule stability, causing hypomyelination with atrophy of the basal ganglia and cerebellum, a distinct hypomyelinating leukodystrophy.[61] The OL cytoskeleton is not only critical for oligodendrocyte maturation but for myelination as microtubules mediate the transport of *Mbp* mRNA to process terminals.[62] Cytoskeletal proteins are also regulated, including by the myelin protein CNPase which has been shown to drive microtubule assembly, enabling OL process outgrowth, with OLs derived from CNP-deficient mice demonstrating smaller and fewer complex processes *in vitro.*[63] It is possible that in the face of reduced Pol III expression, immature OLs are unable to meet the high protein synthesis demands of cytoskeletal and/or myelin proteins, impacting the development and function of these cells.

The data herein suggests the Pol III siRNA-treated OLs experiencing impaired differentiation are not subjected to apoptosis, a process known to regulate the maturation of OL-lineage cells. This finding is consistent with previous work revealing that cleaved caspase-3 levels were not upregulated in brain lysates of a *Polr3a* mutant mice.[29] Crucially, this would suggest

that oligodendrocytes exhibiting defective differentiation in the context of decreased Pol III could be capable of therapeutic rescuing, though importantly, various *in vivo* mechanisms might be at play that are not seen in our experimental conditions.

In summary, our study identifies a role for Pol III in oligodendrocyte development, with decreased Pol III expression leading to defects at the level of oligodendrocyte maturation, including an accelerated rate of proliferation of oligodendrocytes in the precursor stage, impaired differentiation into mature oligodendrocytes and myelination. In turn, our results shed light on pathophysiological mechanisms underlying hypomyelination in POLR3-related leukodystrophy, providing foundational knowledge relevant to the field's advancement in developing therapeutics.

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4.9 Supplemental Information

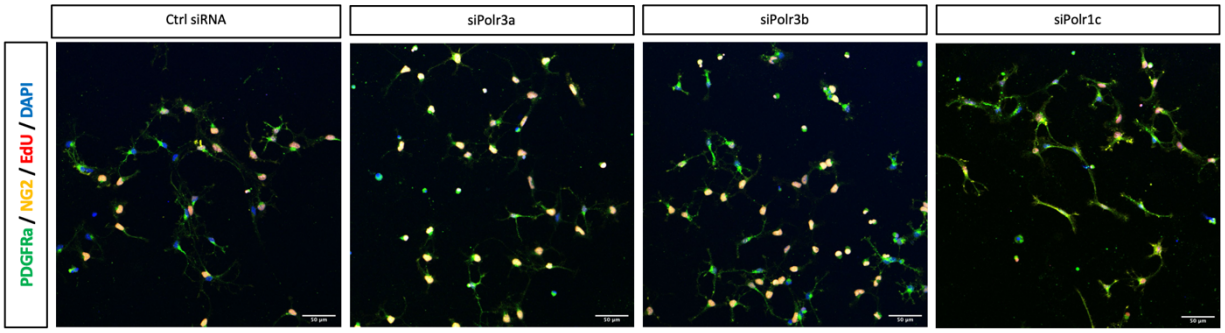

Figure S4.1: Representative immunofluorescence images (from three independent experiments with similar results) of siRNA-treated OPCs in proliferation media labeled with EdU and showing PDGFRα (green) and NG2 (yellow). Nuclei stained with DAPI. Scale bar, 50 µm.

Table S4.1: Antibody information

Anti-Ki67 (1:200)	Novus	AF7649
Anti-PDGFRα (ICC 1:100, IP 1:250)	BD	558774
Anti-NG2 (1:200)	Milipore	AB5320
Anti-MOG (1:300)	R&D	MAB2439
Anti-MBP (1:100-1:500)	Novus	NB600-717
Anti-Calbindin D28-k (1:5000)	Swant	CB38
Anti-Cleaved caspase 3 (1:250)	CST	9661
AlexaFluor 488 donkey anti-rat (1:1000)	Fisher Scientific	A21208
AlexaFluor 647 donkey anti-rabbit (1:300-1:500)	Jackson ImmunoResearch	711-605-152
AlexaFluor 594 donkey anti-sheep (1:500)	Abcam	713-585-147
AlexaFluor 555 donkey anti-goat (1:500)	Invitrogen	A-21432
Goat anti-rat IgG (IP 1: 333)	Jackson ImmunoResearch	115-005-167

Table S4.2: RT-qPCR primers

Polr3a	F: CATGTCGGGTACTTCAGGGCT
	R: TAATGTGGCAGCAGGTTTTGCAG
Polr3b	F: TTGGAGCCTCAGTTACCAGC
	R: GAATCGACCCTGTTTCACGG
Polr1c	F: GTCCACACCACGGACTTTC
	R: CCACACGGAAATTCTTCTCGA
Pdgfrα	F: CATCTATGTACCAGACCCAGAC
	R: AAGGTATGATGGCAGAGTCATCC
Cspg4	F: CCTTCCTACAAGTGACCATTGCC
	R: TCCTGGACCACCTCTAGCT

Fyn	F: GAAGCCCGCTCCTTGACA
	R: CCAAGTTTTCCAAAGTACCACTCTT
Nfasc	F: AATCCGAGTCCTGAACAGCA
	R: TTCCCTCCAGTAGTAAGCTCGATA
Kif19a	F: GCTGAGTGACAAGGGCAGTAA
	R: GTTCCCGCCTAGTGAATCCTTC
Mbp	F: TCAAGAACATTGTGACACCTCG
	R: AGCTAAATCTGCTGAGGGAC
Mog	F: GCCTGTTTGTGGAGCTTCTCTTG
	R: CCTATCACTCTGAATTGTCCTGCAT
Cnp	F: CTGAGACCCTCCGAAAAGCTG
	R: TCCAGAAATAAAGTGTCGAAGCTCT
5s	F: GCCATACCACCCTGAACGC
	R: TATTCCCAGGCGGTCTCCC
7sl	F: GGAGTTCTGGGCTGTAGTGC
	R: TTTGACCTGCTCCGTTTCCG
U6	F: CGCTTCGGCAGCACATATAC
	R: TTCACGAATTTGCGTGTCAT
18s	F: GTAACCCGTTGAACCCCATT
	R: CCATCCAATCGGTAGTAGCG
Gusb	F: GTGGTATGAACGGGAAGCAAT
	R: AACTGCATAATAATGGGCACTGT
Pgk1	F: TGGTGGGTGTGAATCTGCC
	R: ACTTTAGCGCCTCCCAAGATA

Table S4.3: Materials and Reagents

Reagent	**Source**	**Identifier**
Griffonia (Bandeiraea) simplicifolia lectin I GSL I, BSL I	Biolynx	VECTL1100
Papain	Worthington	LS003126
DNase I	Worthington	LS002007
L-cysteine	Sigma	C7477
Insulin from bovine pancreas	Sigma	I6634
Ovomucoid	Worthington	LS003086
Bovine Serum Albumin	Sigma	A3912
Transferrin	Sigma	T1147
Putrescine	Sigma	221732
Progesterone	Sigma	P8783
Sodium selenite	Sigma	S5261
Penicillin-Streptomycin	Wisent	450-201-EL
Sodium pyruvate	Gibco	11360070
Glutamax	Gibco	35050061

N-acetyl-L-cysteine	Sigma	A7250
Forskolin	Sigma	F3917
D-Biotin	Sigma	B4639
Trace Elements B	Fisher Scientific	MT99175Cl
B-27 without vitamin A	ThermoFisher	12587010
Recombinant human PDGF-AA	Preprotech	100-13A
Recombinant human bFGF	Preprotech	100-18B
Triiodothyronine	Sigma	T6397-100mg
D-PBS with Mg^{2+} Ca^{2+}	ThermoFisher	14287080
Earle's Balanced Salt Solution	Wisent	311210CL
Dubleco's Modified Eagle's Medium	Wisent	319-005-CL
Minimum Essential Medium	Sigma	51412C-1000ML
DMEM-F12 1% GlutaMAX	ThermoFisher	10565018
Fetal Bovine Serum	Wisent	089-150
Heat-Inactivated horse serum	ThermoFisher	26050088
Glucose	ThermoFisher	A2494001
Fungizone	ThermoFisher	15290018
B-27 Supplement (50X), serum free	ThermoFisher	17504044
N-2 Supplement (100X)	ThermoFisher	17502048
Poly-d-Lysine hydrobromide	Sigma	P8099
Trypsin/EDTA	Wisent	325.043-cl
Corning™ Transwell™ Multiple Well Plate with Permeable Polycarbonate Membrane Inserts	Fisher	07-200-150
ReadyProbes™ Cell Viability Imaging Kit, Blue/Green	Invitrogen	R37609
TRIzol™ reagent	ThermoFisher	15596018
miRNeasy Mini Kit	Qiagen	217004
RNAse free DNAse kit	Qiagen	79254
Oligo(d)T	ThermoFisher	18418020
Random Hexamers	IDT	51-01-18-25
RNasin® Ribonuclease Inhibitors	Promega	N2611
MMLV RT	Promega	M1705
MMLV 5x reaction buffer	Promega	M5313
SsoAdvanced Universal SYBR® Green Supermix	Bio-Rad Laboratories	1725274
GoTaq® Master Mixes	Promega	M7122
Extracta™ DNA Prep for PCR	QuantaBio	CA97065-350
MilliporeSigma™ Millicell™ Culture Plate Inserts	Fisher	PICM03050
Pre-Treated German Glass Coverslips 15mm #1	Electron Microscopy Sciences	72291-04

Thermo Scientific™ Shandon™ Immu-Mount™	Fisher Scientific	9990402
Click-iT™ EdU Cell Proliferation Kit for Imaging	ThermoFisher	C10340
siGENOME mouse Polr3a siRNA - SMARTPool 5nmol	Dharmacon	M-160178-00-0005
siGENOME mouse Polr3b siRNA - SMARTPool 5nmol	Dharmacon	M-064863-00-0005
siGENOME mouse Polr1c siRNA - SMART Pool, 5nmol	Dharmacon	M-046056-01-0005
siGENOME Non-Targeting siRNA Control Pools	Dharmcon	D-001206-14-05
Lipofectamine	Invitrogen	13778150

Chapter 5: Discussion, Conclusion and Future Directions

5.1 Preface

This thesis focuses on an *in vitro* approach to explore oligodendrocyte biology, and specifically, its application to the study of cellular pathophysiology in POLR3-related leukodystrophy. At the onset of this research project, our aim was to identify how reducing Pol III function in oligodendrocyte-lineage cells might impact their development. In **Chapter 3**, we customized an immunopanning approach for the isolation of PDGFRa+ oligodendrocyte precursor cells. Our modifications led to a method optimized for a high yield and high purity isolation of cells at an early stage of the lineage. In turn, this enabled us to perform a comprehensive study of the fundamental aspects of OL-lineage biology, from the precursor stage to the myelinating stage, *in vitro*. In **Chapter 4**, we put this method into practice by developing a cellular model of POLR3-related leukodystrophy, based on a knockdown approach. As the hundreds of variants seen in POLR3-related leukodystrophy are predicted to share a common hypomorphic outcome, a knockdown approach allowed us to model the disease in a way that we believe is broadly applicable to the disease state. This led to an expanded understanding of the cellular biology of hypomyelination in POLR3-related leukodystrophy. This concluding chapter will act as a more detailed discussion for the cellular pathophysiology findings documented in this thesis, integrating the limitations of this work and potential future directions.

5.2 Novel insights into oligodendrocyte development in POLR3-related leukodystrophy

In this thesis, we sought to provide insight into the hypomyelination seen in POLR3-related leukodystrophy, at a cellular level. We found that in oligodendrocytes with reduced expression of Pol III subunits, defects could be seen both at the precursor stage and in the

maturation of oligodendrocytes from precursors to mature cells. Indeed, the latter could be seen both at the expression of stage-specific OL transcripts and as evidenced by their immature branching morphology. In line with this, our findings from ongoing myelination experiments suggest this defect impacts the ability of these cells to myelinate.

As the biallelic pathogenic variants seen in patients are predicted to be hypomorphic, we opted to use a knockdown approach when developing our cellular model of POLR3-related leukodystrophy.[7,10,63,126] Additionally, since mouse models of POLR3-related leukodystrophy harboring patient mutations have shown variable success in recapitulating a myelin phenotype, we believed a knockdown approach would overcome potential species differences that might account for the differential vulnerability to mutations between mice and humans.[127,129,130] It is unclear in patients what level of RNA expression may be present for the affected Pol III subunit. As parents of those affected do not show clinical features of the disease, haplosufficiency is presumed to be at play in carriers. Additionally, work at the protein level has shown a range of decreased expression of the affected Pol III subunit, from as little as 6.8% reduced (lysates of cortex, individuals with a typical disease course) to upwards of 84.7% reduced (gray matter, individuals with a severe disease course), depending on the tissue type being studied and the severity of the clinical course for the individual from which the tissue was derived.[7,115] Further, protein levels may not faithfully reflect RNA dosage.[180] We were able to achieve a downregulation fairly consistent between the three subunits studied, ranging from a 59-63% reduction. As past work in HEK293 cells with a knock-in of the *POLR3A* M852V mutation demonstrated roughly 20-60% knockdown of *POLR3A* mRNA (based on whether the missense mutation was in combination with a frameshift mutation or null allele), the level of knockdown

achieved here should model a diseased state.[123] Importantly, as a deficit could be seen with only a 60% reduction in Pol III subunits, our results suggest that >40% of Pol III subunit expression is required for normal oligodendrocyte development.

While our *in vitro* approach was highly beneficial in enabling us to dissect OL-maturation into its various components and comprehensively study OL development, various limitations of this approach should be mentioned. For one, we noted that our Pol III siRNA-treated OLs remain alive, even in the face of a presumed maturation block. While this is in fact consistent with past *in vivo* work, it is possible a more complex situation could be at play that our approach does not account for.[130] Specifically, working with an OL-specific culture means we could not account for the possibility of cross-talk between other cell type, such as astrocytes and microglia, and how these may play a role in the regulation of OL survival.[181]

Further, our approach took an oligodendrocyte-central perspective when studying the disease mechanisms of hypomyelination. While various lines of work support an OL-predominant role in POLR3-related leukodystrophy, neuronal-intrinsic problems could lead to compromised axonal integrity and subsequent white matter damage.[102,182] Further, POLR3-related leukodystrophy caused by specific variants in *POLR3A,* and which run a particularly severe disease course, are thought to have a predominantly neuronal pathophysiology.[115,117-119]

It remains crucial to recapitulate our findings of impaired oligodendrocyte development using an *in vivo* approach. While our approach allowed for a more detailed study of OL development, animal models remain a critical aspect of studying disease mechanisms. The methodologies used in this thesis could be applied to a transgenic mouse model, by isolating and recapitulating these experiments to enable a more detailed study of these mice, but the advent

of a model itself would allow for a broader perspective of disease mechanisms. Indeed, a model organism is critical for a more holistic approach of studying the underlying biology of a disease, while also serving as a prerequisite for the future exploration of therapeutic options.

Moreover, while this work is an important advancement for our understanding of pathophysiological mechanisms in this disease, many unanswered questions remain. A preliminary RT-qPCR assessment of three Pol III transcript (i.e., *5S, 7SL* and *U6),* each representing a promoter type, was unsuccessful in revealing what sort of perturbed Pol III function may be occurring molecularly to explain the cellular defects seen. Based on the two predominant disease hypotheses for POLR3-related leukodystrophy, it is possible that tRNAs or ncRNA(s) are relevant to the disease state.[93] One ncRNA that has previously been suggested in the disease pathophysiology is BC200, however RNA-Seq databases suggest this gene is not expressed in OPCs.[123,183] As such, while BC200 holds a potential role in neuronal aspect of this disease, it is unlikely to explain our OL-specific findings and we did not study this. Additionally, tRNAs have traditionally posed difficulties for analysis via RT-qPCR due to their short sizes, extensive post-transcriptional modifications and highly-stable structures.[184] One interesting follow-up experiment would be to perform a SUnSET assay to look at the translational capacity of these Pol III siRNA-treated cells and to determine whether protein synthesis is in fact impacted in a disease state.[185] Additionally, a more detailed transcriptomics study would hold great promise for providing insight into the molecular pathophysiology of this condition.

5.3 Concluding remarks

In summary, in this thesis, we use an optimized method for the isolation of primary mouse OPCs as the basis of a cellular model to identify potential pathophysiological mechanisms of

POLR3-related leukodystrophy. As neurological manifestations are the most debilitating aspect of the disease and arise from hypomyelination, how white matter is particularly impacted by reduced activity of Pol III is an especially intriguing area of study. One would imagine that a deficit of myelination could arise from a defect in oligodendrocyte development but where this defect arises, in the ability of precursor oligodendrocytes to migrate or proliferate, their differentiation or their myelination ability, has been poorly delineated.

Here, we demonstrate a vulnerability of oligodendrocytes to reduced Pol III activity. Specifically, we identify that OPCs with decreased endogenous transcript levels of leukodystrophy-associated Pol III subunits demonstrate increased levels of proliferation, suggestive of a paradoxically larger population of OPCs in patients. In comparison, we found no change in the migration of these cells, putting forth the possibility that OPCs in affected individuals are present at myelination sites. Differentiation of these cells demonstrated Pol III as important for their maturation into mature, myelinating oligodendrocytes and concurrently, their myelination ability. We also showed that survival of oligodendrocytes was unaffected by the maturation defect seen. Ultimately, this research provides valuable insight into the cellular mechanisms underlying hypomyelination in POLR3-related leukodystrophy and imparts foundational knowledge for future studies on the pathophysiology of this disorder.

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