REVIEW

The Healthy and Diseased Microenvironments Regulate Oligodendrocyte Properties *Implications for Regenerative Medicine*

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Accepted for publication
August 1, 2017.

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White matter disorders are characterized by deficient myelin or myelin loss, lead to a range of neurologic dysfunctions, and can result in early death. Oligodendrocytes, which are responsible for white matter formation, are the first targets for treatment. However, many studies indicate that failure of white matter repair goes beyond the intrinsic incapacity of oligodendrocytes to (re)generate myelin and that failed interactions with neighboring cells or factors in the diseased microenvironment can underlie white matter defects. Moreover, most of the white matter disorders show specific white matter pathology caused by different disease mechanisms. Herein, we review the factors within the cellular and the extracellular microenvironment regulating oligodendrocyte properties and discuss stem cell tools to identify microenvironmental factors of importance to the development of improved regenerative medicine for patients with white matter disorders. (*Am J Pathol* 2018, 188: 39–52; <https://doi.org/10.1016/j.ajpath.2017.08.030>)

The pathology of white matter disorders (WMDs) is characterized by deficient white matter (WM) or myelin loss. WMDs have different causes, such as injury, inflammation, or genetic mutations. Currently, there is an urgent need for better treatment for patients with WMDs.¹ Cell replacement therapy is seen as one of the promising new treatment options. Because oligodendrocytes (OLs) are responsible for the generation of myelin in the brain, many studies focus on the development of OL replacement therapies.^{2,3} Transplantation of glial progenitor cells in rodent models of myelin disease resulted in improved pathology and clinical phenotype.^{4–6} However, although transplanted cells successfully spread throughout the brain, recovery is often insufficient. And in cases of successful transplantation, recovery was also ascribed to factors secreted by the transplanted cells, rather than cell replacement.^{7,8} Moreover, brain pathology of WMD patients shows involvement of different neural cell types. Therefore, therapeutic strategies to regenerate WM should comprise more than OL replacement.⁹

WM structure and function is determined by constant interplay between the OL intracellular properties and local signals in the brain microenvironment. As a result, WMDs can be caused by an affected microenvironment, even though the OLs are intrinsically normal. On the other hand, malfunctioning OLs can lead to a changed microenvironment, which could cause WMDs. In other words, as defected OLs disrupt normal interactions with their local milieu, the brain microenvironment will get into a diseased state over time as well. In this way, WMD pathology becomes multicellular. Hence, microenvironmental

Supported by ZonMw (The Netherlands Organisation for Health Research and Development) VIDI (part of The Innovational Research Incentives Scheme) research grant 91712343 (V.M.H.), ZonMw TAS IDB (part of the Translational Adult Stem Cell Research Program) project 116005006 (V.M.H.), E-Rare Joint Call project 9003037601 (V.M.H.), and European Leukodystrophy Association Research grant 2014-012L1 (V.M.H.).

Disclosures: None declared.

This article is part of a review series on neural regeneration and developmental biology in health and disease.

factors are important in the pathology of all WMDs. Thus, before we can start designing new therapeutic strategies, we do not only need insight into basic disease mechanisms underlying WMDs, we also need understanding of how the microenvironment is primarily and secondarily involved. Although some of these aversive microenvironmental factors easily reverse, others might need active modulation and should be resolved for successful treatment.

Herein, we give an overview of important components in the microenvironment that are known to regulate OL development and function. Furthermore, to identify new microenvironmental factors that influence OL properties and that are of importance in WM defects of particular WMDs, we discuss the use of new stem cell tools.

WMD Cell Therapy Candidates

Cell replacement therapies have prospects for many WMDs, especially for the genetic WMDs because the OLs (and other neural cell types) carry genetic mutations. Before we describe microenvironmental components of importance to WM health, we first briefly discuss examples of genetic WMDs that are early candidates of future cell-based therapies.

Pelizaeus-Merzbacher Disease

Pelizaeus-Merzbacher disease (PMD; Online Mendelian Inheritance in Man no. 312080) is characterized by diffuse hypomyelination or a tigroid pattern of dysmyelination and is caused by genetic changes in the X-linked proteolipid protein 1 (*PLP1*) gene, which is involved in the adherence and stabilization of compact myelin.¹⁰ Most patients show duplications of the *PLP1* gene, which results in accumulation of PLP1 proteins in the lysosome affecting the viability of the OLs.¹¹ Missense mutations in *PLP1* cause a more severe type of PMD, in which PLP proteins accumulate in the endoplasmic reticulum, leading to reduced myelin formation and to increased apoptosis of OLs.¹² In a first clinical trial, transplantation of human central nervous system stem cells in PMD patients showed no adverse effects, which gives prospects for the following clinical studies.¹³

Alexander Disease

Many genetic WMDs are caused by mutations in genes that are non-OL-lineage specific, including Alexander disease (Online Mendelian Inheritance in Man no. 203450), which is caused by mutations in glial fibrillary acidic protein (*GFAP*), a gene mainly expressed in astrocytes.¹⁴ These mutations result in Rosenthal fibers, which are accumulations of clustered intermediate filaments in astrocytes.¹⁵ Astrocyte dysfunction eventually leads to characteristic symmetric white matter abnormalities with frontal lobe predominance. Because Alexander disease is a typical WMD with cell-specific defects, cell replacement might be an attractive therapeutic strategy. However, the exact mechanism remains

unknown. Dysfunction in glutamate uptake and potassium buffering, as well as production of inflammatory molecules and the activation of microglia, are suggested.¹⁶

Vanishing White Matter Disease

Vanishing white matter disease (VWM; Online Mendelian Inheritance in Man no. 603896) is a progressive WMD and results from mutations in the *EIF2B1-5* genes, which play a role in translation initiation and protein regulation.¹⁷ Patients show diffuse myelin defects leading to cystic degeneration. Although both oligodendrocytes and astrocytes look abnormal, there is increasing evidence that astrocytes are the primary affected cell type, eventually resulting in myelin loss.^{18,19} VWM is suggested as an early candidate for cell replacement therapy.¹ However, like other WMDs, VWM pathology is complex and involves changes in the microenvironment.¹⁹ To design cell therapies, we need a better understanding of how the microenvironment affects OL development and myelin formation.

Microenvironment Regulates Oligodendrocyte Identity

During OL development, the processes of differentiation, migration, and myelination comprise specific programs of gene expression, which have been studied and reviewed extensively.^{20,21} These intracellular OL programs are influenced by many factors that come from outside the OL. For instance, generation of myelin sheaths is not only dependent on the developmental state of the OL but also on the maturational state of the axon.²² In addition, OLs show different myelination properties, depending on the location in the postnatal cortex.²³ OLs in the deeper cortical layers are more mature and produce more myelin than OLs in the superficial cortical areas.²³ Similarly, WM regions stimulate maturation of OLs more than gray matter regions, regardless of the regional origin of the OLs.²⁴ Thus, different factors in the local microenvironment guide OL myelination.

Then again, different studies indicate that OLs have different intrinsic properties. Dorsally and ventrally derived OLs have different migration and settling patterns,²⁵ and cortical- and spinal cord-derived OLs produce different myelin sheet lengths.²⁶ Furthermore, when transplanted into the gray matter, WM-derived OLs have higher maturation efficiency than gray matter-derived OLs.²⁴ This suggests that the early microenvironment to which the OL progenitors have been exposed also influences OL properties in adulthood. Indeed, OL progenitor populations emerge from different regions and at different time points in development.²⁷ During brain patterning, different morphogens are secreted in a regionally and temporally restricted manner, resulting in specific anteroposterior and rostral-caudal gradients. These gradients induce expression of particular transcription factors, generating unique progenitor domains,

where specific populations of progenitor cells emerge, including OL progenitor populations.²⁷ In summary, many OL populations exist in the brain, each with their own intrinsic capabilities, which are regulated by microenvironmental factors during early and late development.

To understand disease mechanisms underlying WMDs and to facilitate the development of regenerative medicine, we need better insight into mechanisms involved in OL development and WM formation. The microenvironmental factors regulating WM health (ie, OL functioning) involve connections with local cells, the extracellular matrix (ECM), and cellular- and blood-derived factors. The cellular microenvironment involves neural cell types, including cell types of neuronal, astrocytic, and microglial cell lineages. The ECM surrounds the developing and mature OLs. The cellular microenvironment and the ECM, as well as the vasculature, provide physical organization and signaling molecules, such as growth factors and neurotrophins. All these factors together are crucial for proper OL development and determine OL properties in adulthood. In WMDs, these microenvironmental factors might be affected, which lead to affected OL function and subsequently failed WM (re)generation.

Cellular Microenvironment

Neurons

Because the main function of OLs is myelination of axons, which is necessary for the efficient and rapid transmission of action potentials, the role of neurons on OL development has been explored extensively.²⁸ Many studies showed that the number of neurons (ie, axons) directly influences OL proliferation, survival, and differentiation.^{28–30} Next to cellular contact, neuronal activity can modulate the numbers of OL progenitors and the amount of myelin formed.^{31,32} Changes in the activity of the neurons affect the release of neurotransmitters. When unmyelinated axons fire action potentials, neurons can release the neurotransmitter glutamate, which, on binding of glutamate receptors on OLs, can induce myelination processes.³³ However, glutamate receptor properties depend on the developmental stage of the OL and on the location in the brain.³⁴ It has been shown that prolonged glutamate receptor activation on OL progenitors causes calcium overload and eventually cell death, whereas mature OLs stay unaffected.³⁵ Therefore, because different glutamate receptors have specific regulating roles, glutamate is suggested to have dynamic functions during OL development.³⁶ Next to glutamate, other neurotransmitters also affect OL progenitor development (Table 1). For example, several studies demonstrated the regulating actions of GABA during OL development.³⁷ It has been shown that GABA signaling can modulate synaptic communications with hippocampal interneurons³⁸ and OL progenitor proliferation and migration *in vitro*.³⁹ Apart from cellular contact, and neuronal activity, neurons secrete many other factors that regulate OL development via direct and/or indirect signaling (Table 2).

Astrocytes

Astrocytes are responsible for many different functions in the brain, including regulating the blood-brain barrier (BBB), neurotransmitter uptake, synaptic transmission, and the extracellular ion concentration.⁴⁰ It is widely accepted that astrocytes also have direct influences on OL development and functioning.⁴¹ Astrocytes and OLs are directly connected via gap junctions,⁴² formed by connexins (Cx), like the Cx26-Cx32 and the Cx30/Cx43-Cx47 channel.⁴³ Multiple studies showed that mutations in Cx47 and Cx32 result in myelin pathology.^{44,45} The supportive roles of astrocytes for OLs have also been illustrated by Alexander disease, in which dominant gain-of-function mutations in *GFAP* cause myelin defects.⁴⁶ In addition, astrocytes regulate OL development via many secreted factors, including glycoprotein growth factors and neurotrophins (discussed later) (Table 2).

Microglia

Microglia have different roles in the central nervous system. The quiescent anti-inflammatory/immunoregulatory microglia remove damaged cells by phagocytosis after neuronal damage. However, the activated microglia, so-called proinflammatory microglia, display an inflammatory response after injury. Chronically activated microglia can cause damage to their surrounding cells via the release of potentially cytotoxic molecules, including proinflammatory cytokines, reactive oxygen intermediates, proteinases, and complement proteins.⁴⁷ Microglia also regulate OL development, myelination, and WM injury.⁴⁸ Anti-inflammatory/immunoregulatory microglia protect OL progenitors from apoptosis and enhance OL maturation and myelination via the secretion of soluble factors^{41,49} (Table 2), possibly by stimulating the synthesis of sulfatide.⁵⁰ Furthermore, they are involved in the promotion of myelination and axon regeneration (Table 2). During remyelination, microglia clear myelin debris,⁵¹ and their recruitment is regulated by astrocytes.⁵² On the other hand, proinflammatory microglia lower insulin-like growth factor-1 and ciliary neurotrophic factor production, secrete nitric oxide, and inhibit OL progenitor survival and OL differentiation.^{53–55} Thus, microglia have both positive and negative effects on OLs, depending on OL developmental stages.⁵³

Extracellular Matrix

The ECM is a network of proteins and glycans that provides structural and biochemical support to surrounding cells.⁵⁶ The ECM is composed of factors that are secreted by surrounding cells and consists of three compartments: the basement membrane, the perineuronal nets, and the neural interstitial matrix. The basement membrane is positioned between the vasculature and the astrocyte end feet, plays important roles in maintaining the BBB, and consists of molecular components, like collagen, laminin, fibronectin, and dystroglycan. The perineuronal nets surround neuronal cell structures, are responsible for synaptic

Table 1 An Overview of the Microenvironmental Factors Predominantly Derived from Neurons or Blood, That Have Been Reported to Regulate OL Progenitor Characteristics

Name	Binding partner	OL progenitor specification	Proliferation	Migration	Maturation	Survival	Myelination
Glutamate	GluR, AMPAR, KAR, NMDAR, GLT-1, GLAST		+/-	+	+	-	+
γ -Aminobutyric acid	GABA _A R, GABA _B R		+/-	+/-	+		+
Serotonin	5-HT2AR				-	-	-
Adenosine	A _{1A} R, A _{2A} R			+			+
Acetylcholine	nAChR, mAChR		+	+	+/-	+	
Dopamine	DRD2, DRD3	-	+			+	
LINGO-1	NgR, p75				-		-
Nogo-A	NgR				+		-
Semaphorins	Plexins			+/-	+/-		+/-
Netrin-1	DCC, UNC5H1			-	+		+/-
Ephrins	EphA, EphB receptors			+			
Thyroxine, triiodothyronine	NR1A1, NR1A2				+		+
Iron	Transferrin, ferritin	+	+		+	+	+
ILs	IL receptors	+	+/-		+	+/-	+
LIF	LIFR	+	+		+	+	+
TNF- α	TNFR1, TNFR2		+		-	-	+/-
IFN- γ	IFNGR1, IFNGR2		+/-		-	+/-	-

This overview is based on 100 experimental studies, which showed regulating effects of the presented neuron- or blood-derived factors on processes of OL development. This is not a complete list; many other studies have been performed.

+, Positive regulation; -, negative regulation; A_{1A}R, adenosine receptor 1A; A_{2A}R, adenosine receptor 2A; AMPAR, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor; DCC, deleted in colorectal cancer; DRD, dopamine receptor D; Eph, erythropoietin-producing human hepatocellular; GABA_AR, GABA_A receptor; GABA_BR, GABA_B receptor; GLAST, glutamate aspartate transporter; GLT, glutamate transporter; GluR, glutamate receptor; 5-HT2AR, 5-hydroxytryptamine receptor 2A; IFN- γ , interferon- γ ; IFNGR, IFN- γ receptor; KAR, kainic acid receptor; LIF, leukemia inhibitory factor; LIFR, LIF receptor; LINGO, leucine rich repeat and immunoglobulin-like domain-containing protein; mAChR, muscarinic acetylcholine receptor; nAChR, nicotinic acetylcholine receptor; NgR, Nogo receptor; OL, oligodendrocyte; NMDAR, N-methyl-D-aspartate receptor; NR1A1, nuclear receptor subfamily 1, group A, member 1; NR1A2, nuclear receptor subfamily 1, group A, member 2; TNF- α , tumor necrosis factor- α ; TNFR, TNF- α receptor; UNC5H1, uncoordinated-5 homology 1.

stabilization, and consist of dense structures of proteoglycans. The neural interstitial matrix, on the other hand, is not strongly connected to the basement membrane or the perineuronal nets, has multiple ECM functions, and is a more loose network of proteoglycans (eg, tenascin), glycosaminoglycan (eg, hyaluronan), proteins (eg, collagen and elastin), and glycoproteins (eg, laminin and fibronectin).

The ECM facilitates cell-to-cell communication and plays a role in the migration, differentiation, and proliferation of neural cells during development.⁵⁶ Neural cells, including early OL progenitors and myelinating OLs, can bind to the ECM via cell-surface cellular adhesion molecules, like integrins and cadherins, which are connected to intermediate filaments in their cytoskeleton.^{57–59} Integrins have the ability to bind to proteins (eg, collagen) and glycoproteins (eg, fibronectin or laminin) in the ECM and to integrins on other cells. Cadherins are calcium-dependent glycoproteins, which bind to the cytoskeleton through specific linking proteins called catenins, and tend to cluster at the intermediate cell junctions. Because integrins and cadherins have an extracellular, a transmembrane, and an intracellular domain, they have essential functions in mediating extracellular signals to intracellular signaling pathways.⁶⁰ Apart from the physical interaction, the ECM can also support cell

growth and survival by quick release of stored signaling molecules at specific time points and in particular regions of the brain.⁶¹ For example, protease activities can be triggered on physiological changes without de novo synthesis, like matrix metalloproteinase 9 or 2, which, in high amounts, can cause basement membrane detachment and a compromised BBB.

Different proteins and glycoproteins in the ECM control OLs.⁶² During development, N-cadherin is important for OL progenitor migration,⁶³ and laminin stimulates postnatal oligodendrogenesis in the subventricular zone, as shown in mice.⁶⁴ α 2-Laminin-deficient mice show delayed OL maturation and accumulation of OL progenitors during the active myelination period.⁶⁵ Also in humans, mutations in laminin genes lead to WM defects. Children with mutations in the α 2-laminin gene *LAMA2* show WM defects, next to defects in brain size.⁶⁶ The interactions between laminin and cellular adhesion molecules, like dystroglycan and integrin, may play important roles, because cleavage by metalloproteinase increases the number of OL progenitors⁶⁷ and administration of antisulfatide antibodies that modulate laminin-integrin interactions impedes OL differentiation and (re)myelination *in vitro*.⁶⁸ It is further shown that laminin acts on dystroglycan receptors to regulate processes of OL

Table 2 An Overview of the Glycoprotein Growth Factors and Neurotrophins, Derived from Neurons, Microglia, and Astrocytes, That Have Been Reported to Regulate OL Progenitor Characteristics

Name	Receptor	OL progenitor specification	Proliferation	Migration	Maturation	Survival	Myelination
PDGF-AA	PDGF α R	+	+	+	—		+
IGF-1	IGF1R	+	+		+	+	+
FGF2	FGFR1-4	+	+		+/-		+/-
EGF	EGFR	+	+	+	+/-	+	+
CNTF	CNTF α R		+		+	+	+/-
BDNF	TrkB, p75		+/-		+		+
GDNF	GFR α 1	+	+		+		+
NGF	TrkA, p75		+	+		+/-	—
NT3	TrkB, TrkC		+		+	+	+/-
NRG	ErbB	+/-	+	+	+/-	+	+
SDF-1	CXCR4			+	+		+

This overview is based on 82 experimental studies, which showed regulating effects of the presented microenvironmental factors on processes of OL development. This is not a complete list; many other studies have been performed.

+, Positive regulation; —, negative regulation; BDNF, brain-derived neurotrophic factor; CNTF, ciliary neurotrophic factor; CNTF α R, CNTF- α receptor; CXCR, C-X-C chemokine receptor; EGF, epidermal growth factor; EGFR, EGF receptor; ErbB, ErbB tyrosin kinase receptor; FGF, fibroblast growth factor; FGFR, FGF receptor; GDNF, glia-derived neurotrophic factor; GFR α 1, GDNF family receptor α 1; IGF-1, insulin-like growth factor-1; IGF1R, IGF-1 receptor; NGF, nerve growth factor; NRG, neuregulin; NT, neurotrophic factor; OL, oligodendrocyte; PDGF-AA, platelet-derived growth factor AA; PDGF α R, platelet-derived growth factor- α receptor; SDF, stromal cell-derived factor; Trk, tyrosine kinase.

outgrowth and branching,⁶⁹ as well as myelin production and stability.⁷⁰ Fibronectin is another glycoprotein that is well studied for its function during OL development. It mediates proliferation of OL progenitors but can form aggregates during inflammation-induced forms of demyelination, thereby impairing remyelination.⁷¹ Furthermore, fibronectin can impair OL differentiation by inhibiting morphologic changes in OLs that are required for the production of myelin sheets,⁷² possibly via mislocalization of matrix metalloproteinase 9 activity.⁷³ The glycoprotein tenascin-C can inhibit processes of OL progenitor proliferation and maturation.^{74,75}

Another ECM molecule with clear regulating actions on OLs is the glycosaminoglycan hyaluronan. Hyaluronan is a

large polysaccharide that forms complexes with proteoglycans in the ECM and exists in a high-molecular-weight (HMW) and a low-molecular-weight form. Increased levels of hyaluronan in the neural interstitial matrix are described for WMDs, like multiple sclerosis (MS) and VWM. Patients with VWM, who show defects in OL progenitor maturation,¹⁸ have increased levels of HMW hyaluronan.⁷⁶ The HMW hyaluronan receptor CD44 regulates the cellular responses to hyaluronan, regulates the assembly of hyaluronan-rich extracellular matrices, and mediates internalization of hyaluronan. Postmortem brains of VWM patients also show increased levels of astrocytes expressing the CD44 receptor.⁷⁶ This indicates that astrocytes contribute to WM defects found in VWM patients, as was

Table 3 An Overview of the ECM Components That Have Been Reported to Regulate OL Progenitor Characteristics

Name	Binding partner	OL progenitor specification	Proliferation	Migration	Maturation	Survival	Myelination
Chondroitin sulfate proteoglycan	Protein tyrosine phosphatase σ receptor				—	—	—
Hyaluronan	TLR2, CD44				—		—
Laminin	Dystroglycan, integrins		+		+	+	+
Osteopontin	Integrins		+			+	+
Tenascin-C	Integrins		—	—	—	+	+
Fibronectin	Integrins		+		—		+/-
Vitronectin	Integrins	+	+				+
Matrix metalloproteinases					+		+/-
Tissue inhibitor of metalloproteinase 1	CD63, integrins	+			+		+
Polysialylated—neural cell adhesion molecule				+			—

This overview is based on 38 experimental studies, which showed regulating effects of the presented ECM factors on processes of OL development. This is not a complete list; many other studies have been performed.

+, Positive regulation; —, negative regulation; ECM, extracellular matrix; OL, oligodendrocyte; TLR, Toll-like receptor.

recently confirmed.¹⁹ Also, others have shown that increased HMW hyaluronan can inhibit OL progenitor maturation *in vitro*.⁷⁷ After central nervous system injury, HMW hyaluronan is rapidly degraded into low-molecular-weight hyaluronan. Studies in MS patients showed that the low-molecular-weight hyaluronan can inhibit OL progenitor maturation via the Toll-like receptor 2.⁷⁸

In conclusion, because ECM molecules have important regulating functions during WM (re)generation, the composition of the ECM is an important determinant of whether (re)myelination is successful. More ECM components that are known to influence OLs are listed in Table 3.

Growth Factors

Patterning Factors

Many growth factors play an important role in regulating the precise control of OL development from early to late stages. Studies in rodents and chicks provided much insight into the actions of different growth factors, their role at certain developmental stages, and their interactions via signaling pathways. During early development, several morphogens (alias patterning factors) are crucial for neural patterning and proper emergence of OL progenitor populations, like sonic hedgehog, WNT, retinoic acid, and bone morphogenetic proteins.⁷⁹ These specific sets of patterning factors generate OL progenitor populations with unique expression profiles. It is, however, unclear to what extent these initial identities are affecting OL functions later in life.

These patterning factors also show important regulatory functions at later stages of OL development. For example, although sonic hedgehog is important in the specification and proliferation of early OL progenitors,⁸⁰ it also promotes the differentiation toward OLs⁸¹ and is a potent stimulator of remyelination.⁸² On the other hand, bone morphogenetic proteins can inhibit OL differentiation,⁸³ work as a chemorepellent in migration,^{84,85} and impair remyelination.⁸⁶ Although WNT is a negative regulator of OL specification,⁸⁷ its signaling is required for the timing of OL maturation and (re)myelination.⁸⁸ Retinoic acid also has dual roles, because it can inhibit OL progenitor proliferation and differentiation⁸⁹ but has shown a positive effect on remyelination.⁹⁰

Glycoprotein Growth Factors

Growth factors classically refer to the glycoprotein families, including platelet-derived growth factor (PDGF), insulin-like growth factor, epidermal growth factor, and fibroblast growth factors (FGFs). Binding of the growth factor to its receptor activates downstream signaling pathways that often overlap or interact with other signaling pathways. Many of these growth factors function in OL progenitor proliferation, maturation, differentiation, and myelin formation, and are secreted by astrocytes, microglia, and/or neurons (Table 2).

Of the PDGF family, PDGF-AA is most known for its functions in OL development. PDGF-AA binds to the PDGF α receptor that is expressed by many OL progenitors.⁹¹ Mice lacking PDGF α receptors demonstrate defects in OL progenitor proliferation and migration and show precocious OL differentiation and myelination.^{92,93} Furthermore, many FGF family members, which act via four FGF tyrosine kinase receptors (receptors 1 to 4), also have various functions during OL development, starting during early neural patterning. Of the FGF family, FGF2 is studied most and shows, next to many other functions, important effects on OL proliferation, migration, and myelination. Interestingly, mice lacking FGF2 show no post-natal peak period of oligodendrogenesis but have a normal OL density in adulthood.⁹⁴ This indicates that, although FGF2 has important functions in OL development, other signaling cascades can compensate for FGF signaling. Furthermore, similar to the FGFs, epidermal growth factor has many effects on proliferation and differentiation of neural stem cells into OLs, both during embryonic development and in adulthood.⁹⁵ In addition, insulin-like growth factor-1 signaling can increase OL progenitor proliferation and maturation, as well as late-stage OL differentiation and myelination.⁹⁶ Overexpression of insulin-like growth factor-1 can increase brain size and myelination of axons and can result in an increased number of myelinated axons.^{97,98} In conclusion, growth factors have many important actions on OLs that differ depending on the developmental stage.

Neurotrophins

Neurotrophins are a class of growth factors secreted by astrocytes, microglia, and/or neurons, which can activate the p75 neurotrophin receptor and the family of tyrosine kinase (Trk) receptors consisting of TrkA, TrkB, and TrkC. The neurotrophins that have been shown to regulate OL development involve brain-derived neurotrophic factor, neurotrophic factor 3, nerve growth factor, and neuregulin. Brain-derived neurotrophic factor has clear effects on myelination, because it can increase expression of myelin proteins *in vitro*; when brain-derived neurotrophic factor is absent, this results in reduced expression of myelin proteins and delayed myelination *in vivo*.⁹⁹ Nerve growth factor can bind TrkA or p75 neurotrophin receptor and, depending on downstream interactions with other signaling pathways, can either encourage survival or induce cell death of OLs.¹⁰⁰ Furthermore, because neuronal activity can regulate regional secretion of neurotrophins, neurotrophins could also have crucial roles in deciding whether or when OLs myelinate certain axons.¹⁰¹

Blood-Derived Factors

The vasculature transports many factors from the periphery to the brain, and from one brain region to another. The passage of the blood-derived factors into the brain edema is strictly

regulated by the BBB, which is composed of endothelial cells, astrocyte end feet, pericytes, and the basement membrane. The cerebral endothelial cells have specialized tight junctions to limit the diffusion of toxic substances and carry specific transporters with properties to selectively transport certain nutrients into the brain.¹⁰² The astrocyte end feet interact with the endothelium via gap and adherens junctions and are thought to help determine BBB function. The pericytes are, however, thought to be predominantly responsible for endothelial proliferation, vascular branching, and the regulation of endothelial cell junction sites. Pericytes are flat contractile cells that develop around capillary walls and are embedded in the basement membrane. The basement membrane provides the mechanical barrier that avoids the passage of macromolecules and can support in functions like cell migration.

One class of the signaling molecules produced in the periphery with functions in the brain is the thyroid hormones, like triiodothyronine (T3). T3 is derived from the less active prohormone thyroxine that, on its turn, is produced by the follicular cells of the thyroid. The production of T3 and thyroxine is regulated by thyroid-stimulating hormone secreted from the pituitary gland. Iodothyronine transporters regulate T3 and thyroxine transport over membranes. On binding of the nuclear thyroid hormone receptors TR α and TR β , thyroid hormones regulate gene expression, including those genes that are involved in OL development.¹⁰³ Several studies indicated that T3 is important in regulating the switch from OL progenitor proliferation to OL differentiation.¹⁰⁴ Furthermore, thyroid hormones promote the maturation of postmitotic OLs by increasing the number of committed OL progenitors and by up-regulating the expression of various myelin genes.^{105,106} Mice lacking the T3 receptor never switch from OL progenitor proliferation to OL progenitor maturation and develop myelin defects.¹⁰⁷ Furthermore, it was shown that treatment with TR β agonists increases differentiation of human OL progenitors *in vitro* and can enhance myelination of the developing mouse brain *in vivo*.¹⁰⁸ In conclusion, thyroid hormones have important functions in stimulating, and especially timing of, OL differentiation (Table 1).

Apart from the physical barrier and transportation functions, the vasculature regulates local secretion of signaling molecules, such as chemokines, by endothelial cells. Although the presence of chemokines on vascular endothelium is extensively studied for its role in leukocyte extravasation, new studies also indicate a role for chemokines in OL development. Stromal cell–derived factor 1 binds C-X-C chemokine receptor 4, which is highly expressed on membranes of migrating OLs.¹⁰⁹ When C-X-C chemokine receptor 4 function in mice is lost, OL progenitors are not attracted to the vasculature and thereby lose migration behavior, because their physical support for movement is absent.¹⁰⁹ Also, other chemokine receptors are expressed on developing OLs, like CCR3, which binds eotaxin chemokine C-C motif ligand 11. Chemokine C-C motif ligand 11 readily crosses the BBB and has been shown to increase OL progenitor proliferation.¹¹⁰ As more and more studies indicate a role for

chemokines in OL development,¹¹¹ it is expected that modulation of cytokine levels can contribute to a supportive microenvironment for myelin (re)generation.

Toward Improved Regenerative Medicine

Identification of Microenvironmental Factors in WMDs

Because many studies indicate that the microenvironmental factors are important regulators of proper WM development, it is expected that in states of WM disease, changes in the microenvironment will determine successful or failed WM (re)generation. Indeed, all WMDs show small to large changes in the microenvironment.

One of the WMDs that is extensively studied for the involvement of microenvironmental factors is MS. MS shows high numbers of OL progenitors in active demyelinating lesions but low OL progenitor numbers and complete demyelination in chronic-inactive lesions, implying that during the disease course the MS microenvironment is not successful in supporting OL maturation and myelination. Many immune cells, as well as astrocytes and microglial cells, infiltrate the lesion sites, which show active morphologies and changed expression profiles.⁴⁹ Active lesions are filled with proinflammatory microglia opposing remyelination, whereas in chronic-active plaques, anti-inflammatory/immunoregulatory microglia are present that secrete proregenerative factors. Chronic-active lesions in MS patients are further characterized by a changed ECM, such as increased levels of fibronectin and hyaluronan, in which activated astrocytes play an important role.⁴⁹

Another WMD that shows clear involvements of the microenvironment is VWM. As described above, recent studies confirm a major role for astrocytes in WM pathology of patients with VWM.¹⁹ *In vitro*, OLs of VWM mouse models develop normally in the presence of healthy astrocytes, but healthy OLs show impaired maturation when co-cultured with astrocytes carrying VWM mutations.¹⁹ This suggests that, in prospective cell therapies, grafts of healthy OLs would counter an aversive diseased microenvironment and, therefore, might fail successful myelination. Therefore, a better understanding of the microenvironmental factors during normal OL development and WM formation, and how these interplay with intracellular signals, will be of utmost importance in understanding why WM (re)generation fails in WMDs.

Mouse Modeling

There are different approaches to identify microenvironmental factors. Pathologic studies on patient postmortem tissue have been informative but lack insight into the microenvironmental state during early disease states. Transgenic animal models carrying mutations in genes involved in functions of either intracellular OLs or microenvironmental signaling gave many new insights into OL and WM development.¹¹² There are animal models that representatively mimic the human disease and

Figure 1 Schematic overview of induced pluripotent stem cell (iPSC) technology in regenerative medicine and important considerations in studies of white matter disorders (WMDs) with microenvironmental involvements. iPSC technology is broadly used to generate patient cells of interest *in vitro*, to study disease mechanisms using functional assays, co-culture systems, and profile screening, and to explore treatment options using compound screening and transplantation assays. However, existing protocols often do not take microenvironmental factors into consideration, which play important roles in WMD pathology. Furthermore, oligodendrocyte (OL) heterogeneity and non-OL-specific processes are involved. Therefore, *in vitro* stem cell protocols would benefit from the latest advances in the stem cell field, such as co-culture, three-dimensional (3D), and nanosubstrate techniques, toward the development of improved regenerative medicine for WMDs.

have been helpful in identifying new microenvironmental factors.¹⁹ However, many disease mutations in rodent models do not mimic typical characteristics of human pathology. For example, mouse models for PMD, like the jimpy mouse that has a spontaneous X-linked intronic deletion or transgenic mice with increased copy numbers of *Plp1*, show impaired myelin formation.^{113,114} However, *Plp* knockouts show almost no myelin defects, whereas *PLP*-null mutations in humans result in PMD.¹¹⁵ Furthermore, a mouse model for Alexander disease, overexpressing the human mutant *GFAP*, only resulted in Rosenthal fibers after a 30% increase of the mutant protein and did not resemble other pathologic or clinical aspects of Alexander disease.¹¹⁶ Another transgenic mouse model harboring human knock-in mutations of Alexander disease showed defects in neurogenesis and cognition, because the mutant *GFAP* is also expressed in neural stem cells.¹¹⁷ However, although the mice developed Rosenthal fibers, the white matter architecture and myelination were unaffected, even when *GFAP* expression was increased to a lethal dose.¹¹⁸ Furthermore, several mouse models for X-linked adrenoleukodystrophy (X-ALD), a lipid storage disorder that affects central nervous system myelin in humans, showed no neurologic symptoms or myelin lesions in mice.¹¹⁹

In conclusion, the species differences make *in vivo* modeling of human WMDs in rodents still challenging. Furthermore, although in some cases experimental manipulation of the pathway led to an abnormal phenotype in mice, it is the question if this is the same pathologic causality and mechanism in human patients. On the other hand, although

modeling of genetic WMDs is often dependent on transgenic techniques in mice, models of WMDs that are caused by injury or inflammation can be accomplished in larger animals (eg, pigs or primates). Because the brains of these animals are larger and have more convolutions, more WM, and a higher complexity, the results are also more translatable to humans.

iPSC-Based Modeling

Another strategy in dissecting microenvironmental factors involves *in vitro* modeling. Unfortunately, the isolation of human primary cells is restricted by the availability of tissue, and the variability between subjects is high. However, the use of human induced pluripotent stem cell (iPSC) technologies overcomes many of these issues. The invention of iPSC technology gave prospects for generating human *in vitro* models for WMDs. For example, iPSC-derived OL cultures generated from PMD patients showed reduced numbers of OL progenitors and abnormally branched mature OLs compared with controls.^{12,120} Furthermore, iPSC-derived astrocytes from Alexander disease patients, with either the early onset or the later onset variant of the disease, showed GFAP-positive aggregates, similar to (early-stage) Rosenthal fibers present in the patient brain. In addition, Alexander disease iPSC-derived astrocyte cultures suggested increased mammalian target of rapamycin activation and up-regulated cytokine secretion.¹²¹ Also for X-ALD, iPSC-derived cultures showed typical human pathology.¹²² In X-ALD patient iPSC-derived OLs, accumulation of the long fatty acid chains was observed, whereas X-ALD patient iPSC-derived neurons were unaffected.¹²² Furthermore, X-ALD iPSC models could discriminate between the more and less severe phenotypes.¹²³

Although iPSC technology gives promising results, it is still challenged by high variability, and many iPSC models lack robust correlation between iPSC-derived OL defects and disease severity in patients. Also, current iPSC cultures often do not capture correct regional or functional subtypes of OLs or do not model the different microenvironmental factors present in the diseased brain. Furthermore, enriched culturing conditions could mask disease phenotypes, and monocultures lack the interactions with their (pathologic) environment.

Future of New Stem Cell Technologies

Although iPSC technology accelerated the development of new *in vitro* OL differentiation protocols,^{124–126} these differ in culturing methods, such as media compositions and growth additives during early neural induction up to late OL maturation. Many of the stem cell-derived OL populations are tested in myelination assays, including co-cultures with neurons, *ex vivo* brain slices, and/or *in vivo* demyelination models.^{4,127,128} However, because not all iPSC-derived OL populations are analyzed with the same assays and with standardized readouts, it is unclear what the differences are

between the populations. Besides, the brain shows OL heterogeneity.^{26,129,130} Differences in OL proliferation and maturation and the properties of myelin, including myelin thickness, intermodal length, and axonal thickness of myelinated axons, are all reported between OL populations *in vivo*.^{23,131} Furthermore, many WMDs show WM degeneration in particular patterns, suggesting that subpopulations of OLs are affected.¹³² For that reason, we need specific iPSC-derived OL populations to study certain WMDs (Figure 1). It is possible that the existing protocols generate stem cell–derived OL populations that mimic certain characteristics or even specific OL subpopulations found *in vivo*. To model disease-specific OL (re)generation failure and to produce OL populations that can replace regional-specific OLs, we need a better understanding of how *in vitro* procedures affect OL identity and properties. Thus, standardized assays are required, including expression profiling of specific gene sets and detailed analysis of the myelination properties (Figure 1).

Many *in vitro* stem cell protocols focus on optimizing culture conditions to generate OL monocultures. These monocultures are not suitable for disease modeling or identifying interactive mechanisms between OLs and microenvironmental factors. Some disease phenotypes only become evident in complex or multicellular systems and appear after failed interactions with the microenvironment. Furthermore, many protocols use multiple supplements to nurture the stem cell–derived OL products. It is possible that these well-fortified conditions mask disease-associated phenotypes in patient-derived cultures. Therefore, to model such WMD phenotypes *in vitro*, we need dynamic culture systems that allow dissection of cell-intrinsic from cell-extrinsic aspects (Figure 1).

Because the importance of proper neuron-OL communications in myelination has already been appreciated for decades, there are many different neuron-OL co-cultures.^{133,134} However, the evaluation of myelin formation in these cultures is still challenging, for several reasons. At first, mixed cultures with random distribution of neural cell bodies and cellular processes are hard to control and analyze. Furthermore, it is hard to manipulate cell-specific pathways, to quantify neuronal- versus OL-related changes, and to uncouple direct from indirect (axonal-OL interaction) effects in traditional cultures. Last, most of these cultures contain one or more cellular products that are nonhuman. To our knowledge, we still lack reproducible co-cultures of human OLs and human neurons. In addition, many other microenvironmental factors determine OL functions, which should be included in these cultures. Therefore, we need human multicellular culture systems that are suitable to study WMDs and that are controllable and quantifiable (Figure 1).

Artificial nanosubstrates helped the development of new tools to study myelination in complex cultures. For example, to study the effects of the biophysical properties of the axon on myelin formation, a neuron-free culture system was generated. OLs were cultured in the presence of nanofibers, which substituted the axons and provided a substrate for myelinating OLs.¹³⁵ Next to the fact that OLs successfully myelinate

nanofibers, it was shown that varying fiber diameters can regulate myelination processes.¹³⁵ Because no neurons were present in these cultures, other axonal/neuronal signals were not responsible for the observed effects. Therefore, this *in vitro* system gave new methods to manipulate the biophysical properties of the axons and can be used to study how administration of different factors affect myelination via direct OL signaling. Furthermore, this nanofiber-based culture is useful in modeling the regulating actions of ECM factors. It was shown that myelination of larger-fiber diameters increased when nanofibers were coated with ECM glycoprotein laminin 2.^{26,135} In summary, although co-cultures give insight into complex intercellular signaling, fabrication of nanofibers can help to dissect OL intrinsic factors in the presence or absence of different signaling molecules (Figure 1).

Furthermore, the use of artificial nanosubstrates also gave new ways to compartmentalize different cell types and structures into separate chambers, while allowing communication and interaction.¹³⁶ By separating neuronal cell bodies from axons, microfluidic chambers give the possibility of studying OL-axon interactions without interference of neuronal cell bodies. It was shown that these myelinating co-cultures together with microfluidic technology are able to follow OL process extension and retraction, OL anchoring to the axon, and myelin wrapping over long periods of time.¹³⁷ Thus, different artificial nanosubstrates can be used to investigate the effects of microenvironmental factors in a controlled time- and location-specific manner (Figure 1).

Many of the latest technologies to improve stem cell cultures involve three-dimensional (3D) conditions. 3D cultures more closely resemble the *in vivo* differentiation and microenvironment than the widely used flat two-dimensional culture methods, by generating complex interactions between the neural cells.¹³⁸ One of the 3D culture approaches involves embedding of cells in a thick gel of different kinds of ECM proteins (eg, Matrigel; Corning, Corning, New York).^{139,140} It was shown that 3D conditions encourage OL maturation.¹⁴¹ The mechanical stiffness of the gel matrix is one of the properties that influences cellular processes. It was shown before that soft brain-like matrices (1.5 kPa) enhanced OL progenitor branching compared with more rigid substrates (30 kPa).¹⁴² Another 3D culture method involves free-floating aggregate structures (alias cerebral organoids or spheroids). In cerebral organoids, the pluripotent stem cells differentiate into complex 3D structures with organization of different progenitor domains and brain regions.¹⁴³ Although current 3D cerebral organoids receive more attention for the modeling of early neural and neuronal structures, these 3D cultures also contain radial glial stem cells¹⁴⁴ and glial cells¹⁴⁵ and, therefore, have potential to study OL development. Interestingly, recent 3D models not only show presence of neuronal and glial cell types but also the presence of endothelial cells.¹⁴⁶ Therefore, capillary-like networks in neural cultures containing developing OLs may aid the study of blood-derived factors on myelination. Because 3D cultures have complex interactions with the cellular and extracellular microenvironment

that resemble important aspects of the *in vivo* situation, these are interesting tools in the study of microenvironmental factors. Next to 3D analysis *in vitro*, different studies show transplantation of stem cell–derived 3D products into animal models.¹⁴⁷ Monitoring of grafted patient-derived cells in a brain gives the possibility of studying cellular processes in a living multifaceted microenvironment and over longer time periods. Although standardization of 3D cultures is challenging, even more so than two-dimensional cultures, these developments illustrate how 3D and complex culture techniques promise new tools to dissect and modulate microenvironmental factors in OL development (Figure 1).

The design of good readouts for complex multicellular cultures to study OL–microenvironmental interactions is also not straightforward. However, upscaling to high throughput seems possible with the introduction of multiwell-based platforms, including those for 3D culture.¹⁴⁸ Because superresolution microscopy has been evolving for years, the ability of screening complex cultures at high resolution will make imaging of different OL–microenvironmental interactions possible. Because myelin is necessary for the efficient and rapid transmission of action potentials, properties of neuronal activity are another way to analyze (re)generation of myelin. Integrated systems with multielectrode arrays and optical imaging tools are in development and have interesting potentials to study OL actions on neuronal network functionality. Furthermore, evolution in RNA sequencing of single cells has only started and will aid in the identification of cell-specific pathways in complex systems. Overall, although the use of stem cell techniques in the studies of WMDs increased enormously, recent developments of different technologies promise new tools to help us investigate the interactions between OLs and microenvironment in health and disease (Figure 1).

Conclusions

It is known that the microenvironment, including connections with neighboring cells, factors provided by local cells, the ECM, and blood-derived factors, is an important regulator of basic processes of OL development and function. The long list of known factors underlines the complex and dynamic interplay between the microenvironment and the intracellular state of OLs, which results in OL heterogeneity.^{26,129,130} Also in the pathology of WMDs, the microenvironment is involved. To facilitate the development of new treatments for WMDs, it is of utmost importance to study how microenvironmental factors regulate normal and diseased WM development. Although future OL replacement therapies give great promises, these can only be successful if the host microenvironment is permissive for functional integration of the transplanted cells. New stem cell protocols and culture techniques can be helpful in dissecting and modulating microenvironmental factors and in testing new therapeutic targets, but they need standardization to evaluate OL products for functionality and subtype

specificity. To design better therapeutic strategies, we need to identify microenvironmental factors involved in the failure of proper OL development and WM (re)generation in patients with WMDs.

Acknowledgments

We apologize to all authors whose important work could not mention because of space limitations.

P.S.L. and V.M.H. conceptualized, drafted, and edited the manuscript.

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