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·Review·

Regulation of the timing of oligodendrocyte differentiation: mechanisms and perspectives

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Axonal myelination is an essential process for normal functioning of the vertebrate central nervous system. Proper formation of myelin sheaths around axons depends on the timely differentiation of oligodendrocytes. This differentiation occurs on a predictable schedule both in culture and during development. However, the timing mechanisms for oligodendrocyte differentiation during normal development have not been fully uncovered. Recent studies have identified a large number of regulatory factors, including cell-intrinsic factors and extracellular signals, that could control the timing of oligodendrocyte differentiation. Here we provide a mechanistic and critical review of the timing control of oligodendrocyte differentiation.

Keywords: oligodendrocytes; differentiation; timing; remyelination

Introduction

Multicellular animals develop on a predictable schedule that depends on both cell-intrinsic regulation and cell-cell interactions mediated by extracellular signals. During animal development, precursor cells in many tissues divide a limited number of times before terminally differentiating into post-mitotic mature cell types. An underlying timing mechanism controls not only the onset of cell differentiation, but also the size (total number of cells) of the tissues. In the developing vertebrate central nervous system (CNS), the timing mechanism has been well documented and extensively studied in cells of the oligodendrocyte lineage[1].

Evidence for the Timing Control of Oligodendrocyte Differentiation

About three decades ago, Temple *et al.* demonstrated that oligodendrocyte differentiation is related to the number of cell divisions in culture[2]. Later, Gao *et al.* revisited the concept by culturing oligodendrocyte precursor cells (OPCs) separately at 33°C and 37°C in the presence of mitogens [platelet-derived growth factor (PDGF) and neurotrophin-3] and the absence of thyroid hormone (TH), and showed that OPCs cultured at 33°C divide more slowly, but stop dividing and differentiate sooner after fewer cell divisions. These results suggested that there exist mechanisms that measure time but do not count OPC divisions before differentiation[3].

Does the timing mechanism also operate during oligodendrocyte differentiation *in vivo*? In the developing CNS, most OPCs appear to differentiate and myelinate axons on a predictable schedule. For instance, in the white matter of the mouse spinal cord, oligodendrocyte differentiation mainly occurs during early postnatal stages, peaking at around postnatal day 3. Recent molecular and genetic studies have suggested a strong correlation between OPC birth date and the time of their terminal differentiation, and delayed generation of OPCs in several genetic mutants is invariantly associated with delayed differentiation. In the spinal cord, most OPCs are produced from the ventral motor neuron progenitor (pMN) domain of neuroepithelium from embryonic day 12.5 (E12.5) to E14.5[4]; most of the

ventrally-derived OPCs start to differentiate in the white matter from postnatal day 0 (P0) to P5. In Nkx6.1 and Gli2-null mutant mice, the production of early OPCs from the ventral spinal cord is reduced and delayed, leading to a delay in OPCs occupying the entire spinal cord. In both cases, a significant delay in the terminal differentiation of oligodendrocytes also occurs in the mutants[5,6].

In contrast, in the developing spinal cord, only about 10% of OPCs are derived from the dorsal neuroepithelium[7]. The dorsal OPCs are produced at ~E15, 2–3 days later than their ventral counterparts. These dorsal OPCs do not differentiate until several days after birth. In Nkx6.1/Nkx6.2 double mutants and Dicer1$^{flox/flox}$/Olig1^{Cre} conditional mutants[6-8], the generation of ventral OPCs from the pMN domain is largely inhibited, and the vast majority of OPCs are of dorsal origin. Although the mutant spinal cords have numbers and densities of OPCs similar to controls at birth, oligodendrocyte differentiation and myelin gene expression do not occur in the newborn mutants.

The close associations between OPC birthdate and the onset of their maturation strongly suggest that a timing mechanism indeed operates *in vivo* for OPCs of both ventral and dorsal origin. However, it is uncertain whether the *in vivo* timing mechanism counts the number of cell divisions or measures the passage of time. The presence of a small number of immature OPCs in the adult CNS suggests the former. It is conceivable that these adult OPCs may have undergone fewer cell divisions than their differentiated siblings, and could cycle several more rounds prior to differentiation in later life.

Although the molecular basis of the counting mechanisms is not fully understood, it is known that the timing of oligodendrocyte differentiation is controlled by both intrinsic factors and extracellular signals (Fig. 1). To date, a large number of molecules have been implicated. Here, we provide a critical review to update the developmental roles of key regulatory molecules in the timing control of oligodendrocyte differentiation.

Intracellular Signals that Regulate the Timing of Oligodendrocyte Differentiation

In the past two decades, great progress has been made in the identification and characterization of a plethora of intrinsic factors that regulate oligodendrocyte differentiation. Some inhibit the differentiation process and are therefore

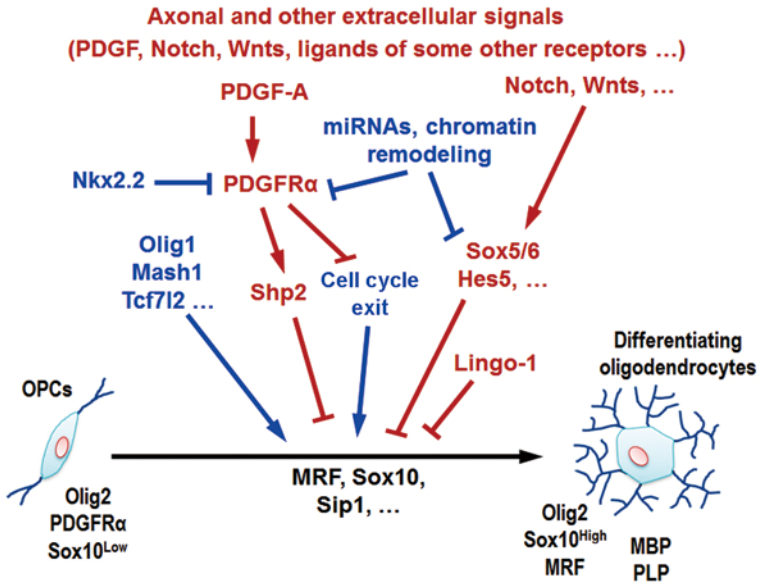

Fig. 1. Schematic of the molecular control of oligodendrocyte differentiation. Extracellular pathways such as PDGF-A/PDGFRα (red) inhibit differentiation by repressing the function or expression of differentiation-promoting factors such as Sox10, MRF and Sip1, while the cell-intrinsic regulators (blue) including Nkx2.2 and Olig1 enhance the function of those transcription factors directly and/or indirectly and initiate differentiation. The cross-talk of these factors determines the onset of differentiation.

considered to be negative factors, whereas others have the opposite function and are therefore considered to be positive factors. The negative factors are commonly expressed in immature OPCs and subsequently down-regulate during oligodendrocyte differentiation. In contrast, the positive factors are generally up-regulated in differentiating oligodendrocytes with little expression or function in immature OPCs.

Cell-Cycle-Dependent Kinase Inhibitors

Early studies demonstrated that several cell-cycle-dependent kinase (CDK) inhibitors, such as p18/INK, p21/Cip1, p27/Kip1, and p57/Kip2, gradually accumulate in dividing OPCs in culture, and overexpression of some of these proteins causes them to cease cycling and differentiate prematurely[9-11]. Thus, these inhibitors function as positive differentiation regulators. The increased level of CDK inhibitors in dividing OPCs and their daughter cells has been proposed to be part of the timing mechanism governing oligodendrocyte differentiation. However, the evidence obtained so far mainly comes from *in vitro* observations, and it remains uncertain to what extent these inhibitors are responsible for the timed differentiation during development *in vivo*. For instance, p27/Kip1 was suggested to be a major timing component in the transition from OPC proliferation to differentiation, based on *in vitro* studies. However, loss of p27/Kip1 expression in mice does not affect the timing of OPC differentiation, despite the increased number of OPC divisions[12]. This result demonstrates that inhibition of cell cycling can be uncoupled from the onset of oligodendrocyte differentiation. One explanation for this somewhat unexpected result is that p27/Kip1 controls cell division, while the initiation of differentiation involves other CDK inhibitors. Therefore, future studies are needed to investigate the unique and redundant roles of CDK inhibitors in the developmental control of the timing of oligodendrocyte differentiation, using transgenic and conditional knockout approaches.

Transcription Factors

The past decade has witnessed significant advances in understanding the transcriptional control of oligodendrocyte differentiation and CNS myelination. A large number of transcription factors of multiple classes have been implicated in the regulation of oligodendrocyte maturation, including the negative factors Hes5, ID2/4, and Sox5/Sox6[13-16] and the positive factors Sox10, Nkx2.2, MRF, Zfp191, Zfp488 and Sip1[17-22].

Negative Differentiation Regulators

Hes5, Id2 and Id4 have been suggested as major negative regulators of oligodendrocyte differentiation. Hes5 is expressed in immature OPCs but down-regulated in mature oligodendrocytes in the developing CNS. Over-expression of Hes5 in purified OPCs inhibits their differentiation[13], and conversely, its inactivation results in a mild increase in myelin gene expression[23]. In the same study[23], it was shown that Hes5 binds to the Sox10 promoter and suppresses its expression *in vitro*. Thus, Hes5 appears to have a small impact on the timing of oligodendrocyte differentiation by negatively regulating Sox10 expression.

The differentiation inhibitors Id2 and Id4 have been perceived as major components of the timing mechanism[14,15]. In dissociated culture, enforced expression of either stimulates OPC proliferation and blocks differentiation, possibly by binding to Olig2 and Olig1 proteins and consequently inhibiting their functions[14,15]. However, our recent studies showed that neither Id2 nor Id4 is significantly expressed in OPCs in the developing CNS, and inactivation of their genes has little or no apparent effect on oligodendrocyte differentiation in the spinal cord (unpublished data). Thus, the physiological roles of Id2 and Id4 in the control of oligodendrocyte differentiation during development remain debatable.

Sox5 and Sox6 are another two negative regulators that influence the timing of oligodendrocyte differentiation. It has been reported that oligodendrocytes express several Sox transcription factors during their development, including Sox8/Sox9/Sox10[17, 24-26] of group E that promote oligodendrocyte differentiation and Sox5/Sox6 of group D that have the opposite function[16]. Sox5 and Sox6 are expressed in PDGFRa$^+$ OPCs but down-regulated prior to differentiation. Loss of function of these two genes induces the precocious differentiation of oligodendrocytes *in vivo*[16]. The same studies showed that SoxD proteins bind to several Sox10 response elements in the promoters of myelin-specific genes[16]. Together, these observations suggest that SoxD proteins play a role in the timing of oligodendrocyte differentiation by antagonizing the function of SoxE proteins.

Intriguingly, all these negative regulators are initially

expressed in the ventricular zone along the entire dorsal–ventral axis during early gliogenic stages. At later stages, Hes5, Sox5 and Sox6 are expressed in OPCs and astrocyte precursor cells as well, suggesting that these genes may also play a role in regulating the differentiation of astrocytes.

Positive Differentiation Regulators

Among the positive transcription factors, Sox10, MRF and Sip1 appear to be absolutely required for oligodendrocyte differentiation and myelination, and thereby are unlikely to be involved in the timing process.

Increasing evidence indicates that the Nkx2.2 homeodomain transcriptional factor is a key timing component of oligodendrocyte differentiation. Our previous work demonstrated that Nkx2.2 expression is up-regulated in differentiating OPCs, and disruption of the Nkx2.2 gene leads to a dramatic inhibition of oligodendrocyte differentiation and myelin production throughout the CNS[22,27]. Our recent study using Nkx2.2 conditional mutants suggested that Nkx2.2 is not essential for oligodendrocyte differentiation; instead it controls the timing of differentiation by directly repressing the expression of PDGFRα in OPCs (unpublished data), possibly by recruiting a repressor complex including histone deacetylases (HDACs) and the DNA methyltransferase Dnmt3a[28]. Consistently, co-expression of Nkx2.2 and Olig2 induces ectopic and precocious oligodendrocyte differentiation in the embryonic chick spinal cord[29]. Based on these observations, Nkx2.2 appears to function as a major cell-intrinsic factor that switches on the differentiation program in oligodendrocytes in the CNS.

The Olig genes were identified as key regulatory transcription factors for the development of the oligodendrocyte lineage[30]. Olig2 functions to specify the fate of the oligodendroglial lineage, while Olig1 appears to play an important role in timing oligodendrocyte differentiation[31,32]. Olig1 and Sox10 have synergistic actions in promoting myelin gene expression[33,34]. In Olig1-null mice that retain the PGK-neo cassette, there is a mild delay in the onset of oligodendrocyte differentiation[32]. Paradoxically, Olig1 mutants without the PGK-neo cassette display a much more severe phenotype with an almost complete loss of mature oligodendrocytes[35]. Recently, we noted a marked decrease in the number of OPCs in the newborn spinal cord compared with normal control (unpublished data), suggesting that the more severe phenotype in these mutants may be attributed to an early defect in OPC generation. Interestingly, phosphorylated Olig1 switches its intracellular sublocalization from nucleus to cytoplasm during differentiation and in remyelinating oligodendrocytes after lesions[36,37]. It is conceivable that, during nuclear re-localization, Olig1 transports negative regulatory factors out of the nucleus to facilitate oligodendrocyte differentiation and myelination.

Also, Mash1 promotes the differentiation of cultured OPCs together with Nkx2.2 and Olig2[38]. Enforced expression of Mash1 in purified OPCs somewhat accelerates the increase of TRβ1 protein[13], the thyroid hormone receptor involved in oligodendrocyte differentiation, while loss of Mash1 function inhibits oligodendrocyte differentiation in the spinal cord[38]. However, due to the neonatal lethality of Mash1 mutants, it remains unclear whether Mash1 is absolutely required for oligodendrocyte differentiation, or simply regulates the timing of differentiation. Since Mash1 functions upstream of Hes5[39], it is possible that Mash1 regulates the timing of oligodendrocyte differentiation partly by repressing the function of Hes5.

Epigenetic Factors

Epigenetic chromatin remodeling events are crucial for many biological processes, including cell differentiation[40]. It was recently reported that HDACs interact with Tcf4/Tcf7l2 to promote oligodendrocyte differentiation by competing with β-catenin[41] and are required for differentiation during a critical time window[42]. In addition, HDACs and the DNA methyltransferase Dnmt3a may also participate in the Nkx2.2 repression of PDGFRα expression[28]. Thus, epigenetic regulation appears to play an important and perhaps permissive role in oligodendrocyte differentiation and myelin gene expression.

MicroRNAs have been demonstrated to influence oligodendrocyte differentiation post-transcriptionally by down-regulating the differentiation inhibitors. In $CNP^{cre}/Dicer^{flox/flox}$ conditional mutants, there is a mild delay of oligodendrocyte differentiation[43], suggesting that miRNAs fine-tune its timing[44]. Paradoxically, in $Olig1^{cre}/Dicer^{flox/flox}$ and $Olig2^{cre}/Dicer^{flox/flox}$ mutants, there is a more dramatic inhibition of myelin gene expression[43,45]. However, OPC generation is also reduced and delayed in $Olig1^{cre}/Dicer^{flox/flox}$ and perhaps in $Olig2^{cre}/Dicer^{flox/flox}$ mutants as well, and this early defect may contribute to the more severe phenotype in both mu-

tants considering the close association between OPC birth date and the timing of differentiation. miR-219 and miR-338 are defined miRNAs that are expressed in differentiating oligodendrocytes and function to repress the expression of three negative differentiation regulators, PDGFRα, Sox6 and Hes5[45,46]. Therefore, it is not surprising that disruption of miRNA formation in the Dicer gene results in a slight delay in the timing of oligodendrocyte differentiation.

Extracellular Signals Involved in the Timing of Oligodendrocyte Differentiation

Intercellular signaling plays cardinal roles in a variety of cellular processes including fate specification and cell differentiation in nearly all developing tissues. It is likely that extracellular signals derived from neurons or astrocytes may regulate the expression of intracellular factors involved in the process of oligodendrocyte differentiation. So far, several extracellular signals and/or their receptors have been implicated in the timing of oligodendrocyte differentiation.

PDGF Signaling

Early studies indicated that PDGF is required to maintain OPC division and inhibit differentiation, and removing it from the culture medium promotes the onset of oligodendrocyte differentiation[47,48]. Consistent with the *in vitro* data, in PDGF-A$^{-/-}$ mutants, the proliferation and migration of OPCs are strongly blocked. More importantly, OPCs differentiate prematurely, albeit the myelination is severely reduced at postnatal stages due to a much-reduced population of OPCs[49,50]. Similarly, conditional ablation of PDGFRα (PDGF receptor a) also exhibits a reduced population of OPCs and their precocious differentiation (Zheng and Qiu, unpublished observations). Based on these observations, PDGF is likely to be a key extracellular factor that maintains OPC proliferation but inhibits differentiation, and inactivation of PDGF signaling triggers the onset of oligodendrocyte differentiation.

In further support of this concept, loss of Shp2 protein tyrosine phosphatase, a downstream target of a variety of receptor tyrosine kinases including PDGFR[51], causes phenotypes similar to those observed in PDGF-A null mice[52]. This raises the possibility that PDGF-A/PDGFRα signals inhibit oligodendrocyte differentiation *via* the Shp2 pathway. In addition, another receptor tyrosine kinase target gene, Erk2, has been implicated as a regulator of the timing of oligodendrocyte differentiation[53]. At this stage, it remains unclear how the PDGFRα signaling pathway interacts with the above intrinsic factors to regulate the timing of oligodendrocyte maturation.

Thyroid Hormone Signaling

TH has been widely used to promote oligodendrocyte differentiation in culture[54]. It has been proposed that the timing control of oligodendrocyte differentiation involves both PDGF, which measures time as a timing component, and TH which initiates differentiation at the appropriate time as an effector[1,55]. Consistently, optic nerve myelination is delayed in hypothyroid animals during the early postnatal weeks. It was previously shown that the receptor TRα1 is responsible for TH-dependent oligodendrocyte differentiation[56]. Moreover, deletion of all thyroid receptors results in the incomplete differentiation of OPCs in adult optic nerve[57]. These observations provide strong evidence for the involvement of TH signaling in the control of oligodendrocyte differentiation and myelin formation, but the underlying mechanism remains largely unknown.

Notch Signaling

As expected, Notch signaling, the upstream regulator of Hes5, has also been shown to regulate the differentiation of oligodendrocytes. Notch1 is known to be an inhibitor of oligodendrocyte differentiation *in vitro*[58]. A deficiency in Notch1 leads to an increased abundance of the products of specific myelin genes during the first two weeks of postnatal life[59]. In Notch1 conditional knockout mice, a small population of premature and ectopic oligodendrocytes are found in the E17.5 spinal cord[60], similar to the phenotype of Hes5 mutants[23]. Notably, a separate study showed that Notch1 signaling plays a role in regulating precursor differentiation during CNS remyelination[61]. Thereby, the Notch signaling pathway is partly required for the correct spatial and temporal regulation of oligodendrocyte differentiation during development and in injuries.

Wnt/β-Catenin Signaling Pathway

The Wnt/β-catenin pathway is a key regulator of different stages of oligodendrocyte development[41,62]. Over-expression of Wnt protein or constitutive activation of β-catenin at an early stage of neural development significantly inhibits the expression of mature oligodendrocyte markers. Para-

doxically, Tcf4/Tcf7l2, an activated β-catenin effector, is transiently up-regulated in post-mitotic oligodendrocytes and in adult white-matter lesions[62,63]. Mutation of Tcf7l2 blocks oligodendrocyte differentiation without disturbing OPC proliferation[41]. These contradictory findings can be simply explained by the finding that OPC generation is markedly reduced in animals with constitutive activation of β-catenin or Wnt protein[41,62,64], perhaps by antagonizing Shh signaling, and the early defect in OPC production results in a significant delay of oligodendrocyte differentiation and myelin gene expression as in many other genetic mutants described earlier.

Considering the up-regulation of the β-catenin antagonist APC at later stages of oligodendrocyte development, it is conceivable that activated Wnt/β-catenin signals are required for the initiation of differentiation, but prevent subsequent stages of maturation and myelination in developing tissues and lesions. There is now a general consensus that dysregulation of the Wnt/β-catenin pathway profoundly disturbs the oligodendrocyte differentiation process and CNS myelin repair.

Other Possible Signaling Pathways

Accumulating evidence suggests that oligodendrocyte differentiation and maturation can be influenced by other intercellular signaling pathways. For instance, the G protein-coupled receptor Gpr17 has been proposed as an intrinsic timer of myelination[65]. It is up-regulated in differentiating oligodendrocytes at early postnatal stages, and then down-regulated after terminal differentiation. Sustained expression of Gpr17 in oligodendrocyte lineage cells causes severe myelination disorders and stalls differentiation at early stages. Conversely, *in vitro* data showed that loss of function of Gpr17 causes premature oligodendrocyte myelination with an accelerated expression of MBP**.** These experiments indicate that Gpr17 functions as a negative regulator of oligodendrocyte differentiation and myelination during development. However, unlike PDGFRα and other negative regulators, Gpr17 is not expressed in immature OPCs; instead, its expression pattern is similar to other positive factors with regard to up-regulation during oligodendrocyte differentiation. Also, precocious expression of mature oligodendrocyte markers has not been reported in mutant or transgenic tissues. In addition, an independent study showed a contradictory result that treatment with the Gpr17 ligand UDP-glucose promotes oligodendrocyte differentiation[66]. Despite the important role of Gpr17 in the myelination process, its involvement in the timing control of oligodendrocyte differentiation remains uncertain.

Another negative regulator of oligodendrocyte differentiation and myelination is Lingo-1, a surface molecule containing the luceine-rich repeat and Ig domains. Lingo-1 is expressed in both oligodendrocytes and neurons, inhibits axonal growth *via* RhoA, and prevents oligodendrocyte differentiation[67]. Lingo-1 conditional knockout mice exhibit an early onset of CNS myelination[68]. Moreover, a Lingo-1 antagonist promotes spinal cord remyelination in myelin oligodendrocyte glycoprotein-induced experimental autoimmune encephalomyelitis[69]. Currently, it is not clear whether Lingo-1 functions autonomously or non-autonomously to regulate oligodendrocyte differentiation, as it is expressed in both neuronal axons and OPCs. If it functions non-autonomously, these findings may provide insights into negative regulation by axonal signaling for the temporal control of oligodendrocyte differentiation.

The serine/threonine kinase bone morphogenetic protein receptors (BMPRs) have also been implicated as negative regulators of oligodendrocyte myelination through the Smads (Smad1/5/8)[70,71]. Consistent with this, deletion of Smad7, an inhibitor of Smads in the BMP pathway, delays oligodendrocyte differentiation[19]. It has been proposed that Smad7 promotes oligodendrocyte differentiation by suppressing the expression of Hes5 and Id2/Id4[19]. However, BMPR knockouts do not appear to have an obvious inhibitory effect on oligodendrocyte differentiation in the CNS[72,73], consistent with the lack of significant expression and function of ID (inhibitor of differentiation) genes in OPC development *in vivo*. Thus, the role of BMP signaling in the timing of oligodendrocyte differentiation requires further investigation.

Perspectives and Challenges

Temporal control of oligodendrocyte differentiation is essential for proper axonal myelination and functioning of the vertebrate CNS. The timing of oligodendrocyte differentiation and myelination is likely regulated by intercellular signaling but realized by the activation of intrinsic factors. Extensive research in the past decades has identified many extracel-

lular and intracellular factors that regulate oligodendrocyte development by distinct molecular mechanisms including signal transduction, transcriptional control, protein interaction and epigenetic regulation. While some of these play an instructive role in the control of oligodendrocyte differentiation, others may simply serve a permissive role, possibly along with other cell types. With the advent of high-throughput DNA/RNA sequencing, proteomics and gene expression analysis[65,74], it is likely that many more regulatory molecules will be identified as involved in the oligodendrocyte differentiation process. One future challenge will be to determine their biological roles in oligodendrocyte development using a combination of molecular, cellular, transgenic and knockout approaches. Strict criteria must be used to assign their physiological functions, and *in vitro* functional assays need to be combined with *in vivo* analyses, while expression analysis should be verified by both immunostaining and RNA *in situ* hybridization. It has been noted that some of the regulatory molecules that were thought to be important for oligodendrocyte differentiation and myelination are not even expressed in cells of the oligodendrocyte lineage and null mutants display minimal phenotypes.

Another related challenge is to determine the working mechanisms of these differentiation factors and distinguish oligodendrocyte-specific from generic cellular functions, such as energy metabolism or cell survival. With the sophisticated Cre/loxP technology, it is possible to disrupt an irrelevant or ubiquitously-expressed gene in the oligodendrocyte lineage and cause a severe phenotype in oligodendrocyte lineage development and CNS myelination.

Perhaps a greater challenge will be to determine the epistatic relationships of the regulatory factors during the differentiation process. As described above, many intrinsic factors and extracellular signals are known to regulate oligodendrocyte differentiation. However, cross-regulation or genetic interactions among these differentiation factors have not been defined. Also, it is not clear how the activators and inhibitors maintain a balance in promoting and repressing oligodendrocyte differentiation during the orchestration of oligodendrocyte development.

The ultimate challenges will be translating the knowledge of oligodendrocyte development into therapeutic approaches that promote remyelination in human CNS injuries and diseases. It is known that OPCs are present in the adult CNS and manage to migrate to lesions after injury, but fail to differentiate and effectively form myelin, suggesting that the developmental timing is blocked at a pre-differentiation stage in demyelinating lesions[75-78]. It will be of interest and importance to investigate what keeps the intrinsic clock from ticking in adult OPCs. Exploration of the timing control of oligodendrocyte differentiation during development will help us better understand the myelination/remyelination process and discover potential therapeutic targets for CNS myelin repair.

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