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Review

Updates and challenges of axon regeneration in the mammalian central nervous system

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Axon regeneration in the mammalian central nervous system (CNS) has been a long-standing and highly challenging issue. Successful CNS axon regeneration will benefit many human diseases involving axonal damage, such as spinal cord injury, traumatic brain injury, glaucoma, and neurodegenerative diseases. The current consensus is that the diminished intrinsic regenerative ability in mature CNS neurons and the presence of extrinsic inhibitors blocking axon regrowth are two major barriers for axon regeneration. During the past decade, studies targeting the intrinsic axon growth ability via regulation of gene transcription have produced very promising results in optic nerve and/or spinal cord regeneration. Manipulations of various signaling pathways or the nuclear transcription factors directly have been shown to sufficiently drive CNS axon regrowth. Converging evidence reveals that some pro-regenerative transcriptomic states, which are commonly accomplished by more comprehensive epigenetic regulations, exist to orchestrate the complex tasks of injury sensing and axon regeneration. Moreover, genetic reprogramming achieved via transcriptome and epigenome modifications provides novel mechanisms for enhancing axon regeneration. Recent studies also highlighted the important roles of remodeling neuronal cytoskeleton in overcoming the extrinsic inhibitory cues. However, our knowledge about the cellular and molecular mechanisms by which neurons regulate their intrinsic axon regeneration ability and response to extrinsic inhibitory cues is still fragmented. Here, we provide an update about recent research progress in axon regeneration and discuss major remaining challenges for long-distance axon regeneration and the subsequent functional recovery.

Keywords: axon regeneration, optic nerve regeneration, spinal cord regeneration, epigenetic, transcription factors, reprogramming, glaucoma

Introduction

Long-distance axon regeneration is one of the most important aspects for successful functional recovery after neural injuries in the central nervous system (CNS). There are two major reasons that neurons in the mature mammalian CNS fail to regenerate their axons. One is the diminished intrinsic neural regeneration ability of mature CNS neurons, which is regulated by gene transcriptional and epigenetic programs. The other is the presence of extrinsic inhibitors blocking axon growth at the nerve growth cone. Great progress has been made during the past decade with single and combined approaches of gene network regulation and overcoming extrinsic factors to promote post-injury axon regeneration in the CNS. Here, we will provide concise updates of recent progress in CNS axon regeneration research and discuss existing barriers and challenges the field still faces.

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Transcriptional regulation of intrinsic axon regeneration ability

Axon regeneration is a highly coordinating process related to various cellular events, including but not restricted to the injury sensing, axonal transport, the synthesis of macromolecules, cellular energy homeostasis, and cytoskeletal organization. It is thus a consensus that the regulation of a single terminal gene may not be sufficient to drive post-injury axon regeneration, especially across a long distance (Figure 1). Indeed, the transition of transcriptomes in neurons toward regenerative states is usually initiated from the nuclear hubs of

transcriptional factors (TFs), which in turn coordinate the expression of multiple downstream regeneration-associated genes (RAGs). In support, many previous studies have used either selected real-time quantitative polymerase reaction (qPCR) or unbiased whole-genome sequencing to assess the transcriptional profiles of regenerating neurons. The results indicated that most identified axon regeneration promoting approaches to date, such as upregulation of ciliary neurotrophic factor (CNTF), c-Myc, Kruppel-like factors 6/7 (Klf6/7), Lin28, oncomodulin/cAMP, p53, Stat3, Sry-related HMG box 11 (Sox11), Akt, and dual leucine zipper kinase (Dlk) and downregulation of Klf4, phosphatase and tensin homolog (Pten), glycogen synthase kinase 3β (Gsk-3β) , suppressor of cytokine signaling 3 (Socs3), and murine double minute 2 and 4 (Mdm2/4), effectively changed the transcriptome of CNS neurons toward pro-regenerative states via modulation of gene transcription (Table 1). Among these molecules, some of them are TFs directly regulating gene transcription and the others are signaling regulators acting upstream of gene transcription (Figure 1).

During the past decade, the roles of hub TFs have been investigated in axon regeneration. Neurons in the peripheral nerve system (PNS) regenerate their axons spontaneously after axotomy, and several TFs, such as c-Jun, Atf3, Stat3, and mothers against decapentaplegic homolog 1 (Smad1), have been shown to be important for PNS axon regeneration (Raivich et al., 2004; Seijffers et al., 2007; Saijilafu et al., 2011, 2013). In the CNS, overexpression of Stat3, a key TF in the cytokine pathway, could significantly promote axon regeneration after the optic nerve or spinal cord injury (Luo et al., 2016; Mehta et al., 2016). Real-time qPCR data revealed that Stat3 regulated the transcription of various RAGs, including Sprr1a, and TFs, including Atf3, Socs3, and itself (Luo et al., 2016; Mehta et al., 2016). The Smad1-mediated bone morphogenetic protein (BMP) signaling is required for the axotomy-initiated sensory neuron axon regeneration and is developmentally downregulated, which is associated with the aging-dependent decline of regenerative ability. Indeed, direct activation of Smad1 selectively in adult sensory neurons has been shown to enhance the dorsal column axon regeneration after the spinal cord injury (Parikh et al., 2011). A later study demonstrated that Smad1, as a hub TF, worked with histone-modifying enzymes to regulate the transcription of RAGs (Finelli et al., 2013). Also, BMP4 overexpression has been shown to promote retinal ganglion cell (RGC) survival and axon regeneration (Thompson et al., 2019). The Klf family of TFs is well-known for its association with CNS development and regeneration. The developmentally downregulated TFs Klf6/7 have been shown to enhance optic nerve and cortical spinal tract (CST) regeneration when overexpressed (Moore et al., 2009; Blackmore et al., 2012), whereas deleting the developmentally upregulated Klf4 also resulted in enhanced optic nerve regeneration (Moore et al., 2009). RNA-seq approach has been applied to interrogate the transcriptomic transition in cultured P5 mouse cortical neurons upon Klf6 overexpression, showing 250 significantly upregulated genes and 204 significantly downregulated genes (Wang et al., 2018b). It is worthy of note that manipulations of single or some combinatory terminal genes downstream of Klf6 failed to mimic Klf6 overexpression-induced promotion of axon regeneration, reemphasizing the necessary roles of high-hierarchy transcriptional hubs in various comprehensive cellular programs such as the axon growth. Beside RNA-seq of P5 mouse cortical neurons, microarray analysis (sub-genome) of purified RGCs from P4 mice has also been adopted to explore the pro-regenerative transcriptome with Klf7 overexpression or the anti-regenerative transcriptome with Klf9 or Klf16 overexpression (Galvao et al., 2018). Interestingly, Klf4 has been reported to interact with Stat3 and suppressing Stat3-dependent gene expression by blocking its DNA-binding activity (Qin et al., 2013). In addition to Klf family TFs, the developmentally downregulated Sox11, when overexpressed in mature neurons, has been shown to sufficiently promote axon regeneration in both optic nerve and spinal cord injury and regeneration model systems (Wang et al., 2015; Norsworthy et al., 2017). Whole-genome transcriptome profiling using RNA-seq of purified post-injury (3d) RGCs revealed 2797 differential genes (0.1 FDR cut-off) when comparing Sox11 overexpression with the control (Norsworthy et al., 2017). Key developmentally declined TF, c-Myc, was able to promote neuronal survival and axon regeneration after optic nerve injury when ectopically upregulated (Belin et al., 2015). Although not assessed at transcriptional level, the ingenuity pathway analysis gave high rank to c-Myc in regulating 62 major altered proteins in the purified injured RGCs compared with the naïve group (Belin et al., 2015). Among these 62 proteins, the downregulated proteins in injured RGCs functioned in the organization of cytoplasm and cytoskeleton, suggesting a rational mechanism of the weak intrinsic regenerative capacity in RGCs. Tumor suppressor p53, widely known for its role as a TF, has been shown to promote optic nerve regeneration downstream of c-Myc (Ma et al., 2019). The potential mechanism may be attributable to several p53 gene targets that are highly related to axon regeneration, including Gap43, Coronin 1b, and Sprr1a (Di Giovanni et al., 2006; Gaub et al., 2011). Furthermore, p53 has been identified to be the key mediator in optic nerve and corticospinal tract regeneration-induced by knocking out the ubiquitin ligases Mdm2/4 (Joshi et al., 2015). From the mechanistic point of view, the deletion of Mdm2/4 promoted axon regeneration by transactivating p53 and upregulated p53-dependent RAGs (Joshi et al., 2015). Similar to c-Myc, Sox, and Klf families, which are well-known for their roles in regulation of cell lineage reprogramming, reprogramming factor Lin28 has been shown to induce significant axon regeneration after optic nerve or spinal cord injury (Wang et al., 2018a; Zhang et al., 2019; Nathan et al., 2020). Although it was not conducted in CNS sources, real-time qPCR revealed that Lin28 overexpression in mouse dorsal root ganglion (DRG) neurons upregulated genes c-Myc and ten-eleven translocation 3 (Tet3), two broad-spectrum epigenetic and transcriptional regulators shown related to axon regeneration (Belin et al., 2015; Weng et al., 2017; Wang et al., 2018a). Moreover, besides acting as RNA-binding proteins, a recent

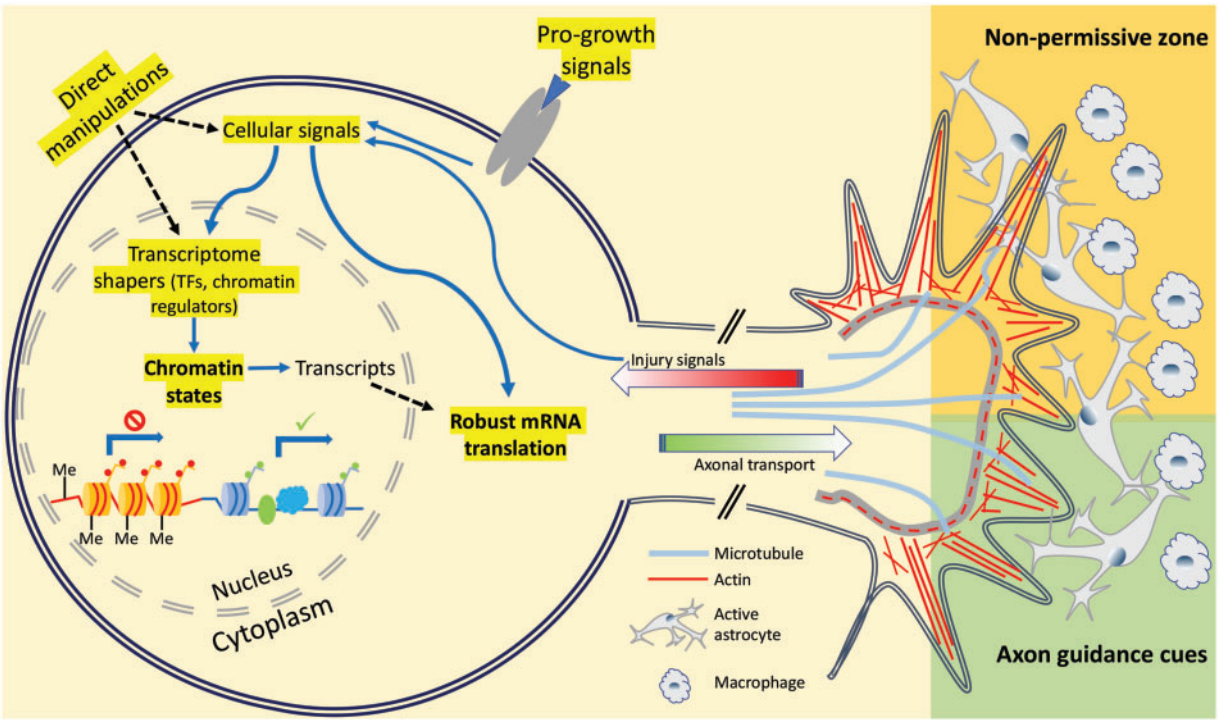

Figure 1 Representative post-injury CNS neuron in a robustly regenerating state. Acting as the effective driving force of CNS axon regeneration, some pro-growth receptor ligands (growth factors such as CNTF, etc.) coordinate with intracellular retrograde injury-sensing signaling pathways (the Stat3–Socs3 pathway), convergingly activating TFs and/or chromatin regulators to transmit the cellular signals into the cell nucleus. Direct manipulations on these nuclear elements (Sox11-OE, Klf6/7-OE, or p300-OE) could result in similar outcomes. The hub TFs and chromatin regulators may establish specific pro-regeneration chromatin states by influencing both the local accessibility and the distal *cis* interaction of the whole genome. DNA methylation is represented by '-Me' in the diagram. Histone modifications leading to the closed heterochromatin are represented by red dots, whereas histone modifications leading to the open euchromatin are represented by green dots. Such epigenome eventually results in a pro-regeneration transcriptional program. Meanwhile, the most efficient manipulation should also activate robust ribosomal protein synthesis to translate such pro-regeneration transcriptome into an entity of functional effector proteins, some for the upstream pathways and some for the transcriptional, and other terminal effector proteins transported to the growth cone and functioning to rebuild a penetrating growth cone, which could effectively reextend through the inhibitory environment around the injury site, and guide the axon toward certain temporal pathfinding cues.

study showed that Lin28 could directly bind to DNA to regulate gene transcription (Zeng et al., 2016).

Our knowledge about how these TFs are regulated by upstream signaling cascades during axon regeneration is very limited. Inducible intraocular inflammation with lens injury, Zymosan injection, or macrophage-derived factor oncomodulin (Ocm) has been shown to promote RGC axon regeneration after optic nerve injury (Leon et al., 2000; Yin et al., 2006, 2009). The pro-regenerative effect of Ocm was shown to be sensitive to the general transcriptional inhibitor actinomycin D (Yin et al., 2006), suggesting that it act in a transcription-dependent manner to enhance axon regeneration. Indeed, intravitreal injection of Ocm significantly increased the active phosphorylation of the cAMP-response element-binding protein (CREB) in RGCs (Yin et al., 2006), suggesting CREB to be a potential downstream TF. Another effector in the Zymosan or lens injury-induced optic nerve regeneration has been identified to be the astrocyte-derived CNTF (Muller et al., 2007). Direct application of CNTF was able to promote axon regeneration after optic nerve or CST injuries (Muller et al., 2007; Luo et al., 2013; Anderson et al., 2018). It is well-known that the Janus kinase (Jak) and Stat3 act downstream of CNTF to regulate gene transcription. Conversely, deletion of Socs3, the negative regulator of the Jak–Stat pathway, promoted optic nerve axon regeneration (Smith et al., 2009). Deletion of Pten has been shown to promote by far the strongest axon regeneration in the optic nerves (Park et al., 2008) or spinal cords (Liu et al., 2010). Moreover, delayed deletion of Pten after chronical CST injury also led to CST axon regeneration (Du et al., 2015). Transcritptional profile assessed by the microarray assay of purified post-injury RGCs showed that Pten deletion resulted in broad changes in gene transcription (Sun et al., 2011). Pten deletion is known to lead to Akt activation and Gsk-3β inactivation, both of which have been shown to enhance optic nerve regeneration (Guo et al., 2016; Miao et al., 2016). Although not directly examined in CNS, a potential TF downstream of Pten/Akt/Gsk-3β signaling is Smad1 based on results from sensory axon regeneration (Saijilafu et al., 2013; Zhang et al., 2014).

Table 1 Manipulations promoting CNS axon regeneration via transcriptional regulation.

Pro-regenerative targets	CNS injury and regeneration model system	Real-time qPCR or microarray availability	RNA-seq availability	References[a]
Stat3	ONI, CSTI	Yes	No	Luo et al. (2016); Mehta et al. (2016)
Klf6/7	ONI, CSTI	Yes	Yes	Galvao et al. (2018); Wang et al. (2018b)
Klf4/9/16 deletion	ONI	Yes	No	Galvao et al. (2018)
Sox11	ONI, CSTI	No	Yes	Norsworthy et al. (2017)
c-Myc	ONI	No	No[b]	Belin et al. (2015)
p53	ONI	Yes	No	Gaub et al. (2011)
Mdm2/4 deletion	ONI, CSTI	No	Yes	Joshi et al. (2015)
Lin28	ONI	Yes[c]	No	Wang et al. (2018a)
Ocm/cAMP	ONI	No	No	Yin et al. (2006)
CNTF	ONI, CSTI	No	No	Muller et al. (2007)
Socs3 deletion	ONI, CSTI	Yes	No	Sun et al. (2011)
Pten deletion	ONI, CSTI	Yes	No	Sun et al. (2011)
Akt3	ONI	No	No	Guo et al. (2016); Miao et al. (2016)
Gsk-3β deletion	ONI	No	No	Guo et al. (2016); Miao et al. (2016)
Dlk	ONI	Yes	No	Watkins et al. (2013)
p300	ONI	Yes	No	Gaub et al. (2011)
Hdac5	ONI	No	Yes	Pita-Thomas et al. (2019)

[a]If both real-time qPCR and RNA-seq are unavailable, the first study identified specific gene target is cited herein.
[b]The proteomics in purified RGCs is available in the reference.
[c]The real-time qPCR data are available in DRGs.
CSTI, cortical spinal tract injury; ONI, optic nerve injury.

Importantly, co-deletion of Pten and Socs3 generated synergistic promoting effect on optic nerve regeneration (Sun et al., 2011) and enhanced CST axon sprouting (Jin et al., 2015), indicating that Pten and Jak–Stat3 acted through different signaling mechanisms. Furthermore, MAP kinase kinase kinase (MAP3K12), also known as DLK, has been shown to play important roles in regulation of CNS axon regeneration and neuronal apoptosis (Asghari Adib et al., 2018). Based on DLK knockout experiments, the transcription of 342 genes was shown to be DLK-dependent in RGCs in response to optic nerve injury, which included hub TFs Klf6 and Atf3 and the terminal RAG Sprr1a (Watkins et al., 2013). A later study using enhanced functional genomic screening approach discovered that leucine zipper kinase acted together with DLK to regulate optic nerve injury-induced RGC death via four TFs Jun, Atf2, Mef2a, and Sox11 (Welsbie et al., 2017).

From the converging evidence, we learned that efficient long-distance axon regrowth can be achieved by artificial manipulation of factor(s) capable of driving the transcriptome toward a pro-regeneration state (Figure 1). A new research strategy is to compare transcriptomic states and seek out novel target(s) underlying such transition. For instance, the DRG neurons in the PNS are a classical model system of spontaneous axon regeneration after peripheral nerve injury, which is driven by DRG's signature pro-regenerative transcriptomic transition in response to injury. In a bioinformatics-driven novel target prediction, 382 microarrays assessing the transcriptional profiles of regeneration DRG neurons from different groups were collected to perform the weighted gene co-expression network analysis of RAGs (Chandran et al., 2016). Based on the list of terminal genes, a motif-based bioinformatics was applied to predict and list the hub TFs that might regulate the promoters of these RAGs. With technical progress in whole-genome transcriptomic profiling, the discrepancies in transcriptomes among different neuron subtypes or between injured naïve mammalian CNS neurons and the axon regeneration-capable species or systems can now be better characterized. Moreover, the deep sequencing-based profiling of function-related transcriptomes enables the prediction of regulatory factors with strong causalities.

Epigenetic landscape underlying axon regeneration

Epigenetic regulations, achieved by microRNAs and genome accessibility alterations, serve as the major mechanisms in shaping certain transcriptomes, without affecting the primary genomic sequence. In terms of the genome accessibility alterations, various post-translational modifications on the histone side chains and DNA methylation establish a 2D barrier and determining the accessibility of TFs and *cis*-regulatory genomic element toward specific gene loci. Furthermore, the large-scale chromatin architectures such as long-distance genome folding function as a 3D mechanism. When adding the temporal dimension (i.e. development, aging, and injury responses), the epigenetic chromatin state transitions exhibit high dynamics to ensure the expression of right genes at the right time.

Similar to the exploration of all other natural phenomena, the discovery of epigenetics–regeneration relationship started with discrete cases of identifying the role played by specific epigenetic regulator in the regeneration-capable system, and then eventually derived into a more systematic profiling of the whole-genome level transition. Several microRNAs (miRs) were first revealed with functions in the PNS axon regeneration of DRG neurons. *In vivo*, miR-138 was identified as a suppressor of axon regeneration, mechanistically by repressing the NAD-dependent histone deacetylase SIRT1. As a developmentally upregulated regeneration barrier, miR-138 is required to be

downregulated automatically in the DRGs to allow spontaneous axon regeneration (Liu et al., 2013). Besides, miR-26a has been shown to support DRG automated axon regeneration by suppressing GSK-3β, and thus activating the downstream axon regeneration-favorable TF Smad1, suggesting that miR-26a acts as a hierarchical hub in the gene expression network (Jiang et al., 2015). In addition to the microRNA-mediated regulation in mRNA turnover, the mRNA post-transcriptional modifications have been shown to be involved in the post-injury axon regeneration. The methylation on the N6 position of mRNA adenosine (N6mA), which has been related to mRNA stability and protein translation efficiency, has been revealed to occur on the transcripts of various RAGs in DRGs after sciatic nerve lesion (Weng et al., 2018). Functionally, such modification on the mRNAs has been further validated to be necessary for the DRG spontaneous axon regeneration, as deletion of either the methyltransferase Mettl14 or the N6mA reader protein Ythdf1 attenuated but not completely blocked the axon regeneration, mechanistically due to the dampening of mRNA-to-protein translation. In addition, this mRNA methylation-dependent maintenance of protein translation has been shown to be required for the Pten deletion-induced optic nerve regeneration (Weng et al., 2018). When moving from the mRNA to the genome accessibility, the methylcytosine dioxygenase Tet3 and 5-hydroxymethylcytosine (5hmC), the intermediate prior to the complete removal of DNA cytosine methylation, have been found elevated in DRGs after sciatic nerve lesion (Weng et al., 2017). Although not profiled with unbiased whole-genome sequencing, real-time qPCR showed the upregulation of several RAGs upon peripheral nerve injury was dependent on Tet3-mediated demethylation. Functionally, Tet3 has been shown to be necessary for the DRG spontaneous post-injury axon regeneration and Pten deletion-mediated optic nerve regeneration (Weng et al., 2017).

Recently, a study with multiomics deep sequencing has characterized the specific signature of chromatin state and correlated gene transcriptional program which supports spontaneous regrowth of the PNS branches of DRG neurons (Palmisano et al., 2019). DRG sensory neurons serve as the gold standard to investigate this issue, as these neurons, with somas in the DRG, project axons and bifurcate to form the regeneration capable peripheral branch into the sciatic nerve, and the central branch into the spinal cord dorsal column, which has weak capacity in regeneration. Notably, it means the same cell nucleus with the initial naïve chromatin state transits into a pro-regeneration state upon PNS injury stimulation, whereas switching into a non-regenerative state upon CNS injury. The analysis of assay of transposase-accessible chromatin sequencing (ATAC-seq) differential peaks showed PNS branch injury significantly switched more genomic loci into euchromatin, comparing with the CNS branch injury. Consistent with the ATAC-seq data, the chromatin immunoprecipitation sequencing (ChIP-seq) showed that the occupancies of acetylation of histone 3 lysine 9 (H3K9ac) (active promoter) and H3K27ac (active enhancer) increased significantly after PNS injury, which was in striking contrast to the outcomes after dorsal column injury. As a result, a certain transcriptome was established, showing increases in several gene ontologies related to axon regeneration, and decreases in genes related to mature neuronal functions, such as synaptic transmissions. Based on the motif-based genome-binding element's footprinting analysis, the chromatin organizer and transcriptional regulator CCCTC-binding factor has been predicted and functionally validated as a regulator required for sensory axon regeneration (Palmisano et al., 2019).

In support, converging evidence showed that the manipulations of some global epigenetic regulators successfully promoted post-injury axon regeneration in the CNS. The developmentally declined histone acetyltransferase p300, when ectopically overexpressed in adult RGCs, has been shown to promote axon regeneration after optic nerve injury (Gaub et al., 2011). Furthermore, chromatin immunoprecipitation (ChIP) coupled with real-time qPCR showed increased interaction between p300 and promoter regions of several RAGs, such as Gap43, coronin 1b, and Sprr1a, supporting the role of p300 in CNS axon regeneration. In addition, the histone deacetylase HDAC5 nuclear export and the increase of histone acetylation on some RAG regions spontaneously occur in the DRG axon regeneration (Cho et al., 2013). Interestingly, in RGCs instead of deleting HDAC5, overexpression of the nuclear active form of HDAC5 promoted cell survival and axon regeneration after optic nerve injury (Pita-Thomas et al., 2019), implying the potential discrepancy between the start-point chromatin states of PNS and CNS neurons.

Reprogramming CNS neurons for axon regeneration

During development, stem cells undergo many steps to turn into differentiated cells. During such process, the whole gene expression profile changes drastically with stem cell-related gene shutting down and only genes relevant for the differentiated cell type expressed. Importantly, differentiated cells (e.g. fibroblast) can be reprogrammed back to induced pluripotent stem cells (iPSC) by overexpressing several reprogramming factors, such as Sox2, c-Myc, Klf4, Oct4, Nanog, and Lin28, which lead to global epigenetic remodeling (Watanabe et al., 2013). Moreover, differentiated non-neuronal cells could also be directly reprogrammed to neurons or oligodendrocyte progenitors (Guo et al., 2014; Liu et al., 2019; Vignoles et al., 2019). These studies highlighted the plasticity of cellular transcriptomics for reprogramming to a different cellular type or state.

Mammalian neurons usually have high capacity to support axon growth during development for neural circuit formation. They gradually lose their intrinsic ability to support axon growth during maturation and switch into a genetic state favorable for synaptic function. Such transition is likely mediated by changed chromatin structure and the transcriptomic pattern. In the PNS, peripheral nerve injury is able to reactivate the intrinsic axon growth ability of neurons to support axon regeneration by switching back to a developmental-like state. If so, can we reprogram mature CNS neurons back to the cellular state of young neurons that supports axon regeneration? The results from several studies suggested

that genetic reprogramming might be a potential new approach for CNS axon regeneration. An early study showed (Moore et al., 2009) that knocking out Klf4 in RGCs promoted optic nerve regeneration. A recent study showed that overexpression of c-Myc was able to induce significant optic nerve regeneration (Belin et al., 2015). Sox11, a TF belonging to the same family of Sox2, has also been shown to induce optic nerve and CST regeneration (Wang et al., 2015; Norsworthy et al., 2017). Furthermore, our recent study (Wang et al., 2018a; Nathan et al., 2020) showed clearly that Lin28 acted to regulate both sensory axon regeneration and optic nerve regeneration *in vivo*. Another study (Zhang et al., 2019) later confirmed that overexpression of Lin28 in RGCs was sufficient to induce optic nerve regeneration. Moreover, specific expression of Lin28 in amacrine cells, together with insulin-like growth factor-1 (IGF-1), could result in even stronger optic nerve regeneration (Zhang et al., 2019).

How these reprogramming factors act to enhance the intrinsic axon regeneration ability of mature CNS neurons is currently unclear. Network analysis of differentially expressed genes upon Klf6 overexpression exhibited five functional modules in the upregulated genes, including cellular energy metabolism, biosynthesis of lipids, regulation of lipid rafts, cytoskeletal reorganization, and cell size regulation, whereas three functional modules identified in the downregulated genes all enriched for synaptic functions related to mature neurons (Wang et al., 2018b). Similarly, RNA-seq analysis of purified RGCs overexpressing Sox11 showed that several gene ontologies enriched in upregulated genes were cell–cell adhesion, neural development, and axonal morphogenesis, all of which were highly related to developmental axon growth. Conversely, downregulated genes upon Sox11 expression were related to synaptic transmission (Norsworthy et al., 2017). These results suggested that Klf6 and Sox11 switch the transcriptome from mature neuron state back to a youthful state favoring axon growth. Because many of these TFs are pioneering TFs, one likely mechanism underlying the switching is that, together with chromatin and/or epigenetic regulators, they modify the chromatin structure and drastically change the transcriptomics, similar to that illustrated during iPSC reprogramming (Zhao et al., 2018; Figure 2). For instance, changes in histone or DNA modification have been shown to play direct and important roles in iPSC reprogramming. Increased methylation of histone 3 lysine 27 (H3K27me3) could enhance reprogramming efficiency, whereas increased H3K79me3 suppressed reprogramming (Onder et al., 2012). H3K27 demethylases UTX and JMJDs have both been shown to regulate iPSC reprogramming (Mansour et al., 2012; Zhao et al., 2013). In consistent, our latest studies (not shown) showed manipulation of H3K27 methyltransferase EZH2 or demethylases UTX was sufficient to induce marked optic nerve regeneration. Future studies using multiomics sequencing techniques, either at bulk or single-cell level, are required to better reveal the molecular steps by which mature CNS neurons are reprogrammed to support axon regeneration (Figure 2). The results will not only help us better understand the mechanisms underlying axon regeneration but also be of great importance for identifying the optimal genes/networks that can be targeted to promote CNS neural regeneration.

Major challenges for long-distance axon regeneration and functional recovery

Although great progress has been made for enhancing CNS axon regeneration, there are still several barriers and challenges for achieving long-distance axon regeneration and the subsequent functional recovery. One challenge is that regenerating axons need to overcome the hostile CNS environment and follow the properly guided pathway to reach their original targets (Figure 1). Pten was the first protein identified to regulate the intrinsic axon regeneration ability of RGCs and cortical motor neurons (Park et al., 2008; Liu et al., 2010), and knocking out Pten produced the strongest promoting effect on CNS axon regeneration with a single gene manipulation. However, a recent study (Geoffroy et al., 2016) showed that Pten deletion-induced regeneration of CST axons beyond the injury site was greatly diminished in aged mice. In the visual system, tissue clearing and 3D imaging studies have revealed that many of the regenerating RGC axons make U-turns in the optic nerve or at the optic chiasm (Luo et al., 2013). When long-distance optic nerve regeneration (e.g. Pten/Socs3 double knockout or Pten knockout combined with application of zymosan and cAMP) was achieved, only a small number of regenerating RGC axons crossed the optic chiasm and almost no axons could reach their targets in the brain (Luo et al., 2013). One major reason is likely due to the inhibitory nature of the optic chiasm, which contains multiple classes of inhibitory molecules (Pernet et al., 2013; Pernet and Schwab, 2014). Neuronal cytoskeleton is not only the major machinery that drives axon growth, but also the converging targets of most, if not all, inhibitory signaling pathways (Hur et al., 2012; Blanquie and Bradke, 2018). Thus, by directly manipulating growth cone cytoskeletal motility it is possible to interfere with how the growth cones respond to multiple inhibitory signals, regardless whether these signals are from different inhibitors or downstream pathways. Indeed, a recent study (Tedeschi et al., 2019) showed that overexpression of cofilin-1, a protein regulating actin turnover at the nerve growth cone, was able to enhance sensory axon regeneration in the spinal cord. Our recent study (Wang et al., 2020) also showed that double knocking out non-muscle myosin-IIA/B in RGCs could induce significant optic nerve regeneration through reducing retraction bulbs and more efficient axon extension. Moreover, combined overexpression of Lin28 and myosin-IIA/B knockout led to much longer optic nerve regeneration, suggesting that modified gene expression in the neuronal soma and cytoskeletal rearrangement at the growth cone might provide an optimal condition for long-distance axon regeneration. Thus, future studies with combined manipulation of gene expression and cytoskeletal dynamics are necessary to allow long-distance axon regeneration over a hostile terrain. Moreover, proper axon guidance, which to date has rarely been explored, is also a key

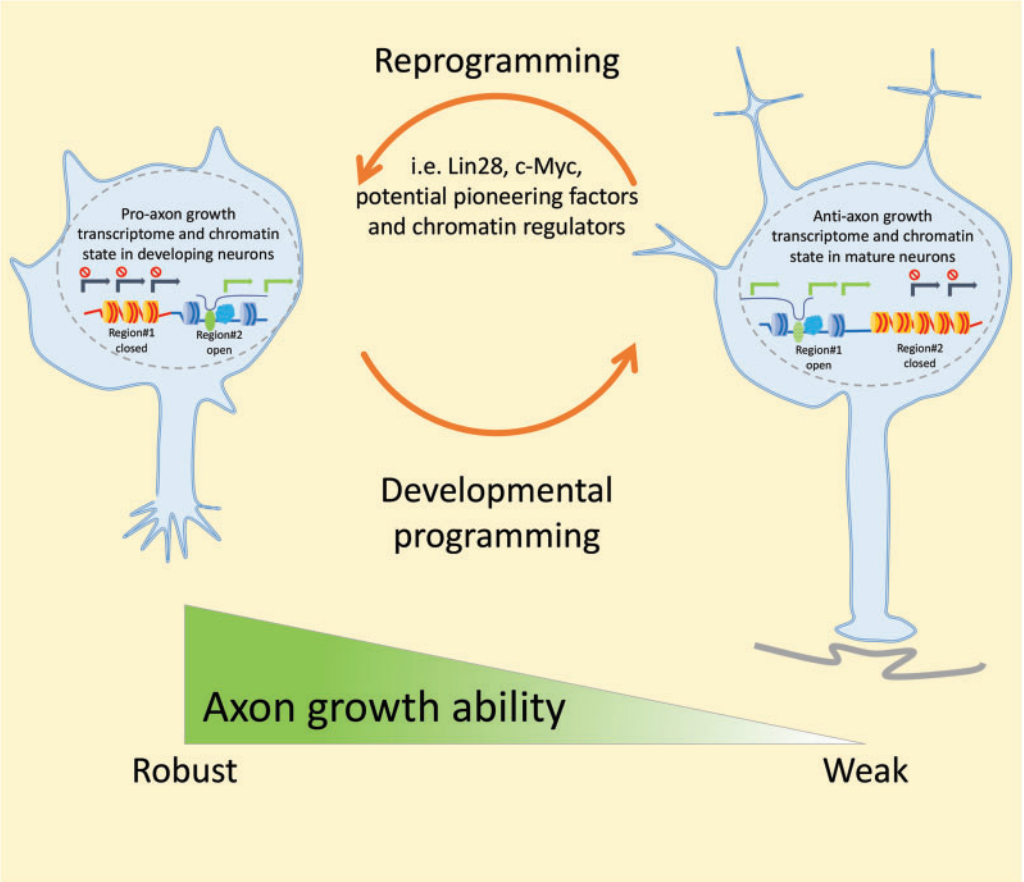

Figure 2 Transcriptomic and chromatin state transition existing between developing and mature neurons. The striking contrast in axonal outgrowth ability between robustly projecting neurons and mature neurons with stopped axon growth after synaptogenesis is attributable to the distinct chromatin states—open and closed genome regions and different *cis*-regulatory folding and TF interaction, which lead to consequential differences in transcriptional programs. Dynamically, the developing young neurons are programmed to transition into mature neurons along with functional alterations. As a therapeutic strategy, the mature neurons (if injured) may be reprogrammed with specific manipulations (Lin28-OE or c-Myc-OE) to re-obtain developing neurons' characteristics in robust axon outgrowth.

step for regenerating axons to find their original targets for functional recovery (Figure 1).

Recent studies (Rheaume et al., 2018; Tran et al., 2019) have shown that RGCs are highly heterogenous with more than 50 subtypes. Importantly, different RGC subtypes respond differently to optic nerve crush and genes enhancing axon regeneration. One study identified that α-RGCs and M1 intrinsic photosensitive RGCs (ipRGCs) preferentially survived and re-extended axons after optic nerve injury with Pten deletion (Duan et al., 2015). The selectivity was because these RGCs have relatively high mTOR activity due to the expression of osteopontin (OPN) and IGF-1 receptor. Although overexpression of OPN and IGF-1 has been shown to enhance optic nerve and CST regeneration (Bei et al., 2016; Liu et al., 2017), their promoting effects on RGCs were still confined to α-RGCs (Duan et al., 2015). When Sox11 was overexpressed to enhance optic nerve regeneration, it selectively promoted regeneration of non-α-RGCs, but killed α-RGCs (Norsworthy et al., 2017). In two different studies (Li et al., 2016; Bray et al., 2019), ipRGCs expressing melanopsin (Opn4) were shown to be more resilient to axonal injury and had better regeneration capacity than other RGC subtypes, e.g. ON–OFF direction-selective RGCs (ooDSGCs), in response to CNTF or Pten deletion. RNA-seq analysis comparing ipRGCs and ooDSGCs has identified thrombospondin-1 (Thbs1) to be selectively highly expressed in ipRGCs. More importantly, ectopic overexpression of Thbs1 promoted regeneration of both ipRGCs and non-ipRGCs. In a latest study (Tran et al., 2019), single-cell RNA-seq was performed to study RGC subtypes' differential resilience to injury. The results showed that different subtypes of RGCs had different resilience to axonal injury with different cell survival rates. Moreover, manipulations of genes that correlated with better cell survival, such as Ucn, Timp2, Crhbp, and Mmp9, were also found to enhance axon regeneration. Lastly, in general more than 80% of RGCs die at 2–4 weeks after optic nerve injury (Wang et al., 2018a), also greatly limiting meaningful visual function recovery. Collectively, these studies provided strong evidence to suggest that different subtypes of RGCs, and

maybe other CNS neurons, might have different cell survival rates and intrinsic axon regeneration capacities after injuries due to their distinct chromatin structures and transcriptomic patterns. Thus, the subtype-to-subtype discrepancy in transcriptome needs to be taken into consideration for promotion of neuronal survival and axon regeneration from more subtypes of CNS neurons. In summary, improved survival and regeneration from multiple neuronal subtypes and proper axon guidance, including overcoming the inhibitory factors and the correct pathfinding, are the near future goals in gaining meaningful function recovery after CNS injuries.

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