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Live, Die, or Regenerate? New insights from multi-omic analyses

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Abstract

In this issue of *Neuron*, three studies established new strategies for efficient retinal neuroprotection and optic nerve regeneration. Tian et al. (2022) carried out a multi-omics screen and identified key transcriptional regulators of axon injury signaling leading to cell death; Jacobi et al. (2022) and Li et al. (2022) combined retrograde tracing and single-cell RNA-Seq (scRNA-Seq) to uncover a new molecular roadmap for axon regeneration.

Permanent impairment following injury to the central nervous system (CNS) results from the inability of axons to regrow and reform functional connections to their original targets. As such, great effort has been made within the field of regenerative medicine to find strategies to enhance CNS regeneration potential. Studies using the optic nerve crush (ONC) model, in which retinal ganglion cell (RGC) axons are transected, have yielded key discoveries surrounding neuron-intrinsic genes that, when manipulated, promote partial neuronal survival and axon regeneration. However, current approaches to identify factors that promote robust optic regeneration remain limited due to the throughput of functional testing—usually one gene at a time. These technical limitations highlight the need for comprehensive approaches to identify genes for neuroprotection and regeneration *in vivo*. In this issue of *Neuron,* three studies leveraged cutting-edge sequencing methods and improved surgical techniques to reveal core transcription programs for neuroprotection and exemplify unbiased approaches for identifying molecular targets for optic nerve regeneration.

Following ONC, signaling pathways are activated from the axon to the cell soma (Welsbie et al., 2017), rapidly triggering deleterious transcriptomic changes. In their *tour de force* study to identify transcriptomic regulators repressing neuronal survival and axon regeneration following ONC, Tian et al. performed a loss-of-function CRISPR-based screen *in vivo* against all known 1,893 mammalian transcription factors (TFs) (Figure 1A) (Tian et al., 2022). Knockout of only a small number of TFs yielded robust phenotypes - 10 were responsible for neuroprotection, 13 were involved in axon regeneration, and one for both processes. By integrating these results with ATAC-Seq and RNA-Seq of RGCs following ONC, the authors pinpointed four TFs – ATF3, ATF4, C/EBPγ, and CHOP/Ddit3 — to be robust regulators of the RGC injury responses *in vivo*. While some of these TFs had

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been implicated in RGC neuroprotection before as part of the cellular injury response, Tian et al. demonstrated that these TFs could be bioinformatically separated into two groups based on shared downstream effectors: 1) ATF3 and CHOP share target genes involved in neuronal responses to extrinsic factors and innate immunity; 2) ATF4 and C/EBPγ have common targets involved in cell-intrinsic stress responses such as cell cycle/DNA damage. Corroborating this bioinformatic prediction, the authors picked one TF from each group and found that co-deletion of ATF3 and C/EBPγ increased RGC survival following ONC. Additionally, they showed that this additive neuroprotection extended to a modified viscobead glaucoma model (Calkins et al., 2018), generalizing the discovery from the ONC model to other forms of optic neuropathies.

Beyond factors affecting neuronal resilience or susceptibility, several master regulators for axon regeneration have also been established using the ONC model. For example, the most potent single-gene strategy for optic nerve regeneration to date has been the deletion of PTEN, a master regulator suppressing PI3K/mTOR signaling (Park et al., 2008). PTEN deletion alone (P_{CKO}) or in combination with other interventions, such as CNTF overexpression (C/P_{CKO}) and SOCS3 deletion (C/PS_{CKO}), leads to synergistic promotion of axon regrowth as well as RGC survival (Williams et al., 2020). While these interventions have been found to increase RGC axon regrowth globally, their effects on individual RGC types have not been thoroughly studied since the multi-allelic genetic crosses needed for RGC type-specific axon tracing onto combinatorial interventions, such as C/P_{CKO} or C/PS_{CKO}, have limited the throughput of testing. As a result, past work primarily focused on P_{CKO}, which was found to promote axon regeneration for a small portion of the RGC types, namely αRGCs and intrinsically photosensitive RGCs (ipRGCs) (Bray et al., 2019; Duan et al., 2015).

There is an increasing demand to identify the general molecular mechanisms underlying axon regeneration and to uncover new regulators of axon regeneration. The next two studies profiled RGCs using scRNA-Seq and developed methods to distinguish regenerating RGCs from the larger cohort of surviving but not regenerating RGCs (Figure 1B&C). To comprehensively survey RGCs types in their survival capacity, Jacobi et al. first recovered RGCs primed by three interventions (P_{cko}, $C/P_{cko,}$ and C/PS_{cko}) and compared their gene expression profiles as well as to those of wildtype RGCs using high-throughput scRNA-Seq. They assigned RGCs into one of 46 types defined in their previously published scRNA-Seq RGC atlas (Tran et al., 2019). By recovering surviving RGCs at multiple time points after ONC for profiling using scRNA-Seq, they found that the survival-promoting effects from all three interventions benefit most RGC types and generally scale with the innate survival potential of each RGC type. In contrast, the axon regeneration effects from these interventions were found to affect cell types differentially. By using a retrograde labeling method in which a fluorescently labeled small molecular-weight dye was stereotaxically injected distal to the ONC site (Zhang et al., 2019), Jacobi et al. collected retrogradely labeled RGCs with regenerating axons for deep scRNA-Seq profiling. PTEN deletion (P_{CKO}) was mainly found to promote αRGC regeneration, and the addition of SOCS3 knockout and CNTF overexpression (C/PS_{CKO}) was found to promote non-αRGC axon regeneration. Jacobi et al. then uncovered gene ontology (GO) patterns modulated by each intervention and compared how GO patterns differ between surviving and regenerating

RGCs. Genes associated with immune responses, particularly interferon and cytokine signaling, contribute to RGC regeneration in C/PS_{CKO} treatment, independent of RGC types, echoing recent advances trying to harness immune responses to promote optic nerve regeneration (Peterson et al., 2021). Interestingly, several genes related to neuropeptide secretion and regulation were also predicted to mediate the pro-regeneration effects of the different interventions and, when overexpressed, were also found here to promote axon regeneration.

Another emerging theme exemplified by Li et al. is the separation between programs for neuroprotection and axon regeneration. Past work has shown that maintaining RGC survival is insufficient for axon regeneration. In an extreme example, Bcl-2 overexpression in the ONC model causes nearly all RGCs to survive, but none regenerate their axons (Goldberg et al., 2002). This raises the question of whether there may be a separate set of molecules that more potently prime RGCs for regeneration. To address this question, Li et al. set out to compare the transcriptional differences between regenerating RGCs from those that merely survive but do not regenerate their axons using deep scRNA-Seq. After priming RGCs to regenerate through PTEN deletion, Li et al. implemented a novel retrograde tracing method in which they performed a lateral canthotomy to deliver a fluorescently labeled dextran distal to the ONC site. This procedure captured more regenerating RGCs for deep sequencing compared to previous stereotaxic methods and was less invasive. By comparing differentially expressed genes between the regenerating and surviving groups, Li et al. identified novel regeneration-associated genes. One of the top hits was Anxa2, which acts synergistically with its ligand, tissue plasminogen activator (tPA), to enhance optic nerve regeneration. Interestingly, administration of tPA alone induced axon regeneration and further potentiated Pten KO-induced axon regeneration. Additionally, Li et al. demonstrated that Anxa2 overexpression protects RGCs and preserves visual function under glaucomatous conditions.

In sum, advancements in sequencing technologies have greatly facilitated the process of identifying molecular candidates to promote neuroprotection and optic nerve regeneration. These three independent studies utilized innovative approaches and revealed largely non-overlapping hits from *in vivo* experiments. One pressing question is how to best integrate data across these diverse studies and generate a new pro-regenerative cocktail for optic nerve regeneration. Parsing through all the multi-omics data gathered by these parallel studies, the field has not yet found an underlying trend as to why certain cell types respond differently to injury and to interventions used to promote optic nerve regrowth. Connecting the TF downstream target genes identified in Tian et al. to the gene regulatory networks from the SCENIC analysis from Jacobi et al. may provide hints as to whether certain RGC types activate distinct transcriptional programs or undergo distinct epigenetic modifications in response to injury. Future work may help examine whether such changes correlate with RGCs' resilience to survive or their selective capabilities to regrow.

Furthermore, it would be interesting to compare the scRNA-Seq data from Jacobi et al. and Li et al. And indeed, some similarities can already be seen: Notably, Anxa2 from Li et al. was a hit in the regenerative module from Jacobi et al., and both studies shared multiple GO pathways, including positive regulation of cell adhesion. There are

some differences, however; even though both studies sequenced regenerating RGCs primed through PTEN deletion, they used different surgical strategies for retrograde labeling. Jacobi et al. retrogradely labeled RGCs with regenerated axons ~1.5 mm distal to the ONC site using a stereotaxic surgery. In contrast, Li et al. labeled RGCs whose axons were ~1 mm distal to the ONC site through their less-invasive lateral canthotomy surgery. Further comparison of these complementary datasets might yield patterns differentiating RGCs that can regenerate shorter versus longer distances.

Lastly, coupling anatomy-based tracing methods to scRNA-Seq has empowered us to revisit fundamental questions in regenerative medicine, as Jacobi et al. and Li et al. specifically illustrated for optic nerve regeneration. While neuroprotection and axonal regeneration are necessary prerequisites for neural repair, the importance of proper synapse reformation must not be overlooked to achieve functional recovery. As interventions become more potent to regenerate axons, clues to whether regenerated axons form correct functional synapses by default (or not) will become clearer. Circuit tracing techniques combining scRNA-Seq in conjunction with anterograde trans-synaptic tracing (Tsai et al., 2022) may be well-suited to examine how synaptic choices from the retina to the brain are made subject to injuries and subsequent neuronal repair. With this information, pro-regenerative cocktails can then be refined to promote accurate synapse reformation following axon regrowth. By tackling these processes for circuit rewiring step by step, achieving circuit repair for functional recovery is in sight.

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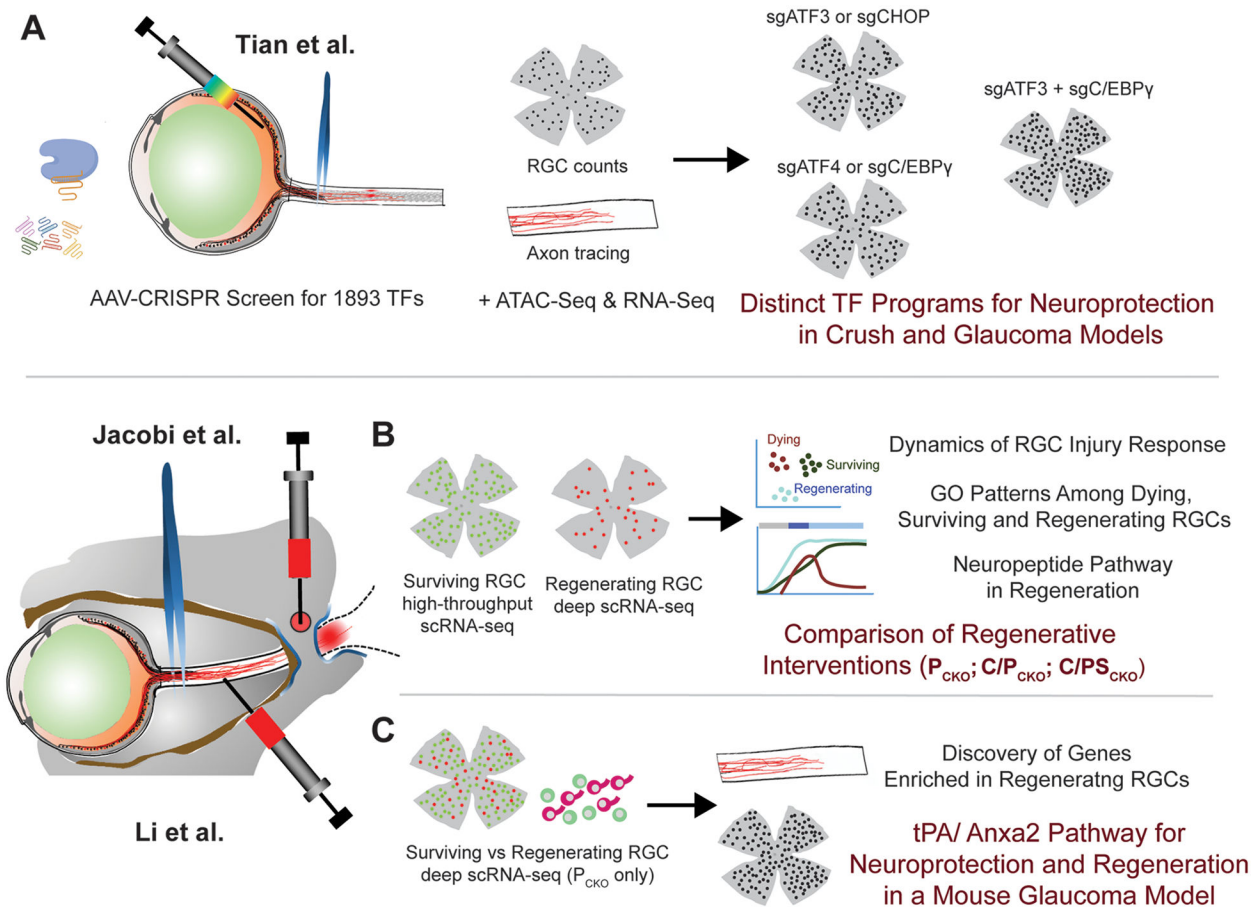

Figure 1. Multi-omics approaches to uncover new transcriptional pathways for neuroprotection and molecular targets for effective axon regeneration.

(**A**) Tian et al. performed an AAV-mediated *in vivo* CRISPR loss-of-function screen against all known mammalian TFs following ONC. RGC counts reflecting neuroprotection and RGC axon regeneration were assessed following the deletion of each TF. By integrating their CRISPR screening results with ATAC-Seq and RNA-Seq of injured RGCs following ONC, the authors uncovered parallel core transcriptional machinery to boost neuroprotection with synergistic effects in ONC and glaucomatous conditions. (**B & C**) Jacobi et al. and Li et al. profiled RGCs primed for regeneration with scRNA-Seq following ONC and used retrograde labeling methods to distinguish regenerating from surviving but non-regenerating RGCs. The two studies used different surgical methods, as shown, to deliver a small-molecular weight fluorescent dye for retrograde labeling. (**B**) Jacobi et al. uncovered RGC taxonomy changes under three different regenerative interventions; differentiated GO patterns among dying, surviving, and regenerating RGCs; and showed that several molecules in neuropeptide signaling are critical for optic nerve regeneration. (**C**) Li et al. compared regenerating RGCs to surviving but non-regenerating RGCs followed by PTEN KO and identified differentially expressed genes between the two RGC groups. They then tested the regeneration-associated genes using an AAV-overexpression screen. They uncovered a role

for the tPA/Anxa2 pathway in promoting axon regeneration and protecting RGC loss under glaucomatous conditions.