

An investigation into the role of circular RNA in zebrafish spinal cord regeneration with focus on circRNA-miRNA interaction

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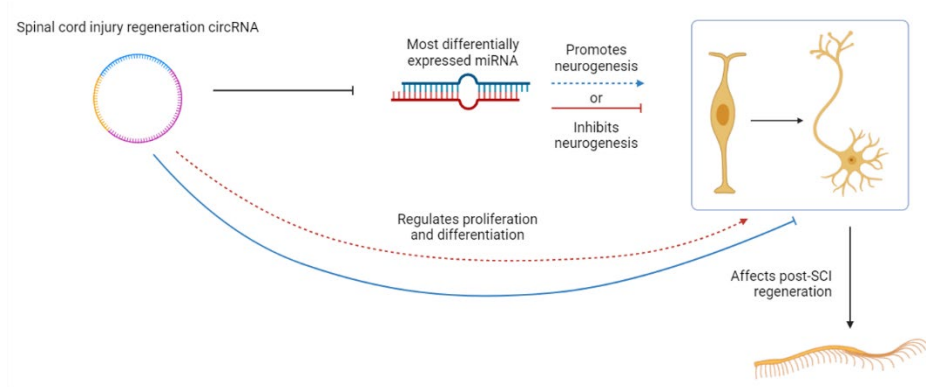
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Abstract. Zebrafish (*Danio rerio*) serve as a valuable model for studying spinal cord regeneration due to their remarkable ability to restore the central nervous system (CNS), challenging the long-standing belief that neurons in the adult CNS cannot regenerate. While current knowledge of spinal cord regeneration primarily centers around signaling pathways like the Wntless/Integrated-1 pathway, emerging evidence suggests that circular RNAs (circRNA) also play a critical role in cellular regeneration. Studies in mouse models and human intestinal epithelium have demonstrated the importance of circRNAs in tissue repair, and significant changes in circRNA expression have been observed in zebrafish spinal cord following injury. However, there has been a lack of investigation into what roles circRNAs play in zebrafish spinal cord regeneration. This proposal seeks to fill this research gap by conducting global RNA sequencing to identify the spinal cord injury regenerative circRNA (SCIR-circRNA) that is most differentially expressed before and after spinal cord transection. The role of this SCIR-circRNA in spinal cord regeneration will then be examined through RNAi knockdown experiments to determine whether it promotes or inhibits the regenerative process. Furthermore, the study will focus on SCIR-circRNA and microRNAs (miRNAs) to elucidate their interaction via luciferase assay and RNA pulldown assay. The expected outcomes of this research will contribute to a deeper understanding of the molecular mechanisms underlying spinal cord regeneration and may identify novel therapeutic targets for treating spinal cord injuries.

Keywords: circular RNA, spinal cord regeneration, zebrafish, micro RNA, motor neuron.

Graphical Abstract



1. Introduction

1.1. CNS injury

Over a century ago, Santiago Ramón y Cajal, the pioneer of modern neuroscience, asserted that neurons in the adult CNS exhibit a fundamental incapacity for regeneration. [1]. This concept is now deeply ingrained in neurobiology, supported by observation that unlike neurons in the peripheral nervous system (PNS) where spontaneous regeneration typically occurs after damage [2], mammalian CNS neurons are often unable to regenerate, leading to irreversible functional loss in conditions such as neurodegenerative diseases and spinal cord injury (SCI). The axonal regrowth in the CNS is impeded, and neuronal circuits are disrupted due to inhibitory factors in the extracellular environment, including glial scar formation [3,4], as well as the intrinsic limited regenerative capacity of mature CNS neurons [5]. Despite advances in neuroregenerative research, efforts to promote functional recovery in the mammalian CNS remain largely unsuccessful, with therapeutic interventions struggling to overcome these barriers to regeneration [6].

1.2. Spinal Cord Regeneration

The spinal cord is a pivotal component of the central nervous system (CNS), essential for relaying signals between the brain and the rest of the body. Spinal cord regeneration is orchestrated through mechanisms such as axonal regrowth and the reformation of neural circuits, processes that are tightly regulated by progenitor cell proliferation and the modulation of inflammation [7]. Among these mechanisms, the Wntless/Integrated-1 (Wnt/ β -catenin) signaling pathway has been identified as a key driver in promoting neural regeneration in zebrafish after spinal cord injury (SCI) [8].

Recent studies focusing on circular RNAs (circRNAs) in zebrafish spinal cords post-SCI have demonstrated significant differential expression patterns [9]. These findings suggest that circRNAs may play a crucial role in modulating inflammation and facilitating neural regeneration during SCI recovery [10].

1.3. Zebrafish as a Model for Studying Spinal Cord Regeneration

In contrast to mammals, certain non-mammalian species demonstrate remarkable regenerative capabilities; for instance, spiny mice retain their spinal cord's regenerative capability until a few days after birth [11], amphibians undergo regenerative process after SCI before metamorphosis [12], and birds display a partial regeneration of the spinal cord at the larval stage [9]. Among those models, zebrafish stand out due to their exceptional ability to regenerate neural tissues, including motor neurons, glial cells, and axons, from the embryonic to adult stages [7, 14]. For instance, following experimentally induced spinal cord injuries, such as transection [15] or mechanical compression [16] affecting one-

third of motor axons, zebrafish can completely restore spinal cord integrity within weeks [17, 18]. Newly generated motor neurons restore normal swimming activity [19].

Zebrafish achieve spinal cord regeneration through coordinated processes that include the formation of a cellular bridge at the injury site, guided neuronal regrowth facilitated by molecular signals, proliferation and differentiation of stem and progenitor cells, and controlled inflammatory responses, as well as specific epigenetic modifications that promote regeneration [20, 21]. It has been shown that in zebrafish, regeneration of neurons depends on differentiation of progenitor cells, specifically the radial glial progenitor cells [11,14] and the ependymo-radial glial cells (ERGs), a class of neural progenitor cells that share some properties with the radial glial cells [22]. Unlike mammalian SCI, in which astrocytic gliosis leads to the formation of a glial scar that obstructs regeneration, zebrafish gliosis is thought to assist axon regrowth and spinal cord regeneration [22]. Given their genetic similarity to humans, zebrafish share orthologous genes with high similarity to human genes [23]. This makes zebrafish an excellent model for investigating molecular mechanisms underlying spinal cord regeneration, with the potential to advance therapeutic approaches for spinal cord injury in the mammalian CNS. However, there is currently a lack of research into the role of circRNAs in zebrafish spinal cord regeneration; hence, to contribute towards filling the research gap of the roles of circular RNAs in zebrafish spinal cord regeneration, this proposal aims to investigate post-SCI circRNA expression levels in zebrafish.

1.4. Circular RNA and its prospect in spinal cord regeneration

Circular RNAs (circRNAs) are single-stranded, covalently bonded RNAs that are found across species, from viruses to mammals. They were initially thought to be viroids [24], and later products of splicing errors [25,26]. Later, circRNAs were found to be a common feature of the transcriptome in humans and many other metazoans. CircRNAs are generated from an alternative splicing event known as back-splicing, when the 3'-end of a pre-mRNA exon ligates to its own 5'-end or that of an upstream exon via a 5'-3' phosphodiester bond, forming a closed, circular structure with a signature back-splicing junction [27]. Their unique circular structure, which lacks 5' caps and 3' polyadenylation tails, significantly enhances their stability by protecting them from exonuclease-mediated degradation [28]. circRNAs were found to perform multiple functions in gene expression, including transcriptional regulation, interacting with and modulating RNA-binding proteins (RBPs), and translation [27]; a property of circRNAs that has received the most attention is their function as micro RNA (miRNA) sponges, through which they upregulate miRNA target mRNAs [27,29]; interestingly, the circRNA *cdrlas* has been shown to upregulate miR-195 by stabilizing pre-miR-195 in the intestine of mice [30] which raises the question that whether other circRNAs have similar functions. In recent years, there has been rising interest in the role of circRNAs' in nerve injury and regeneration; for example, circ-HIPK3 overexpression reduces neuronal cell apoptosis following SCI via regulating the miR-588/DPYSL5 axis in rats [31]. These findings suggest that circRNAs may similarly influence spinal cord regeneration in zebrafish, highlighting their potential significance in enhancing our understanding of regenerative processes across different models. There is great prospect in uncovering circRNA's role in nerve regeneration and potentially applying the findings to regenerative medicine, which this proposal seeks to contribute to by investigating its role in zebrafish spinal cord regeneration.

2. Project Description

The main goal of this project is to identify potential circRNAs that plays a role in post-SCI regeneration of zebrafish, specifically in the differentiation and proliferation of motor neurons, and to focus on investigating the mechanism of one such circRNA, which we will refer to as the Spinal Cord Injury Regenerative Circular RNA (SCIR-circRNA). We hypothesized that the levels of circRNAs that has a role in spinal cord regeneration would be altered following SCI, and that knockdown of which will affect the process of post-SCI regeneration; specifically, we aim to determine whether SCIR-circRNA promotes or inhibits regeneration. We further suspect that the circRNA of interest may regulate spinal cord regeneration via interaction with miRNA, hence knockdown of said circRNA would also result in

the differential expression of its potential target miRNA. As such, this project will identify the most differentially expressed (DE) circRNA out of all circRNAs differentially expressed following SCI, determine its effect on spinal cord regeneration, and find the putative miRNA through which it regulates the process. By characterizing SCIR-circRNA and its regulatory impact, this project seeks to elucidate its contribution to spinal cord repair and identify potential miRNA targets involved in its function.

2.1. Objective 1: identify circRNAs in the spinal cord of zebrafish differentially expressed before and after SCI

Rationale: circRNAs differentially expressed (DE) before and after spinal cord transection in zebrafish likely play roles in spinal cord regeneration.

Methods:

Models: Wildtype adult zebrafish which are raised as standard procedures [14] will be used as model organisms for this experiment. Two conditions, control and SCI, will be used.

Spinal Cord Transection: A previously described procedure will be used: adult wildtype zebrafish will be anesthetized with 0.016% Tricaine (supplied by Sigma-Aldrich); microinjection pipettes will be broken and beveled, then driven into the spinal cord of at the level of the anal pore; the pipette will then be moved dorsally to transect the spinal cord. [14].

circRNA purification: I will extract tissue samples from both groups at the lesion site 3 days post-injury [32] to obtain axotomized nerve fibers [33]. Total RNA from the tissue sample will be extracted with TRIzol reagent (Thermo Fisher Scientific) and treated with RNase-free DNase [34-36] to eliminate residual DNA. Total RNA will then be purified for circRNA by polyadenylating the linear RNAs using a Thermo Fisher polyadenylation kit followed by treatment with RNase R, with Li^+ in place of K^+ in the reaction buffer [36,37].

RNA sequencing and Bioinformatic analysis to identify most differentially expressed circRNA: I will conduct circRNA sequencing following a previously established procedure [34]. A sequencing library for purified circRNA will be prepared using the TruSeq Small RNA Library Prep Kit protocol (Illumina), which will then be loaded on the Illumina NovaSeq sequencer; the Trinity algorithm [34,38] will be used for *de novo* assembly of raw reads, and more accurate mapping outcome can be achieved by Spliced Transcripts Alignment to a Reference (STAR) software [39]. BLAST+ will be used to annotate the sequenced circRNAs against existing zebrafish transcriptome databases [34]. Two bioinformatic algorithms, find-circ and CIRI2, will be used to quantify the sequenced circRNAs to identify the most DE circRNA pre- and post-SCI; two algorithms are used in conjunction here to reduce false-positive results [36].

Expected Outcomes:

Identification of most DE circRNA: we expect there will be one circRNA that is most DE out of all circRNAs in SCI models compared to controls. This hypothetical most DE circRNA potentially plays a role in post-SCI regeneration in zebrafish, hence we will hereafter refer to it as “spinal cord injury regeneration circular RNA” (SCIR-circRNA).

2.2. Objective 2: Investigate the role of SCIR-circRNA in spinal cord regeneration by RNAi knockdown

Rationale: SCIR-circRNA knockdown models would exhibit different post-SCI regeneration characteristics if SCIR-circRNA indeed plays a role in spinal cord regeneration.

Methods:

Model: Tg(HB9:GFP) zebrafish will be used.

Creation of SCIR-circRNA RNAi knockdown zebrafish: Using sequence data from aforementioned RNA seq, I will design a short hairpin RNA (shRNA) targeting the back-splicing junction of the SCIR-circRNA [36]. The shRNA will be delivered to the spinal cord via lentiviral vector siRNA pLKO.1-EGFP-puro plasmid [40]. RT-qPCR protocol adapted from Yang et al. (2024) [36,40] and fluorescein isothiocyanate (FITC)-labeled probes targeting SCIR-circRNA [41] will be used to assess whether SCIR-circRNA level is indeed lowered in knockdown zebrafish.

Assessing motor neuron regeneration: to assess whether knockdown of SCIR-circRNA affects motor neuron (MN) regeneration in the spinal cord, I will conduct RNAi knockdown in both Tg(HB9:GFP) zebrafish, followed by spinal cord transection and tissue sampling as described previously. HB9 (Mnx1) is a spinal cord motor neuron specific marker and a transcription factor critical for its postmitotic differentiation [42]. I will conduct an immunohistochemical assessment to count the number of HB9:GFP⁺ cells in SCIR-circRNA knockdown and control models post-SCI [8,43] to determine how MN regeneration is affected by depletion of SCIR-circRNA. Immunohistochemical assessment of BrdU which is able to label cells in S phase will also be conducted in both knockdown and control groups post-SCI to assess the rate of cell proliferation [8,43].

Expected Outcome

Creation of SCIR-circRNA RNAi knockdown zebrafish: we expect that if RNAi of SCIR-circRNA indeed achieves the desired effect of reducing SCIR-circRNA levels in the spinal cord, RT-qPCR will yield less reads of SCIR-circRNA in addition to less observed amount of FITC⁺ cells in SCIR-circRNA knockdown models compared to control models.

Assessing motor neuron regeneration: we expect SCIR-circRNA to affect post-SCI regeneration in zebrafish, hence we in turn expect its knockdown to affect MN regeneration. If SCIR-circRNA has a promotional effect on MN, there would be less HB9:GFP⁺ cells and BrdU⁺ cells in the spinal cord tissue of SCIR-circRNA knockdown models compared to control models as MN differentiation and proliferation reduces after SCIR-circRNA depletion, and vice versa if SCIR-circRNA has an inhibitory effect on MN regeneration.

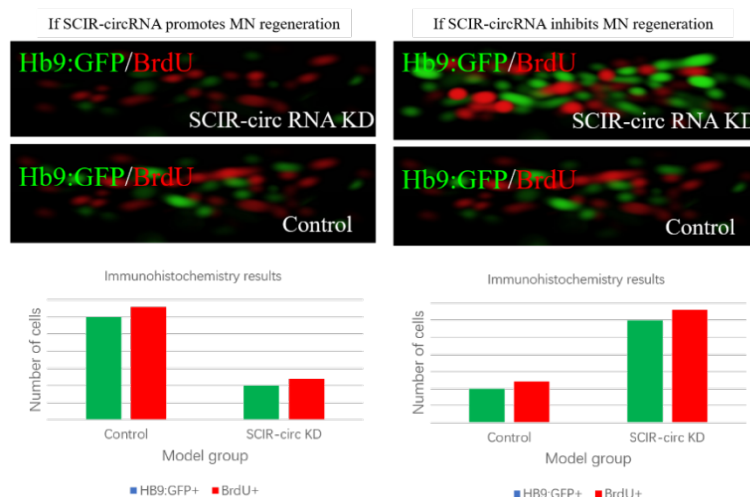


Figure 1. Expected outcome of immunohistochemistry of HB9:GFP-positive cells and BrdU-positive cells in post-SCI tissue samples from SCIR-circR KD and control models.

2.3. Objective 3: Ascertain the miRNA through which SCIR-circRNA influences spinal cord regeneration

Rationale: micro RNA (miRNA) sponging is a prominent feature of circRNA by which it regulates the transcriptome, and it has been shown that some circRNAs regulate spinal cord regeneration via interaction with miRNA [31]. Therefore, it is necessary to ascertain whether SCIR-circRNA also regulates spinal cord regeneration through this mechanism, and if yes, determine which miRNA it interacts with.

Methods:

miRNA isolation: the TRIzol method will be used to extract total RNA from spinal cord tissue sample of lesioned SCIR-circRNA knockdown and control zebrafish, followed by DNase treatment to degrade residual DNA, and subsequently the miRNeasy kit (Qiagen) will be used to isolate miRNA [44,45].

RNA-seq and bioinformatic analysis: I will conduct a bioinformatic analysis of SCIR-circRNA to predict potential miRNA and Argonaut protein binding sites [36]. I will then conduct RNA-seq on the miRNA from SCIR-circRNA knockdown and control groups to quantify miRNAs, thereby identifying the most DE miRNA (MDE-miRNA) before and after SCIR-circRNA depletion that also matches the potential miRNA binding sites on SCIR-circRNA.

FISH confirmation of SCIR-circRNA and MDE-miRNA colocalization: I will conduct fluorescent in-situ hybridization (FISH) assay on both SCIR-circRNA and MDE-miRNA in spinal cord tissues from wildtype zebrafish to confirm co-localization [36,46].

Luciferase assay confirmation of circRNA-miRNA binding: I will create luciferase reporter gene constructs, hereafter referred to as luc-SCIRcirc-WT (where WT refers to wildtype), by inserting the predicted binding site of SCIR-circRNA to miRNA into the 3'UTR of the luciferase gene. Gene constructs with a mutated version of the aforementioned binding site will also be created as a control, hereafter referred to as luc-SCIRcirc-Mut. I will then transfect luc-SCIRcirc-WT or -Mut into zebrafish models alongside MDE-miRNA mimics or negative control (NC) mimics [36,47,48].

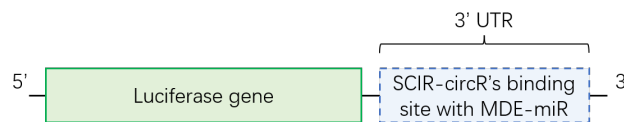


Figure 2. luc-SCIRcirc-WT reporter gene construct

Table 1. Experiment and control groups of luciferase assay

Group 1: zebrafish transfected with luc-SCIRcirc-WT and MDE-miR mimics	Group 3: zebrafish transfected with luc-SCIRcirc-Mut and MDE-miR mimics
Group 2: zebrafish transfected with luc-SCIRcirc-WT and NC mimics	Groups 4: zebrafish transfected with luc-SCIRcirc-Mut and NC mimics

Ex-vivo RNA pulldown assay confirmation of specific binding between MDE-miRNA and SCIR-circRNA: I will use a slightly modified version of a previously described procedure [49]: I will construct biotin-labeled RNA probes according to the predicted MDE-miR binding sites on SCIR-circRNA; the probes will be attached to streptavidin beads for immobilization. Total miRNA from zebrafish spinal cord tissue (obtained with aforementioned isolation procedure) will be incubated with the RNA probes; miRNA that specifically binds to the probes will be eluted while non-specifically binding miRNAs will be washed away and discarded. The specifically binding miRNA will then be sequenced and annotated against existing miRNA databases for identification.

Expected Outcomes:

RNA-seq for MDE-miRNA: I expect the miRNA most differentially expressed before and after SCIR-circRNA knockdown to interact with SCIR-circRNA. I expect there to be one out of two outcomes: either SCIR-circRNA knockdown leads to an increase in the level of MDE-miRNA, which suggests SCIR-circRNA inhibits MDE-miRNA levels and that SCIR-circRNA is an MDE-miRNA sponge, or SCIR-circRNA knockdown leads to a decrease in the level of MDE-miRNA, which suggests SCIR-circRNA upregulates MDE-miRNA levels possibly via stabilizing pre-MDE-miRNA, similar to how CDR1as stabilizes pre-miR-195 [30]. The latter scenario could be subject of future research.

FISH confirmation of colocalization: I expect FISH assay to indicate colocalization of SCIR-circRNA and MDE-miRNA in the same tissue and cells if the two indeed interact. If no colocalization is found, then it is unlikely that the two RNAs are interacting.

Luciferase assay confirmation of circRNA-miRNA binding: if MDE-miRNA indeed binds to the predicted binding sites on SCIR-circRNA, I expect there to be lower levels of luciferase in models transfected with luc-SCIRcirc-WT and MDE-miRNA mimics compared to those transfected with luc-SCIRcirc-WT and NC mimics, because binding of MDE-miRNA to the binding site sequence in luc-SCIRcirc-WT would cause the latter to be degraded, leading to reduced luciferase expression. I also

expect that luciferase levels will have no significant difference in models transfected with luc-SCIRcirc-Mut regardless of whether it was transfected with MDE-miRNA or NC mimics [48].

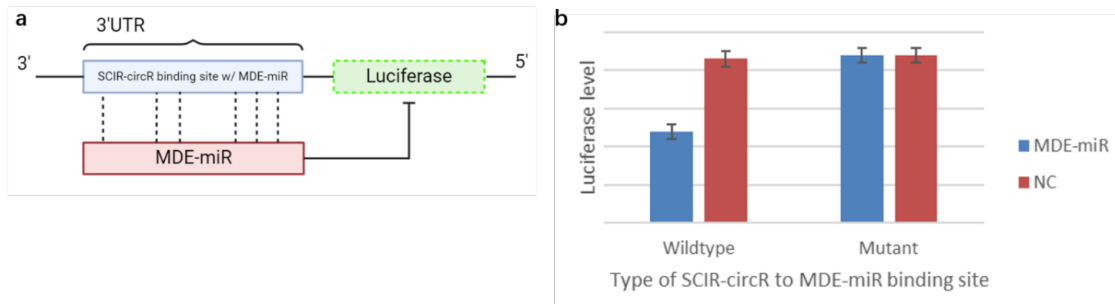


Figure 3. Expected outcomes of luciferase assay. **a)** If MDE-miRNA binds to the SCIR-circRNA binding site downstream of luciferase gene, the gene construct would be degraded and luciferase expression inhibited, **b)** in which case there will be higher level of luciferase in NC models compared to MDE-miRNA models if binding site is wildtype, and no significant difference if binding site is mutated.

RNA-pulldown: if MDE-miRNA indeed specifically binds to SCIR-circRNA, I expect it to bind to the RNA probes that are based on predicted binding sites of SCIR-circRNA to MDE-miRNA; in which case, MDE-miRNA will be immobilized and not washed away with non-specific binding RNAs, and can be eluted, sequenced, and annotated according to existing databases.

3. Conclusion

Summary: The goal of this proposed project is to further elucidate the role of circRNA in spinal cord regeneration in zebrafish, by identifying the most differentially expressed circRNA pre- and post-SCI, determine its effect on motor neuron regeneration by knockdown experiment, and ascertain potential miRNA it interacts with.

Limitation: This proposal only assesses spinal cord regeneration by looking at motor neuron proliferation and differentiation, other critical aspects such as glial cell responses, axonal regrowth, and functional recovery, are not addressed. Also, the complexity of circRNA biology may confine the investigation, like competing for endogenous interactions with multiple miRNAs and RNA-binding proteins that might not be completely presented by these methods.

Future direction: In future studies, the complete molecular pathways and signaling cascades that are involved in SCIR-circRNA mediated effects on spinal cord regeneration should be explored further. If knockdown of SCIR-circRNA is indeed proven to increase the levels of MDE-miRNAs in spinal cord, future studies should also address how exactly increasing SCIR-circRNA can contribute positively towards upregulating that important subset of crucial miRs. This will include investigating whether or not SCIR-circRNA stabilizes MDE-miRNA precursor form and/or inhibits its maturation.

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Zixin Cheng, Hanwen Cui, and Ziqian Wan contributed equally to this work and should be considered co-first authors.

References

- [1] Ramón y Cajal, S. (1928). Degeneration and Regeneration of the Nervous System. Oxford University Press.
- [2] Varadarajan, S. G., Hunyara, J. L., Hamilton, N. R., Kolodkin, A. L., & Huberman, A. D. (2022). Central nervous system regeneration. *Cell*, 185(1), 77–94. <https://doi.org/10.1016/j.cell.2021.10.029>

- [3] Bradke, F., Fawcett, J. W., & Spira, M. E. (2012). Assembly of a new growth cone after axotomy: the precursor to axon regeneration. *Nature reviews. Neuroscience*, 13(3), 183–193. <https://doi.org/10.1038/nrn3176>
- [4] Silver, J., & Miller, J. H. (2004). Regeneration beyond the glial scar. *Nature reviews. Neuroscience*, 5(2), 146–156. <https://doi.org/10.1038/nrn1326>
- [5] He, Z., & Jin, Y. (2016). Intrinsic Control of Axon Regeneration. *Neuron*, 90(3), 437–451. <https://doi.org/10.1016/j.neuron.2016.04.022>
- [6] Liu, K., Tedeschi, A., Park, K. K., & He, Z. (2011). Neuronal Intrinsic Mechanisms of Axon Regeneration. *Annual Review of Neuroscience*, 34, 131-152. doi:10.1146/annurev-neuro-061010-113723.
- [7] Ghosh, S., & Hui, S. P. (2016). "Regeneration of Zebrafish CNS: Adult Neurogenesis." *Neural Plasticity*. doi:10.1155/2016/5815439.
- [8] Reimer, Michell M., Veronika Kuscha, et al. "Sonic Hedgehog Is a Polarized Signal for Motor Neuron Regeneration in Adult Zebrafish." *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, vol. 29, no. 48, 2009, pp. 15073–15082, doi:10.1523/JNEUROSCI.4748-09.2009.
- [9] Shen, W. Y., Luo, Y., Meng, X., Zhang, T., Zhang, M. L., & Yu, J. (2022). Identification of key genes involved in recovery from spinal cord injury in adult zebrafish. *Neural Regeneration Research*, 17(6), 1334-1342. <https://doi.org/10.4103/1673-5374.327360>
- [10] Bie, F., Wang, K., Xu, T., Yuan, J., Ding, H., Lv, B., Liu, Y., & Lan, M. (2021). The potential roles of circular RNAs as modulators in traumatic spinal cord injury. *Biomedicine & Pharmacotherapy*, 141, Article 111826.
- [11] Zeng, C. W., & Tsai, H. J. (2023). The Promising Role of a Zebrafish Model Employed in Neural Regeneration Following a Spinal Cord Injury. *International journal of molecular sciences*, 24(18), 13938. <https://doi.org/10.3390/ijms241813938>
- [12] Seifert, A. W., & Maden, M. (2014). New insights into vertebrate skin regeneration. *International review of cell and molecular Biology*, 310, 129–169. <https://doi.org/10.1016/B978-0-12-800180-6.00004-9>
- [13] Umesono Y, & Agata K. (2009). Evolution and regeneration of the planarian central nervous system. *Dev. Growth. Differ*, 51, 185-195. <https://doi.org/10.1111/j.1440-169X.2009.01099.x>
- [14] Briona, L. K., & Dorsky, R. I. (2014). Radial Glial Progenitors Repair the Zebrafish Spinal Cord Following Transection. *Experimental Neurology*, 256, 81-92. doi:10.1016/j.expneurol.2014.03.017.
- [15] Zeng, C. W., Kamei, Y., Shigenobu, S., Sheu, J. C., & Tsai, H. J. (2021). Injury-induced Cav1-expressing cells at the lesion rostral side play major roles in spinal cord regeneration. *Open Biology*, 11(200304). <https://doi.org/10.1098/rsob.200304>
- [16] Cavone, L., McCann, T., Drake, L. K., Aguzzi, E. A., Oprisoreanu, A. M., Pedersen, E., Sandi, S., Selvarajah, J., Tsarouchas, T. M., Wehner, D., & others. (2021). A unique macrophage subpopulation signals directly to progenitor cells to promote regenerative neurogenesis in the zebrafish spinal cord. *Developmental Cell*, 56(1617-1630). <https://doi.org/10.1016/j.devcel.2021.01.015>
- [17] Bhatt, D. H., Otto, S. J., Depoister, B., & Fetcho, J. R. (2004). Cyclic AMP-induced repair of zebrafish spinal circuits. *Science*, 305(5684), 254-258.
- [18] Becker, T., and Becker, C. G. (2001). Regenerating descending axons preferentially reroute to the gray matter in the presence of a general macrophage/microglial reaction caudal to a spinal transection in adult zebrafish. *J. Comp. Neurol.* 433, 131–147. doi: 10.1002/cne.1131
- [19] Kyritsis, N., Kizil, C., Zocher, S., Kroehne, V., Kaslin, J., Freudenreich, D., Iltzsche, A., & Brand, M. (2012). Acute inflammation initiates the regenerative response in the adult zebrafish brain. *Science*, 338(1353-1356). <https://doi.org/10.1126/science.1227266>

- [20] Tsarouchas, T. M., Wehner, D., Cavone, L., Munir, T., Keatinge, M., Lambertus, M., Oprisoreanu, A. M., Kuznitchev, A., Dong, Z., Bhattarai, P., Shiau, F., Hansen, J. N., Alnassar, N., Randlett, O., ... Becker, T. (2018). Dynamic control of proinflammatory cytokines Il-1 β and Tnf- α by macrophages in zebrafish spinal cord regeneration. *Nature Communications*, 9(1), Article 4670. <https://doi.org/10.1038/s41467-018-07036-w>
- [21] Hui, S. P., Nag, T. C., & Ghosh, S. (2020). Neural cells and their progenitors in regenerating zebrafish spinal cord. *The International Journal of Developmental Biology*, 64(4-5-6), 353–366. <https://doi.org/10.1387/ijdb.190130sg>
- [22] Cigliola, Valentina, et al. "Building Bridges, Not Walls: Spinal Cord Regeneration in Zebrafish." *Disease Models & Mechanisms*, vol. 13, no. 5, 2020, doi:10.1242/dmm.044131.
- [23] Howe, K., Clark, M. D., Torroja, C. F., Torrance, J., Berthelot, C., Muffato, M., Collins, J. E., Humphray, S., McLaren, K., Matthews, L., McLaren, S., Sealy, I., Caccamo, M., Churcher, C., Scott, C., ... Stemple, D. L. (2013). The zebrafish reference genome sequence and its relationship to the human genome. *Nature*, 496(7446), 498–503. <https://doi.org/10.1038/nature12111>
- [24] Sanger, H. L., et al. "Viroids Are Single-Stranded Covalently Closed Circular RNA Molecules Existing as Highly Base-Paired Rod-like Structures." *Proceedings of the National Academy of Sciences of the United States of America*, vol. 73, no. 11, 1976, pp. 3852–3856, doi:10.1073/pnas.73.11.3852.
- [25] Cocquerelle, C., et al. "Mis-Splicing Yields Circular RNA Molecules." *FASEB Journal: Official Publication of the Federation of American Societies for Experimental Biology*, vol. 7, no. 1, 1993, pp. 155–160, doi:10.1096/fasebj.7.1.7678559.
- [26] Nigro, Janice M., et al. "Scrambled Exons." *Cell*, vol. 64, no. 3, 1991, pp. 607–613, doi:10.1016/0092-8674(91)90244-s.
- [27] Zhou, Wei-Yi, et al. "Circular RNA: Metabolism, Functions and Interactions with Proteins." *Molecular Cancer*, vol. 19, no. 1, 2020, doi:10.1186/s12943-020-01286-3.
- [28] Li, X., Yang, L., & Chen, L.-L. (2018). The biogenesis, functions, and challenges of circular RNAs. *Molecular Cell*, 71(3), 428-442. <https://doi.org/10.1016/j.molcel.2018.06.034>
- [29] Panda, Amaresh Chandra. "Circular RNAs Act as miRNA Sponges." *Advances in Experimental Medicine and Biology*, Springer Singapore, 2018, pp. 67–79.
- [30] Chung, Hee Kyoung, et al. "Circular RNA Cdr1as Inhibits Proliferation and Delays Injury-Induced Regeneration of the Intestinal Epithelium." *JCI Insight*, vol. 9, no. 4, 2 2024, doi:10.1172/jci.insight.169716
- [31] Zhao, Jingwei, et al. "A circRNA Derived from Linear HIPK3 Relieves the Neuronal Cell Apoptosis in Spinal Cord Injury via ceRNA Pattern." *Biochemical and Biophysical Research Communications*, vol. 528, no. 2, 2020, pp. 359–367, doi:10.1016/j.bbrc.2020.02.108.
- [32] Ghosh S, Hui SP. Axonal regeneration in zebrafish spinal cord. *Regeneration (Oxf)*. 2018 Apr 22;5(1):43-60. doi: 10.1002/reg2.99. PMID: 29721326; PMCID: PMC5911453.
- [33] Siddiq, Mustafa M., et al. "Spinal Cord Injury Regulates Circular RNA Expression in Axons." *Frontiers in Molecular Neuroscience*, vol. 16, 2023, doi:10.3389/fnmol.2023.1183315.
- [34] Demircan, Turan, and Barış Ethem Süzek. "The Dynamic Landscapes of Circular RNAs in Axolotl, a Regenerative Medicine Model, with Implications for Early Phase of Limb Regeneration." *Omics: A Journal of Integrative Biology*, vol. 27, no. 11, 2023, pp. 526–535, doi:10.1089/omi.2023.0158.
- [35] Guo, Yong, et al. "Circ_0013613 Promotes the Recovery of Spinal Cord Injury by Regulating Mir-370-3p/Caspase1 to Regulate Neuronal Pyroptosis." *Acta Biochimica Polonica*, vol. 70, no. 1, 2023, doi:10.18388/abp.2020_6244.
- [36] Nielsen, Anne F., et al. "Best Practice Standards for Circular RNA Research." *Nature Methods*, vol. 19, no. 10, 2022, pp. 1208–1220, doi:10.1038/s41592-022-01487-2.
- [37] Xiao, Mei-Sheng, and Jeremy E. Wilusz. "An Improved Method for Circular RNA Purification Using RNase R That Efficiently Removes Linear RNAs Containing G-Quadruplexes or

- Structured 3' Ends." *Nucleic Acids Research*, vol. 47, no. 16, 2019, pp. 8755–8769, doi:10.1093/nar/gkz576.
- [38] Grabherr, Manfred G., et al. "Full-Length Transcriptome Assembly from RNA-Seq Data without a Reference Genome." *Nature Biotechnology*, vol. 29, no. 7, 2011, pp. 644–652, doi:10.1038/nbt.1883.
- [39] Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, Batut P, Chaisson M, Gingeras TR. STAR: ultrafast universal RNA-seq aligner. *Bioinformatics*. 2013 Jan 1;29(1):15-21. doi: 10.1093/bioinformatics/bts635. Epub 2012 Oct 25. PMID: 23104886; PMCID: PMC3530905.
- [40] Yang, Hou-Zhi, et al. "Deficiency of Acetyltransferase Nat10 in Zebrafish Causes Developmental Defects in the Visual Function." *Investigative Ophthalmology & Visual Science*, vol. 65, no. 2, 2024, p. 31, doi:10.1167/iovs.65.2.31.
- [41] Hui, Subhra Prakash et al. "Cellular response after crush injury in adult zebrafish spinal cord." *Developmental dynamics : an official publication of the American Association of Anatomists* vol. 239, 11 (2010): 2962-79. doi:10.1002/dvdy.22438
- [42] Letchuman, Sunjay, et al. "Transcription Factor Hb9 Is Expressed in Glial Cell Lineages in the Developing Mouse Spinal Cord." *eNeuro*, vol. 9, no. 6, 2022, p. ENEURO.0214-22.2022, doi: 10.1523/eneuro.0214-22.2022.
- [43] Reimer, Michell M., Inga Sörensen, et al. "Motor Neuron Regeneration in Adult Zebrafish." *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, vol. 28, no. 34, 2008, pp. 8510–8516, doi:10.1523/jneurosci.1189-08.2008.
- [44] Lu, Thomas X., and Marc E. Rothenberg. "MicroRNA." *The Journal of Allergy and Clinical Immunology*, vol. 141, no. 4, 2018, pp. 1202–1207, doi:10.1016/j.jaci.2017.08.034.
- [45] Brown, Rikki A. M., et al. "Total RNA Extraction from Tissues for microRNA and Target Gene Expression Analysis: Not All Kits Are Created Equal." *BMC Biotechnology*, vol. 18, no. 1, 2018, doi:10.1186/s12896-018-0421-6.
- [46] Kristensen, Lasse S., et al. "Spatial Expression Analyses of the Putative Oncogene ciRS-7 in Cancer Reshape the microRNA Sponge Theory." *Nature Communications*, vol. 11, no. 1, 2020, doi:10.1038/s41467-020-18355-2.
- [47] Zheng, Qiupeng et al. "Circular RNA profiling reveals an abundant circHIPK3 that regulates cell growth by sponging multiple miRNAs." *Nature communications* vol. 7 11215. 6 Apr. 2016, doi:10.1038/ncomms11215
- [48] Liu, Jiqing, et al. "CircRNA75 and CircRNA72 Function as the Sponge of MicroRNA-200 to Suppress Coelomocyte Apoptosis Via Targeting Tollip in *Apostichopus Japonicus*." *Frontiers in Immunology*, vol. 12, 2021, doi:10.3389/fimmu.2021.770055.
- [49] Feng, Xin-Yi, et al. "New Insight into circRNAs: Characterization, Strategies, and Biomedical Applications." *Experimental Hematology & Oncology*, vol. 12, no. 1, 2023, doi:10.1186/s40164-023-00451-w.