Proceedings of the 4th International Conference on Biological Engineering and Medical Science
DOI: 10.54254/2753-8818/72/2024.19365

Identifying the role of circular RNA in zebrafish fin regeneration

Junyan Li[1,4,*,†], Xin Yao[2,5,†], Hangchen Liu[3,6]

[1]Bioengineering, Nanjing Agricultural University, Nanjing, 211800, China
[2]Shanghai High School International Division, Shanghai, 200231, China
[3]Haidian Foreign Language Academy, Beijing, 100103, China

[4]kevinli@stu.njau.edu.cn
[5]wxyao88@sina.com
[6]18310300961@163.com
*corresponding author
[†]These authors should be considered as the co-first authors equally.

Abstract. Circular RNA (circRNA) is a class of RNA with circular structures and diverse functions. In recent years, they have emerged as a key regulatory molecule with abilities to regulate stem cell differentiation and other components of the cell cycle including tumor suppression and cell cycle progression. Yet their functions in regeneration are under-investigated. We chose zebrafish as a model organism due to their remarkable regenerative capabilities, and susceptibility to genetic modifications. This study aims to explore the role of circRNAs in zebrafish caudal fin regeneration, with a specific focus on their function as miRNA sponges. Through RNA sequencing (RNA-seq) and cross-linking immunoprecipitation sequencing (CLIP-seq), we will identify differentially expressed circRNAs during the regeneration process and analyze their potential miRNA-binding sites. We will employ small hairpin RNA (shRNA) knockdown experiments to investigate the functional significance of circRNA-miRNA interactions in vivo. This will provide novel insights into the regulatory functions of circRNAs in vertebrate regeneration, with implications for advancing regenerative medicine and therapeutic strategies for human tissue repair.

Keywords: zebrafish, regeneration, circular RNA, miRNA sponges.

1. Introduction

1.1. Zebrafish in regeneration

Mammals have been known to possess comparatively low regeneration abilities, especially in entire appendages[1]. Yet studies of such powerful regeneration are still ongoing projects featuring other species, which could potentially yield solutions to this inability in regenerative medicines. Thus, this proposal will focus on zebrafish, which is a highly regenerative model. As a model organism, zebrafish are susceptible to genetic manipulations such as fluorescence receptor cell lines in the embryonic state. It can regenerate the fin, heart, and parts of the spinal cord[1]. It also has 3428 different circular

© 2025 The Authors. This is an open access article distributed under the terms of the Creative Commons Attribution License 4.0 (https://creativecommons.org/licenses/by/4.0/).

DOI: 10.54254/2753-8818/72/2024.19365

RNAs(circRNA), which may have roles in regulating their regeneration [2]. In this study, we will be choosing the zebrafish fin for its high regenerative ability and accessibility.

1.2. Studying the Zebrafish Fin

In particular, zebrafish fin regeneration is a common study topic due to its accessibility. It also involves the whole appendage, including different tissue types of bone, nerves, blood vessels, and connective tissues [3]. The zebrafish caudal fin is also an ideal material for regeneration research due to its simple structure, ease of surgery, and rapid regeneration. Also, the bi-lobed morphology makes it an optimal material for analysis of the differential growth rate between the dorsal and ventral regions.

There has also been some research on the other mechanisms regulating its regeneration, such as the Wnt signaling pathway and miRNA's functions in inhibition through binding to the 3' UTR region of target mRNAs to downregulate gene expression. For example, silencing key enzymes Dicer involved in miRNA biosynthesis leads to impaired fin regeneration in zebrafish; the Fgf signaling pathway is regulated by miRNAs; Specific miRNAs, such as miR-203 also become downregulated to allow Lef1 expression in the Wnt signaling pathway to allow regeneration by allowing stem cells to differentiate [1]. Yet such research is still left with gaps, such as the regulation of miR 203 has not yet been discovered. Thus, given circRNA's abundance in zebrafish, it is reasonable to propose that circRNA has potential regulatory functions in the regeneration of zebrafish fin. For example, circRNA is known to have a sponge function that may enable it to down-regulate miR 203, and this is not the only known function of circRNA.

1.3. The diverse roles of circRNA

CircRNAs are covalently closed single strands of RNA [4] formed by circularization through back-splicing between the 3' and 5'splice sites [2]. They have diverse functions within the cell, including miRNA sponges, transcriptional regulators, and protein templates and interacting with protein [4]. Their unique function as miRNA sponges depends upon their possession of an array of binding sites for a particular miRNA or miRNA family, allowing it to 'soak' them up [5]. This binding lowers the level of miRNA available to regulate mRNA targets and thus can upregulate genes that a miRNA usually suppresses [4].

CircRNA's protein interactions are also diverse. They can bind with proteins to block their normal activity, stabilize protein-protein interactions, recruit proteins to specific sites, and even transport proteins [4]. Due to their many functions, circRNAs are involved in many processes, including modulating the activities of the cell cycle and stem cell differentiation to maintain cellular balance. For example, circ-FOXO3 can interact with CDK2 to block cell cycle progression. Previous studies have also shown their roles in guiding development and tumor suppression. For instance, CDR1's interactions with miR-1 in zebrafish embryos correlated with brain development. Interactions between circ-sry and miR138 have also been correlated to cancer [6]. However, their roles in regeneration are still under-researched.

1.4. Previous studies of circRNA in regeneration and their limitations

There have been some previous studies on the function of circRNA in regeneration, such as its function in heart, neural, and bone regeneration [7-8]. These studies further justified the importance of circRNA in regeneration. Yet there is no investigation of its role in the regeneration of complete appendages containing multiple tissue types. One of the only related studies was done on circRNA expression in axolotl limb regeneration, which identifies their expression level changes [9], but not functions. Thus, further studies on circRNA in the field of complete appendage regeneration are needed. It could lead to the discovery of more robust roles of circRNA in regeneration, potentially functioning as master regulatory mechanisms. This project seeks to fill this research gap by studying the function of circRNA during zebrafish fin regeneration, which could have much significance.

DOI: 10.54254/2753-8818/72/2024.19365

1.5. The importance of this study

CircRNAs are highly conserved in evolution [10], which makes them key to discovering key regulators of regeneration, in multiple tissues and even species. Giving them a larger possibility of applications for human recipients. Their potential function in appendage regeneration is also lucrative due to the involvement of tissues that are commonly hard to regenerate for humans, like nerve tissues. Not to mention the complexity of regenerating a whole appendage, which requires the simultaneous regulation of multiple tissues [3]. More information about the specific ways circRNA is involved in these difficult regeneration processes could lead to new approaches to aiding human regeneration. Their stability compared to other types of RNA, makes them adapt for being used in medication for regeneration after a wound. Thus, it is key to learn more about their functions in regulation.

1.6. Project design

The main goal of this project is to investigate the role of circRNAs in zebrafish fin regeneration, with a focus on sponge functions. Our goals are as following:

Goal 1: Identify circRNAs that are involved in zebrafish fin regeneration and their role in the process.

Goal 2: Use RNAseq to find the circRNA for which expression levels change in the process, then CLIP-seq and small hairpin RNA (shRNA) knockout experiments to identify functions as miRNA sponges as we hypothesized to exist.

Goal 3: Conduct further research on the other roles of circRNA in regeneration.

1.7. Experimental technologies and their mechanisms

RNAseq can detect the sequence and estimate the expression level of RNA. In this study, it will be used to locate specific circRNAs whose expression levels change significantly in regeneration. Cross-linking immunoprecipitation sequencing, or CLIP-seq, can identify and locate proteins bound to RNA [11]. It will be used to determine the presence of miRNA sponges. Specially designed shRNA will be used to target and knock down circRNA identified from earlier steps for observation of phenotypical differences, and qPCR will be used to double-check the results.

2. Objective 1: Identify circRNAs that are differentially expressed during the regeneration process and match miRNA binding site in circRNA

Rationale: CircRNA involved in the regulation of regeneration will exhibits different expression levels between regenerative tissues and normal tissues. Previous studies suggest that circRNA regulates regeneration by acting as a miRNA sponge. We will initially use databases to match the miRNA binding site on circRNAs.

Methods:

Sampling and Processing:

We will collect tissue from a normal fin (0h after amputation) and tissues regenerating at different time points after fin amputation (12h, 24h,36h,48h,72h,96h). Zebra fish are typically cultured at 27°C, however, since their regeneration rate is approximately double at 33°C compared to 27°C, all zebrafish in this experiment will be cultured at 33°C.

RNA isolation and circRNA sequencing:

We will use TRIzol reagent to isolate the total RNA from tissues collected at step 1. Following previously established protocols, we will extract and purify circRNA[9]. Briefly, we will mix total RNA samples with RiboLock RNase inhibitor and RNase R to eliminate the linear RNA. After phenol-chloroform and sodium acetate precipitation, circRNAs will be purified using a Polyadenylation kit that adds a poly(A) tail to the free hydroxyl group at the 3’ end. The Poly(A) Purist kit will cleave all RNA with a polyA tail at the 3’ ends. Thus, all non-circular RNA will be given a poly-A tail and degraded, while circRNAs will remain unaffected due to their back splicing which results in no 3’ ends.

CircRNA sequencing:

DOI: 10.54254/2753-8818/72/2024.19365

RNA sequencing will be used for circRNA sequencing. This method can directly sequence RNA molecules without prior knowledge of the genome sequence and has high sensitivity, enabling the detection of low-abundance transcripts.

Data analysis:

We will perform data analysis to identify potential binding sites on circRNAs whose expression levels have significantly changed according to the earlier experimental results. The miRNAs of interest will be those identified in prior studies to have altered expression levels during zebrafish fin regeneration. The goal is to determine whether these circRNAs contain miRNA binding sites. If complementary sequences are found, it would suggest that the circRNA has the potential to act as a miRNA sponge.

Expected outcome:

It is likely that several different circRNA will be upregulated and several will be downregulated during regeneration. Their levels of expression should gradually change during zebrafish fin regeneration. By matching the binding sites, we hypothesize that circRNAs with altered expression levels have miRNA binding sites, supporting the idea that circRNAs influence regeneration by acting as miRNA sponges.

3. Objective 2: Identify circRNA acting as miRNA sponges to regulate regeneration in zebrafish fin

Rationale: Earlier papers suggest that miRNA plays a great role in zebrafish regeneration [1]. Yet the regulatory mechanisms of these miRNAs are not yet known. These miRNAs may be regulated by circRNA acting as their sponges. Identifying circRNA acting as sponges to regulate regeneration can allow the understanding of the circRNA involved.

Methods:

Looking for potential sponges:

We will use data-based analysis to look for potential bind sites on the circRNA for which expression levels have significantly changed according to the earlier experiment identifications. The miRNAs that we will focus on will be those identified to have expression level changes in zebrafish fin regeneration as shown by previous studies.

Testing for sponges in the context of the fin itself through CLIP seq:

The sponging ability of the circRNA identified from the last step could be further tested through the CLIP (cross-linking immune precipitation) seq. In the process, sample tissues from regenerating zebrafish skin will again be used to perform CLIP seq. UV light can crosslink RNA with protein in the mixture, meaning that all circRNA with miRNA binding ability should have bound with their corresponding miRNA and argonaute(AGO) proteins that guide these miRNAs. This complex can be isolated out of the mixture through the use of antigens targeting the AGO proteins since these proteins are used to link miRNA to circRNA. After purification and other processes, we perform high-throughput sequencing and result analysis to determine whether circRNA has been precipitated. Specifically, we will look for the predicted groupings from the data analysis section. If the circRNA acts as a sponge, then the corresponding sequence of the circRNA can be identified through high-throughput sequencing results.

Checking the phenotypical effects in regeneration caused by these circRNA:

After determining the sponging function of circRNA, we seek to perform more experiments to see their phenotypical effects to determine if they influence regeneration. Zebrafish will be raised and amputated in environments similar to those earlier mentioned. We will be looking for differences in knocking out and overexpressing the circRNA acting as sponges. ShRNA designed to target back-splicing junctions of the specific circRNA involved can be used to knock out the circRNA without harming any non-circular forms of the same RNA [12]. This is because the sequence of the back-splice junction is specific to the circRNA. The knockdown can be achieved by injecting these designed shRNAs. Meanwhile, the overexpression can be done through the injection of additional circRNA made outside of the zebrafish itself.

DOI: 10.54254/2753-8818/72/2024.19365

The injection can be performed by following the amputation of the zebrafish fin with electroporation (which increases cell permeability and allows the intake of the substances injected) and microinjections [1]. The microinjections should be done 2 days after amputation since microinjection can't be performed directly after amputation. The phenotypical results' difference could be observed 6 days later, which is the time taken for zebrafish fin to regenerate.

The experimental designs are as follows. First, the zebrafish fin will be amputated, and the dorsal and ventral sections will be treated differently. The dorsal section will be treated with the independent variables of shRNA, circRNA, and other treatments, while the ventral section will be left untreated as a group for comparison. Second, there should be 5 different groups of experiments. In the first group, injection of the shRNA should be used to knock out the specific circRNA. In the second group, an injection of circRNA will be used for overexpression. The third group should include no injection. This group can test the regeneration of zebrafish under normal conditions. The fourth should be a rescue group, where shRNA should be co-injected with circRNA genetically modified outside of the zebrafish to have the same function yet no shRNA bind sites. This group can test whether circRNA is the limiting factor. The fifth should act as a mismatch group, where shRNA with no specific target RNA should be injected. It would be able to test if the microinjection process for shRNA itself has any unintended effects on the zebrafish fin. We will measure the phenotypical differences in the form of the area of regenerated fins.

Other controls:

To ensure that electroporation will not affect regeneration, regeneration in a non-electroporated fin should be compared to an electroporated fin. To ensure the success of shRNA's knockdown of circRNA, qPCR can be performed to test for expression level differences in treated and untreated fins.

Performing more investigation into cellular functions:

About the miRNA regulation, if the function of the miRNA is not yet known, then the regeneration of different cell types will be investigated. To determine which specific cell type is affected by circRNA, we can apply fluorescent reporter lines technology. After tissue injury, fluorescent reporter genes can be used to label cells involved in regeneration. By observing changes in the fluorescence signal, the progress of tissue regeneration can be monitored. We can determine at which step circRNA affects regeneration, thereby delving deeper into the associated signaling pathways and regulatory networks.

Expected outcome:

For the first experiments, we expect to find circRNA acting as sponges for miRNA regulating zebrafish fin regeneration such as miR-203, and others for which the expression level changes. In the later experiments, we expect that the control experiments (effect of electroporation, shRNA knockdown successfulness) should prove our experimental methods to be capable and bias-free. Then in the experiment groups, assuming that we target a normally overexpressed circRNA targeting a miRNA that inhibits regeneration and thus needs to be under-expressed, such as miR 203, we expect to find that those with knockdown of circRNA should exhibit repression of regeneration, and overexpression will exhibit proliferation and extra regeneration. For groups 3,4 and 5, it is expected that no injection, rescue, or mismatch should leave no effect on the regenerating fin. We will also likely get to know which tissue is controlled more directly by the circRNA in question. For normally down regulated circRNA that regulates miRNA that is upregulated during regeneration to allow regeneration, we will expect the opposite results for the experimental group and similar results in the rescue, mismatch, and uninjected control groups.

4. Future plan: Evaluate other roles played by circRNA in zebrafish fin regeneration and explore the potential multifunctionality of circRNA

Rationale: CircRNAs have multiple functions, such as binding with RBPs, serving as protein scaffolds to facilitate the localization of substrates and enzymes, or being translated into proteins. Some specific circRNAs have been proven to regulate the differentiation of various types of stem cells through protein sponge effects and signaling pathways such as PI3K-Akt, MAPK, and Wnt. For instance, circBIRC6 and circCORO1C are involved in the regulation of pluripotency and differentiation of human embryonic

stem cells; circZNF91 is involved in the differentiation of epidermal stem cells into keratinocytes.[13]Other studies have indicated that circ-Amotl1 can bind to and activate the AKT signaling pathway, promoting the nuclear translocation of phosphorylated AKT. Additionally, circ-Amotl1 can also promote the nuclear translocation of PDK1, further enhancing the phosphorylation and activation of AKT.[14]The regeneration process involves numerous and complex regulatory networks, so we believe that circRNAs play additional roles in regeneration.

Methods:

We will conduct a comprehensive literature review to integrate other functions of circRNA with the regeneration process, aiming to explore additional roles of circRNA in regeneration.We will also develop experimental designs to test these additional functions.

Expected outcome: We aim to link circRNA with regeneration to uncover more information. Based on the role of circRNA in the regulation of stem cells and regeneration, we will propose new hypotheses to understand the role of circRNA in stem cells and regeneration.

5. Conclusion

Our study aims to investigate the regulatory role of circRNAs in zebrafish fin regeneration, focusing on their function as miRNA sponges. By utilizing RNA-seq and CLIP-seq, we plan to identify circRNAs that exhibit differential expression during the regeneration process and to map their miRNA-binding sites. Subsequent knockdown experiments will be designed to explore the functional significance of these circRNA-miRNA interactions. This research is expected to provide valuable insights into the molecular mechanisms governing vertebrate regeneration and could contribute to the development of novel strategies for human tissue repair and regenerative medicine. Further studies will also explore additional roles of circRNAs, aiming to broaden our understanding of their regulatory functions in tissue regeneration.

References

[1] Thatcher, E. J., Paydar, I., Anderson, K. K. & Patton, J. G. Regulation of zebrafish fin regeneration by microRNAs. Proc. Natl. Acad. Sci. U.S.A. 105, 18384–18389 (2008).
[2] Sharma, D. et al. A genome-wide map of circular RNAs in adult zebrafish. Sci Rep 9, 3432 (2019).
[3] Ribeiro, A. O. et al. MicroRNA roles in regeneration: Multiple lessons from zebrafish. Developmental Dynamics 251, 556–576 (2022).
[4] Zhou, W.-Y. et al. Circular RNA: metabolism, functions and interactions with proteins. Mol Cancer 19, 172 (2020).
[5] Memczak, S. et al. Circular RNAs are a large class of animal RNAs with regulatory potency. Nature 495, 333–338 (2013).
[6] Qu, S. et al. The emerging landscape of circular RNA in life processes. RNA Biology 14, 992–999 (2017).
[7] Mester-Tonczar, J. et al. Circular RNAs in Cardiac Regeneration: Cardiac Cell Proliferation, Differentiation, Survival, and Reprogramming. Front. Physiol. 11, 580465 (2020).
[8] Yao, C. & Yu, B. Role of Long Noncoding RNAs and Circular RNAs in Nerve Regeneration. Front. Mol. Neurosci. 12, 165 (2019).
[9] Demircan, T. & Süzek, B. E. 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 27, 526–535 (2023).
[10] Shen, Y., Guo, X. & Wang, W. Identification and characterization of circular RNAs in zebrafish. FEBS Letters 591, 213–220 (2017).
[11] Hafner, M. CLIP and complementary methods. (2021).
[12] He, A. T., Liu, J., Li, F. & Yang, B. B. Targeting circular RNAs as a therapeutic approach: current strategies and challenges. Sig Transduct Target Ther 6, 185 (2021).
[13] Lin, Z. et al. Functions and mechanisms of circular RNAs in regulating stem cell differentiation. RNA Biology 18, 2136–2149 (2021).

DOI: 10.54254/2753-8818/72/2024.19365

[14] Zeng, Y. et al. A Circular RNA Binds To and Activates AKT Phosphorylation and Nuclear Localization Reducing Apoptosis and Enhancing Cardiac Repair. Theranostics 7, 3842–3855 (2017).