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Comprehensive Spatiotemporal Profiling of circRNA Expression Dynamics and Functional Roles in Regenerating Zebrafish Heart

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Abstract. Cardiovascular diseases are a major cause of mortality globally, primarily due to the limited regeneration ability of human hearts. Zebrafish hearts show high regenerative potential, which makes them a common model for studying mechanisms of heart regeneration. circRNAs have gained increasing interest from researchers as a regulatory molecule. However, their functions in heart regeneration are poorly recognized, especially in zebrafish, where there is a lack of systematic and comprehensive analysis of circRNA expression dynamics and their spatial distribution. This study aims to characterize the dynamics of circRNA expression, including circRNA expression location and distribution at 5 time points of zebrafish heart regeneration, and identify regeneration-associated circRNAs to evaluate their function in zebrafish heart regeneration. The outcomes of this project will provide a foundation for research on circRNAs and the corresponding mechanisms and regulation of zebrafish heart regeneration.

Keywords: circularRNA, zebrafish heart regeneration, spatiotemporal expression profiling, CRISPR functional analysis

1. Introduction

1.1. Heart damage and regeneration

Cardiovascular diseases are the leading cause of fatalities globally [1]. These diseases often cause the loss of cardiomyocytes. The dead cardiomyocytes are replaced by fibrotic scar tissue, this lead to progressive cardiac dysfunction [2,3]. Most adult mammals, including humans, lack heart regenerative capacity, which makes recovery from cardiac injury extremely difficult [4,5]. Meanwhile, certain species including zebrafish exhibit excellent heart regenerative capabilities, and can repair lost tissues after injury [6]. Although heart regeneration has been investigated in many vertebrates, its mechanism remains incompletely understood [7]. Understanding the mechanisms of heart regeneration is essential for developing therapies for cardiovascular diseases.

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1.2. Zebrafish as a model organism

Zebrafish shows high heart regenerative capabilities after injury. Heart tissues are repaired through cardiomyocyte dedifferentiation and proliferation. After injury, mature, differentiated cardiomyocytes near the damaged region will dedifferentiate and lose their specialized characteristics. They then re-enter the cell cycle, and proliferate to replace lost tissues [8]. Their genome shares approximately 70% homology with human genome, and they offer experimental advantages [9], Therefore, zebrafish have become a classical model for studying the mechanisms of heart regeneration.

1.3. circRNA and heart regeneration

Circular RNAs (circRNAs) are non-coding RNAs synthesized through back-splicing [10]. They are notable for their chemical stability, and have diverse functions including acting as miRNA and protein sponges, protein scaffolds, and translational templates [11]. circRNAs have been identified as crucial to heart regeneration of other model organisms, such as mice [12]. However, their functions in zebrafish heart regeneration remain largely unexplored.

1.4. Research gap

Current researches have mapped circRNA expression profiles in both the entire organism and those expressed in the heart [13]. However, systematic analysis of circRNA expression during heart regeneration has not been done, particularly regarding their temporal dynamics and spatial localization. Furthermore, the functional analysis of circRNAs in zebrafish cardiac regeneration has not been conducted. Their roles in zebrafish heart regeneration remains unexplored. These gaps limit the understanding of the regulatory roles of circRNAs in heart regeneration.

2. Project description

The major aim of this project is to systematically identify and compare the circRNAs expressed in healthy (uninjured) and regenerating zebrafish hearts across five time points (1dpa, 3dpa, 7dpa, 14dpa and 28dpa), locate their spatial localization within cardiomyocytes, and determine their functional roles in zebrafish heart regeneration.

2.1. Objective 1: circular RNA sequencing and localization during zebrafish heart regeneration

2.1.1. Methods

(1) Sample design

Adult zebrafish hearts will be treated with ventricular resection [14]. Hearts will be collected at baseline (uninjured) and at 1, 3, 7, 14, and 28 dpa and its cardiomyocyte will be sequenced. These time points are selected based on different stages of regeneration: early injury response (1 dpa), cardiomyocyte dedifferentiation (3 dpa), cardiomyocyte proliferation (7 dpa), cardiomyocyte re-differentiation (14 dpa), and late-stage regeneration with restored myocardial structure and function(28 dpa) [15]. The temporal expression dynamics of circRNA will be captured, so both transiently regulated circRNAs and those with sustained roles in regeneration can be identified.

(2) circRNA preparation and sequencing

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Total RNA will be extracted using the RNeasy Mini Kit (Qiagen, USA) with on-column DNase digestion. Ribosomal RNA will be depleted using Ribo-zero gold rRNA removal probe, followed by RNase R treatment to selectively degrade linear RNAs [15]. Strand-specific libraries will be prepared and sequenced using Illumina paired-end sequencing.

(3) Data analysis pipeline

circRNAs will be identified using CIRI [16]. Identified circRNAs will be cross-referenced with circBase [17] and circAtlas 3.0 [18] databases to be validated. Differential expression analysis will be used to compare regenerating and baseline hearts.

(4) circRNA localization and distribution assays

To identify circRNA distribution, nucleocytoplasmic fractionation will be performed on zebrafish heart tissue, followed by qRT-PCR [19] using primers designed using NCBI dataset, Primer 3 plus and NCBI BLAST. This would estimate and quantify the distribution proportion of the overall circRNAs. Reference genes such as Histone H3 and GAPDH will be used for normalization. For spatial resolution, fluorescence in situ hybridization (FISH) [19] will be performed using probes designed by Chorus 2 and NCBI BLAST. Confocal microscopy will be used to visualize circRNA distribution within cardiomyocytes [20].

(5) Functional annotation

Host genes will be analyzed using Gene Ontology (GO) and KEGG pathway [13] to predict the potential roles of circRNA.

2.1.2. Expected outcome

This objective will produce a comprehensive atlas of circRNAs expressed during zebrafish heart regeneration. The altas will include circRNAs expressed at 1, 3, 7, 14, and 28 dpa, and its distribution and location in the heart. Short-read Illumina sequencing is expected to provide a quantitative profile of circRNAs expressed in zebrafish heart. Through expression analysis across the five regeneration stages (1, 3, 7, 14, 28 dpa), stage-specific circRNAs that are transiently upregulated during regeneration stages, and circRNAs with expression changes throughout the regenerative process will be identified. Localization assays will produce spatial maps of circRNA expression, while functional annotation will suggest their regulatory roles in biological process such as cell cycle regulation. Together, these results will generate testable hypotheses about how circRNAs may regulate regeneration, forming the foundation for downstream functional analysis.

2.2. Objective 2: functional analysis of circRNAs in zebrafish heart regeneration

2.2.1. Candidate selection and transgenic zebrafish generation

circRNAs identified in Objective 1 will be selected for functional validation. To test their functions, transgenic zebrafish with circRNA overexpression or knockdown in cardiomyocyte will be generated via microinjection into zebrafish embryo using the Tol2 transposon system, driven by the cardiomyocyte-specific cmlc2 promoter [21]. dCas9-VP64 will activate circRNA transcription for overexpression, while dCas9-KRAB will repress circRNA formation in knockdown lines. A P2A sequence will allow co-expression of two independent proteins from a single mRNA transcript [22], and MCP-mCherry will serve as a fluorescent reporter to visualize circRNA activity. sgRNAs will act as the navigation RNA of dCas9 protein, and polyA signals will ensure proper transcription termination. The expression level changes of circRNAs will be validated by qPCR.

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2.2.2. Treatment groups experimental design

The experimental design will include three treatment groups: wild-type controls, circRNA overexpression, and circRNA knockdown, with mCherry expression confirming construct activity, as shown in Table 1. After ventricular resection, regeneration will be assessed at time points 1, 3, 7, 14, and 28 dpa. Histological analysis of PCNA in the regenerating region of the heart will indicate whether the circRNA have an impact on heart regeneration: if the mCherry light exsist with PCNA light in same area, the circRNA has effect on heart regenration.

Table 1. Treatment groups

Treatment groups	circRNA OE	circRNA KD	mCherry	What shows
Wild Type Control	(-)	(-)	(-)	The baseline regeneration of zebrafish
Overexpression Group	(+)	(-)	(+)	Increased circRNA expression
Knock Down Group	(-)	(+)	(+)	Reduced circRNA expression

2.3. Expected outcome

This objective is expected to provide evidence of role of circRNAs during zebrafish heart regeneration. Overexpression of pro-regenerative circRNAs is expected to increase PCNA-positive cardiomyocytes, while knockdown of them should impair proliferation and delay tissue repair. Conversely, knockdown of anti-regenerative circRNAs may enhance regeneration, while overexpression of them should inhibit tissue repair. The co-localization of mCherry with PCNA will demonstrate that change in circRNA expression level will directly affects cardiomyocyte regenerative activity. These results will show correlations between circRNA expression and regeneration outcomes, which validate circRNAs as functional regulators.

3. Conclusion

This proposal provides a comprehensive approach for investigating the expression dynamics and functional roles of circRNA in zebrafish heart regeneration. Objective 1 will produce a circRNA expression atlas at five time points. Their location and distribution details in cardiomyocytes will be included. Objective 2 will correlate circRNA expressions with regeneration outcomes. Together, these investigations will enhance understanding of circRNA-mediated regulation in zebrafish heart regeneration, and provide basis for exploring their potential roles in cardiac repair.

3.1. Limitations

Some technical and biological limitations may affect the results. The reliance on bioinformatic pipelines for circRNA identification may result in false positives. During localization of circRNA, reference gene GAPDH may exhibit unstable expression due to lack of oxygen during regeneration, therefore, a better reference gene should be considered. In functional analysis, insufficient structural context of target sequences may lead to off-target effects in sgRNA design, potentially confounding the interpretation of functional outcomes.

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3.2. Future directions

Building on this study, future work will compare circRNA expression profiles across species with varying regenerative capacities. CircRNA expression profiles of zebrafish will be compared with those from other regenerating vertebrates, including neonatal mice and adult spiny mice [7] to identify conserved circRNAs expressed in regenerative hearts. These will then be compared with circRNA datasets of adult humans, where regenerative capacity is limiting [5]. By identifying circRNAs that are present in regenerating hearts but absent in human hearts, molecular deficits underlying limited human cardiac repair may be uncovered, creating foundations for circRNA based therapies to restore regenerative capacity in human heart.

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