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What Specific Mechanisms Do Lignano Stem Cells Regulate after Exposure to Gamma Rays (150 Gy)

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Abstract. The remarkable regenerative abilities of the flatworm Macrostomum lignano arise from a small population of robust and radioresistant stem cells that are capable of surviving acute (150 Gy) gamma irradiation. The in vivo study of these cells, however, is complicated by the intense autofluorescence resulting from the dying tissues that obscures standard fluorescent reporters in living animals. Here we develop a multifaceted approach that employs real-time in vivo imaging combined with a mechanistic molecular dissection. We developed a permanent line of transgenic animals expressing a dual-modality reporter (GeNL) under the control of a potent CAG promoter so that surviving stem cells can be examined in detail through bioluminescent imaging, and their proliferation and migratory behavior tractable through the use of a custom-built tracking microscope. In order to determine the molecular basis of their activation, we conducted single-cell RNA sequencing across a time course of regeneration, identifying key candidate genes from a number of pathways, including Hippo, FoxO and NRF2. These candidate genes were spatially validated by means of HCR RNA-FISH within the stem cells, and their functional significance was probed by RNAi. Our integrative assay reveals a hierarchical regulatory network through which the radioresistant stem cells orchestrate whole-body regeneration, and reveals new insights into stem cell biology and regeneration under stress.

Keywords: Macrostomum lignano, Stem Cells, Radiation, Whole-Body Regeneration

1. Introduction

1.1. Topic selection basis

The regenerative ability of planaria relies on their adult pluripotent stem cells, the neoblasts [1]. Although the majority of neoblasts are sensitive to radiation, research findings of Macrostomum lignano demonstrate that a subpopulation of stem cells that are radioresistant, exist within them, since they are not only able to survive doses up to 150Gy gamma radiation, but also are responsible for complete regeneration of the organism [2,3]. This forms a good experimental basis for the in-depth study of the radioresistant stem cells and their molecular mechanisms in the M. lignano model. The main aim of this study into the central regulatory networks responsible for the stem cell survival, activation and coordination of tissue regeneration under extreme genotoxic stress. By

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utilizing the existing extensive genetic and microscopic tools available for this model it is hoped to shed light of this type of stem cells.

1.2. Research status

However, two big technical bottlenecks hinder research into this process. First, the intense autofluorescence produced by mortality induced by irradiation seriously interferes with the detection of conventional fluorescent reporter signals and with the accurate tracking of the rare surviving stem cells in live animals. Conventional static histological techniques cannot record their major dynamic functions, such as initial division or migration. Second, the molecular mechanisms and dynamic correspondences which relate to the activation of these stem cells are still insufficiently defined, a great deal of significant signaling pathways and fate determining factors await discovery.

1.3. Research framework and innovative highlights

To overcome existing technical bottlenecks, we have constructed an innovative research system that integrates long-term live imaging with functional genomics. This system forms a closed research loop from phenomenon to mechanism through four key steps. In this work, first, we employ a dual-modality reporter system combined with bioluminescence imaging to achieve specific, quantitative monitoring of stem cell survival and positioning in live animals, fundamentally overcoming interference from autofluorescence, coupled with an automated tracking microscope for continuous, high-resolution observation of stem cell behaviors - including division and migration - in freely moving animals over extended periods. Second, utilizing single-cell RNA sequencing technology, we analyze the transcriptomic dynamics of stem cell activation across a complete regeneration timeline, enabling unbiased identification of key candidate regulatory genes and pathways. Subsequently, high-sensitivity HCR RNA-FISH is applied to validate the spatial expression patterns of these candidate genes within their native tissue environment. Finally, RNAi-mediated knockdown experiments are conducted to definitively establish the functional necessity of these genes in regulating stem cell activation and regeneration. The overall experimental workflow is summarized in Figure 1.

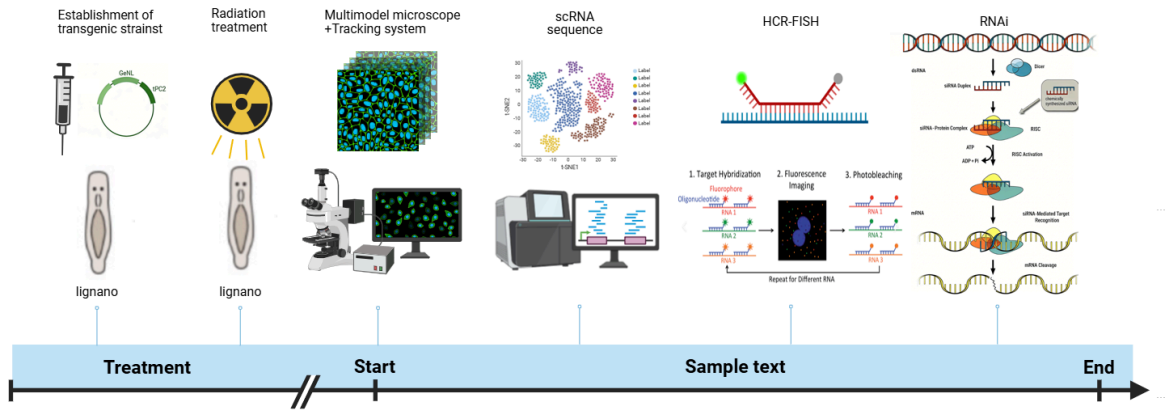

Figure 1. Schematic overview of the experimental workflow

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2. Materials and methods

2.1. Objective 1: the construction, radiation treatment, and monitoring of bioluminescent stem cell reporter strains

Rationale: The damage to tissues caused by high dose gamma irradiation, and the autofluorescence thereafter, conceals the presence of rare, surviving radioresistant stem cells in live M. lignano, thus masking the conventional reporter systems and preventing key activities such as initial division, and subsequent migration from being observed. Therefore, it is of paramount importance to develop a tool for the continuous tracking of these cells in the freely behaving animal to learn more about whole body regeneration [4].

2.1.1. Methods

Construction of reporter strains: A dual-modality reporter construct will be generated by virtue of a fusion of the coding sequences of the fluorescent protein mNeonGreen and the bioluminescent enzyme NanoLuciferase (GeNL) [5]. This fusion gene will be placed under the control of a strong, bidirectional CAG promoter flanked with four copies of the CMV enhancer [6,7] in such a way as to allow a strong, stable expression that is independent of the site of genomic integration and has a strong signal to noise ratio. The final construct, In Figure 2, 5'-[(CMV Enhancer)$_4$ - (CAG Forward) - GeNL - (CAG Reverse) - (CMV Enhancer)$_4$]-tPC2-3' will be confirmed by sequencing and injected into M. lignano by microinjection to give rise to stable transgenic lines.

Figure 2. Schematic of the dual-modality GeNL reporter construct

Irradiation and imaging: Transgenic and wild-type animals will be subjected to 150 Gy whole-body gamma irradiation. Samples will be collected from unirradiated controls and at multiple post-irradiation time points (1hour, 3hours, 6 hours, 24 hours, 7 days, 14 days, 21 days). For imaging, animals will be anesthetized and incubated with the NanoLuciferase substrate furimazine (FFz).

Dual-modality imaging and analysis: Simultaneous fluorescence and bioluminescence imaging will be performed using a multimodal microscope equipped with highly sensitive cooled CMOS cameras. The autofluorescence-independent bioluminescence signal will serve as the definitive marker for viable, GeNL-expressing stem cells. Signal colocalization between channels will generate precise maps of surviving stem cells.

Long-term tracking: An automated tracking microscope system employing real-time image analysis and a feedback-controlled stage will be used to maintain freely moving animals within the field of view, enabling long-term high-resolution time-lapse imaging of stem cell dynamics with a custom system illustrated in Figure 3.

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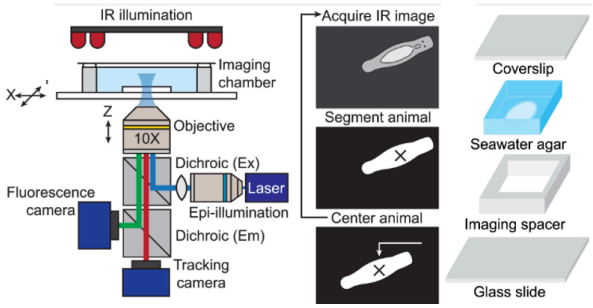

Figure 3. (Left) Diagram of the tracking microscope. (Middle) Overview of the tracking routine involving IR imaging, segmentation, and stage repositioning. (Right) Exploded diagram of the long-term imaging chamber. Reprinted from [3]

2.1.2. Expected outcome

Reporter System Validation: The CAG promoter drives strong, ubiquitous GeNL expression. And bioluminescence (autofluorescence-free) will confirm the specificity of stem cell by co-localization with markers like piwi, while confocal microscopy validates widespread fluorescence.

Stem Cell Dynamics: Bioluminescence imaging tracks survival and proliferation. There will be a sharp signal drop within 24 hours after radiation, which reflects death of radiation-sensitive cells. Then, followed by a slow rise (2-14 days) because of the proliferation of the stem cells, signals plateau at 14-21 days as the stem cell pool is reestablished.

Initial Behavior Quantification: Long-term tracking microscopy will capture the initial division timing, spatial orientation, and daughter cell migration in vivo, thereby establishing a foundational timeline for regeneration initiation, consistent with the regenerative circuitry model proposed in Figure 4.

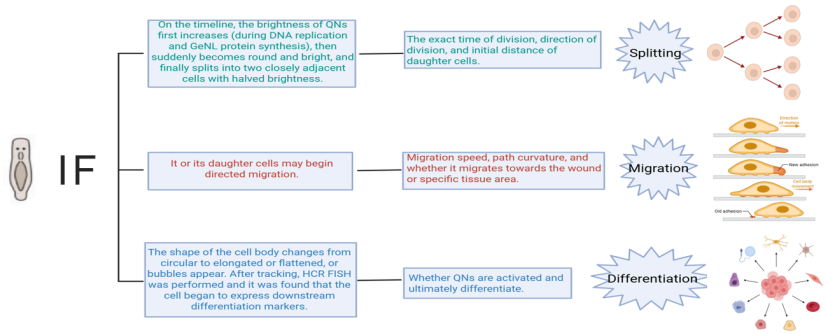

Figure 4. Regenerative circuitry of radioresistant stem cells

2.2. Objective 2: exploration of the molecular mechanisms of activation, regeneration, and differentiation of stem cells after radiation treatment

Rationale: Radiation resistant stem cells exhibit expected activation and differentiation, which is associated with their regulation by specific signaling pathways or transcription factors after 150 Gy irradiation [8]. From observation of cellular behaviors to understanding of molecular basis, profile the transcriptional dynamics of stem cells across a regeneration time course is essential.

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2.2.1. Methods

Collection: M. lignano animals (both wild-type and the pstem cell-specific::H2B-mScarlet reporter) will be exposed to 150 Gy of gamma rays. Collect these cells and non-irradiated control group cells at these points after irradiation (0 hours, 1hour, 3hours, 6 hours, 24 hours, 7 days, 14 days, 21 days).

Cell Dissociation and Sorting: Animals will be mildly digested to create a high-viability single-cell suspension. Using FACS, H2B-mScarlet^{+} cells (enriched for stem cells and their progeny) will be isolated from transgenic animals for downstream sequencing.

Library Preparation and Sequencing: Standard scRNA-seq protocols (10x Genomics) will be used for single-cell capture, barcoding, and cDNA library preparation, followed by high-throughput sequencing on the Illumina platform.

Cell Clustering and Annotation: Data will be processed using CellRanger, Seurat, and Scanpy. Cells will be clustered via PCA and UMAP. stem cells clusters will be identified by high expression of stemness markers (e.g., piwi) and low expression of cell cycle genes (e.g., pcna, histone h3) [9].

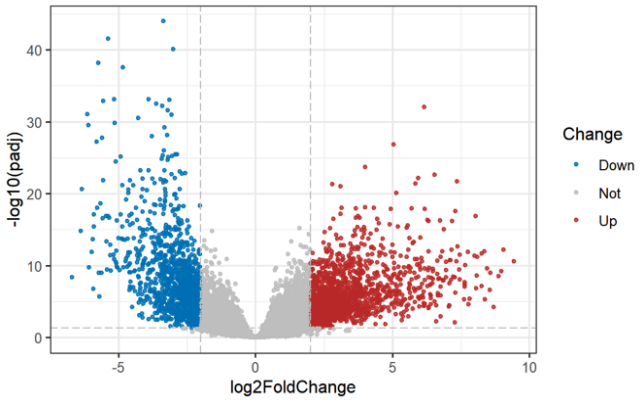

Figure 5. Volcano plot of differentially expressed genes in the stem cells

The volcano plot illustrates the differential expression analysis of genes between samples exposed to radiation and control groups. The x-axis displays the $\log_2$fold changes (upregulation = positive values; Downregulation = negative values) and the y-axis displays statistical significance measured as ($-\log_{10}$adjusted p-value). Genes exhibiting a significant upregulation are depicted using red dots, and genes exhibiting a statistically significant downregulation are shown using blue dots. All other non-significant genes are depicted using grey dots. The dashed lines shown on the graph indicates where typical thresholds of statistical significance may be found from the analysis i.e., where ($|\log_2 FC| \geq 1$) and the adjusted p value is ≤ 0.05, as visualized in a representative volcano plot in Figure 5.

Differential Expression and Pathway Analysis: The stem cells cluster will be extracted for sub-analysis. Differential gene expression analysis will be performed between stem cells from different post-irradiation time points and controls.

Trajectory Inference: Using Monocle3 or PAGA, we will construct a pseudotemporal trajectory from "quiescent" -> "early activated" -> "differentiating" stem cells to identify key regulator genes driving this transition.

Candidate Gene List Generation: Through integrated analysis, we will generate a ranked candidate gene list featuring transcription factors upregulated immediately post-irradiation and key signaling molecules enriched at critical fate decision points in the trajectory.

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Probe Design: Based on the scRNA-seq candidate list, we will design specific HCR v3.0 probe sets for the top candidates from the following 5 high-priority in Table 1, under-explored pathways and other pathways, along with a probe for piwi as a stem cells marker:

Table 1. High probability related pathway list (example)

Pathway/ Mechanism	Key Candidate Molecules	Predicted Role in stem cells Activation
Hippo Pathway	YAP/TAZ	Key mediators linking mechanical cues to proliferation
FoxO Signaling	FoxO	Central regulators of stress resistance and longevity in stem cells
NRF2-mediated Stress Response	NRF2, GST, HO-1	Master regulators of antioxidant defense
BMP/TGF-β Signaling [10]	Noggin, BMP ligands	Potential "quiescence release" signals
Epigenetic Remodeling	KDM5, KDM6 [11]	Putative erasers of repressive chromatin marks during activation

Sample Preparation and Multiplexed HCR: Fixed whole-mount samples from irradiated stem cell-specific::H2B-mScarlet animals will be subjected to multiplexed HCR RNA-FISH.

Imaging and Co-localization Analysis: Samples will be imaged using high-resolution confocal microscopy in three channels:

Channel 1 (Red): H2B-mScarlet signal, marking stem cells nuclei.

Channel 2 (Green): HCR signal for the candidate gene mRNA.

Channel 3 (Far-Red): HCR signal for piwi mRNA, marking stem cells cytoplasm.

Co-localization analysis in Fiji/ImageJ will confirm the presence of candidate gene mRNA within cells defined by both nuclear mScarlet and cytoplasmic piwi signals.

2.2.2. Expected outcome

ScRNA-seq will produce a high-resolution map of the molecular determinants of stem cell activation, yielding a list of ranked candidate genes including signaling molecules and transcription factors. Pseudotemporal trajectory analysis will reconstruct the continuous quiescence → activation → differentiation" transition, revealing key regulatory genes responsible for each stage. Spatial validation of candidate genes using HCR RNA-FISH will provide direct evidence of the expression of candidate genes in stem cells. Evidence for such direct transcriptional activity will come from clear evidence for the colocalization of candidate gene signals with both nuclear and cytoplasmic stem cell markers. This will provide evidence for in situ transcription activity of key signaling candidates such as YAP/TAZ, FoxO and target genes of NRF2 in radioresistant stem cells. Ultimately integrated datasets will form the direct means of revealing the causal relationships between "genes, cellular behaviors and regeneration phenotypes, leading to a mechanistic model of how the Hippo, FoxO and NRF2 pathways are hierarchically organized to drive regeneration.

2.3. Objective 3: functional validation of key regulators in stem cells activation

Rationale: Genes upregulated in stem cells post-irradiation represent strong candidates for driving regeneration. Functional validation is required to establish their causal roles in stem cell activation and systemic recovery.

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Method: Firstly, the top candidate genes selected from objective 2 were counted, and these genes were knocked out using RNAi technology (experimental group). Then, the control group and experimental group were irradiated with 150 Gy. Finally, the phenotype was quantitatively evaluated through real-time imaging and tracking microscopy to compare stem cell activation, regeneration, and behavior. Simultaneously perform H3P staining to evaluate whether gene knockout affects the ability of stem cells to enter the cell cycle.

Expected outcome: Knockout of key genes will result in observable and measurable phenotypes, such as delayed stem cell activation, impaired cell cycle progression, and slowed regeneration.

3. Conclusion

This study provides a framework for investigating the radio resistance and regeneration potential of the quiescent stem cells in M. lignano. The dual reporter system overcomes the complication of post-irradiation autofluorescence, permitting the accurate identification of individual stem cells as well as long-term tracking of their behaviour in freely behaving animals. Transcriptomic analysis across the time course of regeneration is expected to reconstruct their developmental trajectory, and shed light on key candidate genes from pathways including Hippo, FoxO, NRF2, BMP/TGF-β and epigenetic remodeling. Spatial validation via HCR RNA-FISH confirms that they are expressed in stem cells and functional studies via RNAi show that they are required. Our data will provide insight into the hierarchical regulatory network through which radioresistant stem cells coordinate tissue regeneration and by extension provide insights into stem cell biology and stress induced repair. The projected timeline for completing this study is outlined in the Gantt chart shown in Figure 6.

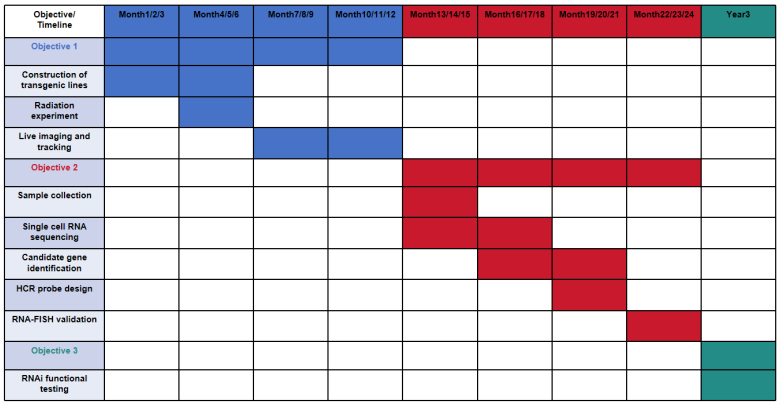

Figure 6. Project timeline (Gantt chart)

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