Proceedings of the 4th International Conference on Modern Medicine and Global Health
DOI: 10.54254/2753-8818/2026.LA32152

Exploring the Regenerative Mechanisms of Macrostomum Lignano Following Exposure to a 150 Gy Dose of Radiation

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Abstract. This article explores the dynamic repair processes of regenerative animals following 150 Gy radiation, with the focus on the Macrostomum lignano, which exhibits strong regenerative capabilities. This organism can regenerate all tissues posterior to the pharynx but lacks the ability to regenerate anterior structures, providing a unique model for study. The article introduces the method of using γ-ray irradiation to investigate its stem cells (neoblasts) and the restoration of cell proliferation and gene expression post-irradiation. The research constructs specific bioluminescent reporter strains and employs dual-mode imaging techniques to track stem cell survival and development, combined with single-cell RNA sequencing to analyze molecular mechanisms after irradiation. Finally, it discusses the roles of metabolic reprogramming, oxidative stress management, and epigenetic reprogramming in regeneration.

Keywords: Macrostomum, Radiation, Neoblasts, Stem cells, Planaria, Regeneration

1. Introduction

In response to injury, regenerative animals initiate a cascade of dynamic processes, including signaling, apoptosis, proliferation, differentiation, and patterning, to restore lost tissues. Closely connected to the stem cells, regeneration ability is various from species to species. It is acknowledged that regeneration ability differs in organs. The number of stem cells and their proliferation activity and decision to differentiate must be tightly controlled during development and homeostasis to avoid tumour formation or premature ageing [1]. Members of the phylum Platyhelminthes are well known for their high regeneration capacity based upon totipotent stem cells. Macrostomum lignano(M. lignano) is capable of regenerating all tissues posterior to the pharynx but not anterior structures, presenting a unique opportunity to compare the molecular and cellular basis of regenerative and non-regenerative outcomes.

Since Wolff and Dubois [2] demonstrated that neoblasts could be specifically eliminated by γ-ray irradiation, radiation exposure has become a method widely used in flatworm stem cell research for testing and confirming various hypotheses. After improvements of tech in decades, irradiation was not only applied in the researches, but also in the clinal treatment. However, humanity show far less radiation residence than some species. Radio resistance is also a cause of concern as it causes failure of radiation therapy and subsequent tumor relapse [3]. Individual cancer cells and the overall tumor have or can develop the ability to overcome the effects of radiation therapy and in some cases,

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potentially become a more aggressive tumor. In an early report, cell proliferation and gene expression in M. lignano were restored within 1 month in a dose dependent manner following exposure to up to 150 Gy irradiation [1]. An accumulating total dose of 120 or 150 Gy caused a significant reduction in cell proliferation activity until 1week post-treatment and gradually regained their cell proliferation ability in 2 weeks.

What remains unknown is the mechanism M. lignano has when suffering 150 Gy irradiation. Exposure of M. lignano to γ−rays caused a drastic effect on the process of regeneration, forming a regeneration blastema and rebuilding their stylet after five days. Now we can establish a modular library of gene parts to enable the rapid assembly of complex expression cassettes, enabling effective procedure. Second, NTR2.0 allow us to track posterior regeneration and identify milestones without the interference of toxins released by dying cells. An epifluorescence tracking mi-croscope to image free-moving animal will be of great help. Making full use of the toolkit created by Nelson and his team [4], M. lignano can become a powerful platform for studies.

2. Results

2.1. Construct specific bioluminescent reporter strains

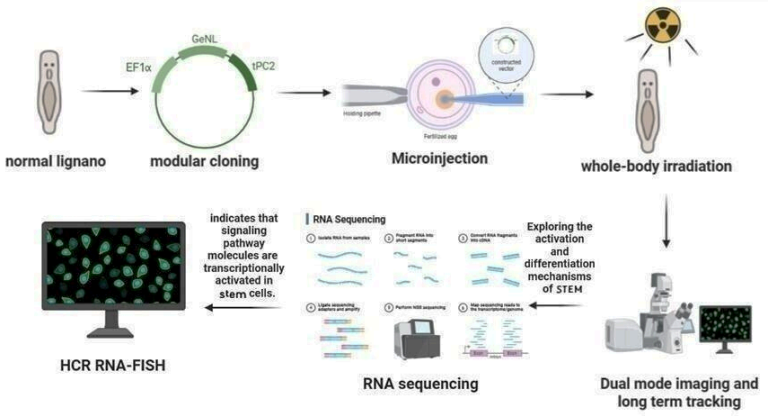

Figure 1. Process of construct, observation and tracking

From the existing single-cell RNA sequencing data of M. lignano, we aim to identify genes that are specifically and highly expressed only in stem cells (which could be specific piwi paralogs, or other unknown marker genes). The promoter can be named as EF1αsc-specific (EF1α promoters showed inconsistent expression, limiting single-cell miRNA sensor use) [5].

In order to observe the whole stem cells better, we can construct a vector: EF1αsc-specific::GeNL::tsc-specific. In Figure 1 it shows that this method breaks down transgenes into "parts"—promoters, genes, and terminators—and hierarchically assembles them through simple reactions while minimizing cloning scar sites [4].

In this method, GeNL is a fusion protein consisting of mNeonGreen (fluorescent) and NanoLuciferase (bioluminescent), which serves as the core of dual-mode imaging. Through microinjection and screening, we can establish a stable transgenic strain. In this strain, stem cells will continuously express the GeNL protein.

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2.2. Observation and tracking of stem cell survival and development following radiation treatment

In order to construct the model, subjecting wild-type and transgenic Macrostomum Lignano to whole-body irradiation with 150 Gy γ-rays. In Figure 1 it shows, setting up control group which is non-irradiated and experiment group suffering 150 Gy γ-rays. Tracking and recording the image at different time points. According to the past research it is believed that 6h, 24h, 7d,14d,21d are ideal time points. For Macrostomum Lignano which suffering irradiation, 14d can be a key point when the potential of regeneration can be totally unleashed, meaning high capability of observing cell survival [1]. Then add the bioluminescent substrate FFz to the samples. FFz, as a substrate for NanoLuc, significantly enhances the sensitivity and durability of in vitro and in vivo bioluminescence imaging [6].

A multimodal microscope integrated with a high-sensitivity cooled CMOS camera is deployed for bioluminescence imaging. The modulated CMOS camera enables fast, accurate widefield fluorescence lifetime imaging [7]. This microscope is capable of simultaneously capturing both fluorescence and bioluminescence signals, and it is equipped with Python control software enabling the microscope to addresses the issue of intense autofluorescence generated by dead tissue following cell ablation, which can obscure fluorescence protein signals. Since bioluminescence signals are not interfered with by autofluorescence, they can clearly and quantitatively display the remaining viable cells, thereby enabling accurate assessment of the ablation effect.

If a signal spot appears simultaneously in both the bioluminescence channel and the fluorescence channel, it indicates a viable cell expressing GeNL. Conversely, if a signal spot is only present in the fluorescence channel but absent in the bioluminescence channel, meaning that it could be a cluster of dead cell debris or structures with autofluorescence. Through this analysis process, mapping the locations accurately of all surviving stem cells amidst the chaotic background following irradiation can be ensure without any errors.

By employing an automatic tracking microscopic imaging system, utilizing infrared illumination, real-time image segmentation algorithms, and a PID (Proportional-Integral-Derivative) feedback control system in this system to drive a motorized stage, enabling it to continuously track freely moving animals (M. lignano in the research) and keep them centered in the sight. Meanwhile, it conducts multi-channel fluorescence imaging, operating continuously for over a week. The extra expose time do harm to the creature, meaning that consider time is important. This achieves high spatiotemporal resolution and long-term imaging of animals under completely free movement, while subjects in their natural physiological states.

The whole process contains several parts:

Division. During this period, stem cells will undergo protein synthesis and DNA replication. GenL allows the microscopic to track the stem cells, presenting increasingly high light. The unique characteristics of cells during the mitotic phase facilitate their localization and allow for direct measurement of: the exact time of mitosis occurrence, the direction of division, and the initial distance between daughter cells.

Migration. Plotting the motion trajectory diagrams of each tracked stem cell can enable direct measurements to be practical: migration speed, path tortuosity, and whether the migration is directed towards a wound or a specific tissue area.

Differentiation. Observe the morphological and molecular changes of a tracked cell and its progeny over several days. After the tracking period, fix the samples and perform HCR FISH. If the cell is observed to present the markers for downstream differentiated cells include specific

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microRNAs like miR-142-3p, which indicate cell fate transitions, it can be the evidence of lineage tracing [8].

2.3. Explore the activation and differentiation mechanisms

scRNA-seq: At multiple key time points after irradiation, taking samples from experiment group that has been treated with 150 Gy γ-rays. Taking samples from non-irradiated control group at the same time point for further analysis. Then make preparation for single-cell suspension: In order to mildly digest animal tissues with the enzymatic digestion method, and employ a 40 μm cell strainer to obtain high-quality single-cell suspensions [9]. Determine cell viability using trypan blue staining. Construction of single-cell sequencing libraries and sequencing machine loading: Employing a standard scRNA-seq platform for single-cell capture, barcoding, and cDNA library construction. Conducting high-throughput sequencing (Illumina) to obtain gene expression data of every single cell. Bioinformatics analysis requiring utilize tools such as Rstudio (R-based tools or RStudio as an integrated development environment), CellRanger, Seurat, or Scanpy for several parts which include raw data alignment, gene quantification, quality control, and normalization.

The specific potential molecular signaling pathways roughly exert their effects in the following aspects:

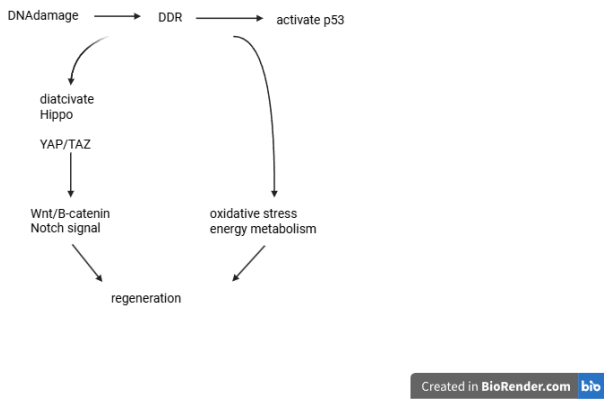

Figure 2. The process of molecular signaling pathway

Sensory Perception and Injury Signaling Pathways: p53: It serves as the "command center" for cellular stress responses. Shown in Figure 2, the p53 protein is a transcription factor that enhances the rate of transcription of six or seven known genes that carry out, at least in part, the p53-dependent functions in a cell [10]. In normal cells, DNA damage triggers p53-mediated apoptosis. Low expression level but high activity might mean its protein is stably and specifically activated, yet the overall level remains relatively low. This enables the initiation of repair programs rather than apoptosis programs.

When it comes to specific target genes, it may not activate pro-apoptotic genes (such as Bax), instead which can preferentially activate DNA repair genes, such as Rad51(RAD51 is central to DNA replication under stress... [It] promotes replication fork reversal, a process in which stalled replication forks are remodelled into a 'chicken-foot' structure) [11], cell cycle checkpoint genes and autophagy-related genes.

Metabolic Reprogramming and Oxidative Stress Management

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NRF2: This is the main regulator of the antioxidant response. The KEAP1-NRF2 pathway is the principal protective response to oxidative and electrophilic stresses [12]. Also, in the stem cells, NRF2 may be stably activated, which in turn upregulate the expression in a series of antioxidant genes (such as glutathione S - transferase and heme oxygenase - 1). This process helps clear reactive oxygen species (ROS) and protects cellular macromolecules. In response to stress, an intricate molecular mechanism facilitated by sensor cysteines within KEAP1 allows NRF2 to escape ubiquitination, accumulate within the cell, and translocate to the nucleus, which might be the same pathway to the reaction to the radiation [12].

Autophagy - related proteins LC3 and p62. LC3 is closely connected with autophagy.

Due to the damage caused by the Irradiation of organelles and macromolecules might activate autophagy, which acts as a "scavenger" mechanism, may be activated to clear damaged mitochondria (mitophagy) and protein aggregates, providing a "clean" internal environment for cell survival and restart [13].

Changes in the expression or activity of metabolic enzymes PKM2 (as a key part of function and expression and regulation) [14] and PGC - 1α (involved in mitochondrial biogenesis) may enable stem cells to rapidly switch from efficient oxidative phosphorylation (a characteristic of the resting state) to glycolysis.

Epigenetic Reprogramming

Histone modifications require demethylation of lysine at position 4 and position 27 on histone H3 (mediated by demethylases such as KDM5 and KDM6) or acetylation (mediated by histone acetyltransferases like p300/CBP), which may occur in the promoter/enhancer regions of activation-related genes. While KDM6 working as a promising therapeutic target in autoimmune disorders and in immune cells, KDM6A and KDM6B fine-tune the transcription of pro- and anti-inflammatory genes, influencing differentiation, polarization, and activation states in monocytes [15]. This process transforms chromatin from a "closed" state to an "open" state.

DNA methylation is a key target in cells circle.Dynamic changes in the global DNA methylation level (regulated by DNMT and TET enzymes) may be involved in the regulation of exiting the resting state and fate-determining genes.

Probe Design - Utilize Molecular Instruments' probe design tool or relevant algorithms to design specific and efficient Hybridization Chain Reaction (HCR) probe sets, based on the mRNA sequences of candidate genes screened via single-cell RNA sequencing (scRNA-seq).

Procedure for HCR RNA-FISH Validation

The detailed procedure for HCR RNA-FISH Validation needs to prepare irradiated transgenic animal samples, which is mentioned in the first part. M. lignano samples from experiment group and control group can be used to conduct experiments on fixed whole-mount slide samples according to the HCR v3.0 RNA-FISH protocol.

Then hybridize the probe mixture targeting candidate genes and piwi overnight with the sample mRNA for hybridization. RNA in situ hybridization methods and immunohistochemistry methods provide biologists with essential tools for elucidating the spatial organization of biological circuitry, enabling imaging of RNA and protein expression in an anatomical context [16].

Signal amplification requires performing a cascade amplification reaction using hairpin DNAs labeled with different fluorophores to generate bright fluorescent signals localized to the mRNA.

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Table 1. Three-channel imaging on the samples using a high-resolution confocal microscope

	Color	Detect	meaning
Channel1	Red	Detect mScarlet signal	the nuclei of stem cells
Channel2	Green	Detect HCR signal	genes
Channel3	Far-red	Detect HCR signal of piwi	marker for the cytoplasm of stem cells

3. Expected results

Determine whether the green signal (candidate gene mRNA) spatially overlaps with the red signal (cells nuclei) and the far-red signal (piwi mRNA, marking stem cells cytoplasm). If distinct punctate signals of the candidate gene are clearly observed within a stem cell (defined by the presence of both nuclear mScarlet and cytoplasmic piwi signals), this provides direct evidence that the signaling pathway molecule is indeed transcriptionally activated within the stem cell.

4. Conclusion

The article describes observing the regenerative processes of Macrostomum lignano following γ-ray irradiation, focusing on the Mechanisms. However, the article might do not clearly specify the sample size or the number of experimental replicates. Adequate sample size and replication are crucial in scientific research to ensure the reliability and reproducibility of results. What's more, we may have disadvantage on Limited Data Presentation: The article primarily relies on qualitative descriptions of experimental results and lacks specific quantitative data, such as cell proliferation rates or gene expression levels. Providing more detailed data and statistical analysis results would enhance the article's persuasiveness.

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