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Identify Potential Anti-radiation Molecule in Macrostomum Lignano

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Abstract. Macrostomum Lignano (M. lignano) has a strong ability to resist radiation up to 210 Gy. Potential anti-radiation molecules in M. lignano can be identified by comparative transcriptomic and proteomic analyses of specimens before and after radiation exposure. The following methods will be employed: RNA sequencing, Gene Ontology (GO) analysis, tandem mass spectrometry (MS/MS)–based proteomic analysis, and fluorescence in situ hybridization (FISH) combined with immunofluorescence. The identified proteins will also be transferred to other cell lines including Hek 293 and SH-SY5Y, which will demonstrate the potential of the newly found protein to help different species, including humans, resist high levels of radiation. This property may be of considerable utility for future space travel as well as for protection during radiotherapy.

Keywords: radiation, RNA, proteomics, genomics, Macrostomum lignano

1. Background

1.1. Introduction to radiation

Radiation is the process of releasing and transmitting energy in the form of electromagnetic waves or high-energy particles (such as X-rays, γ-rays, α particles, or β particles). When an organism is exposed to ionizing radiation, it suffers severe damage due to DNA strand breaks and cellular structural damage mainly caused by reactive oxygen species (ROS). These damages may lead to cell death, gene mutations, and even cancer. Therefore, the exploration of methods to mitigate radiation hazards holds great significance for fields such as cancer radiotherapy, nuclear industry safety, and deep space exploration. Research on the mechanisms of radiation resistance (the survival and repair abilities of organisms under high doses of radiation) provides the prerequisites for developing protective strategies, innovating treatment methods, and ensuring human health in extreme environments.

1.2. The significance and importance of conducting radiation resistance research

Radiation protection is important in organisms. M. lignano can resist strong radiation up to 210 Gy as previously reported [1]. By eliminating the resistance of tumor cells to radiation, the effect of radiotherapy can be enhanced while ensuring the safety of normal cells at the same time. Radio

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protection mechanisms always relate to DNA protection and repair. Research on the mechanisms of DNA repair and protection may provide new ideas for regenerative medicine and anti-aging research.

1.3. Former studies on radiation resistant mechanisms

At present, the research on the mechanism of animal radiation resistance mainly focuses on the following aspects. Firstly, highly effective DNA repair plays a vital role. For example, effective RAD51 protein or Homologous recombination repair (HR) and non-homologous end joining enhance the DNA repair which leads to radio resistance. Secondly, some animals have unique DNA protection proteins, such as Dsup [2] in tardigrades, which can bind to DNA and prevent it from breaking or being damaged, can help them resist radiation directly. Thirdly, high levels of antioxidant enzyme systems, such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), can quickly remove ROS caused by radiation and prevent oxidative damage DNAs. Furthermore, appropriate cell cycle regulation and inhibition of excessive apoptosis have been proved to be important factors contributing to radiation resistance. A recent study has demonstrated that metabolites produced by Lachnospiraceae enhance host resistance to radiation [3].

Evidence shows that some species may use new metabolic pathways or unknown proteins to achieve radiation resistance, but in-depth exploration is lacking. The similarities and differences in the anti-radiation strategies of different species have not been fully clarified, and there is a lack of large-scale phylogenetic and functional studies. Some proteins such as Dsup which has proved to be effective in radiation resistance cannot be used in humans (especially in nerve cells), so finding a protein or regulative gene which can enhance radiation resistance in different systems especially in human (e.g. cell line hek293) is of great importance.

1.4. Reasons for choosing Macrostomum lignano as a model system

Studies shows that, Macrostomum lignano (M. lignano) has a rich stem cell system which accounts for about 6.5% of their total cells [4]. Besides, they are the only cells which can divide in M. lignano. Their transparent body, which is quite easy to observe their cells and tissues, makes them an ideal model for studying radiation resistance. Wudarski's group [5] has developed a stable transgenic method in M. lignano by microinjecting DNA constructs into one-cell stage embryos, which provides an effective method for constructing stable transgenic strains. De's group [1] showed that M. lignano exhibits resistance to ultra-strong radiation up to 210 Gy, mainly due to the presence of quiescent stem cells and efficient DNA double-strand break repair mechanisms. In general, because of these characteristics of M. lignano, it becomes an ideal model animal for further study of its radiation resistance mechanism. The morphology of M. lignano under a microscope is shown in Fig.1.

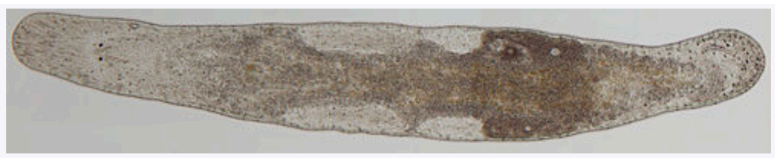

Figure 1. M. lignano as the model system of the experiment [4]

Macrostomum lignano, with its strong regenerative capacity, transparent body for in vivo observation, and well-developed genetic tools, is considered an ideal model organism for studying

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radiation resistance [4].

1.5. Methods: RNA sequencing

RNA sequencing is a high-throughput sequencing based transcriptome analysis method primarily used to identify gene expression and discover novel transcripts. In a recent study [6], they use RNA-seq to analyze gene expression changes in three tardigrade species after irradiation and Bleomycin treatment to identify radiation resistance genes. They also used proteomic analysis to confirm changes at the protein level for genes upregulated at the RNA level. Western Blot experiment will be carried out to detect protein expression of TDR1, XRCC5, XRCC6, and phospho-H2AX and their synthesis dependency. They also carried out Gel Shift Assay to Verify TDR1's direct interaction with DNA. After Functional Experiments in Human Cells, they proved that TDR1 significantly reduced foci number, enhancing DNA damage resistance.

1.6. Experiment plan

The studies above provide great inspiration for work on M. lignano. The goal is to identify radio resistant genes and related proteins (if possible) in M. lignano and verify their ability in radiation-resistance in other systems such as human cell line hek293. The methods to be used include RNA-seq, bioinformatic analysis, western blot experiment just like the experiment before. Knockdown experiments are also being considered to analyze the relevant pathways, and in situ hybridization will be performed to locate the action sites of these genes in M. lignano. If the target gene encode proteins, AlphaFold will be used to model their 3D structures and to analyze specific mechanisms (e.g. binding DNA for protection).

1.7. Prospects and applications

In the future, radiation-resistant genes or proteins may be utilized to develop anti-radiation drugs for humans, aimed at applications in space travel, nuclear pollution protection, and safeguarding body cells during cancer radiotherapy. However, significant challenges remain in identifying these genes and achieving their expression in more advanced life systems. Moreover, methods could be developed to transform radiation-resistant proteins or other organic molecules into drugs that can be administered through injection or oral intake to enhance human resistance to radiation in specific scenarios.

2. Project description

This study aims to screen and validate candidate anti-radiation genes from the model organism Macrostomum lignano through radiation treatment, combined with analysis of transcriptomics and proteomics, RNA-FISH and immunofluorescence localization, gene knockdown experiments, and cross-system expression validation, in order to reveal its molecular mechanisms and explore potential applications in other systems (human cell lines). The general process of the experiment is shown in Fig.2.

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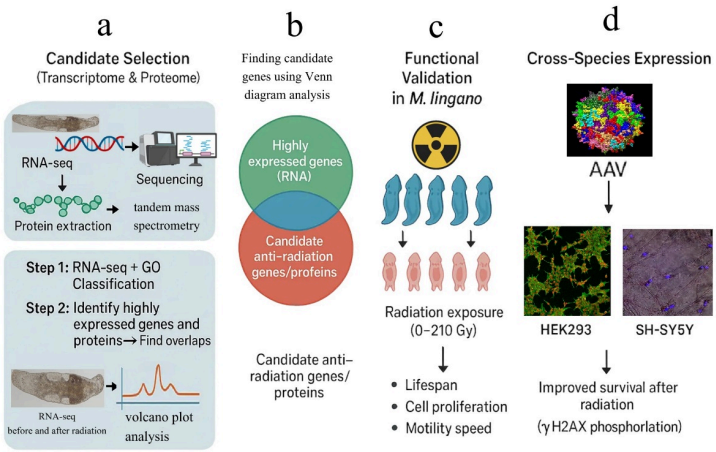

Figure 2. Overview of the experiment (a). Selection of candidate genes. (b). Functional validation in M. lignano. (c). Assessment of post-radiation survival time. (d). Cross species expression

3. Objective1: radiation treatment and grouping experiments for preparation

3.1. Rationale

In order to compare the differences in genes and proteins expression before and after radiation, control experiments need to be conducted. Since M. lignano can withstand radiation doses of up to 210 Gy [1], it is reasonable to conduct comparisons within the radiation dose range from 0 Gy to 210 Gy.

3.2. Method

3.2.1. Control and grouping of radiation dose

The worms will be divided into four groups, receiving 70 Gy, 140 Gy, and 210 Gy of radiation, respectively, with a control group receiving no radiation. This ensures the conduct of controlled experiments while avoiding wasting funds by over-dividing the groups.

3.2.2. Sampling after radiation

After receiving different doses of radiation, the four M. lignano groups will be sampled at four timepoints (pre-irradiation, 6h, 24h, and 72h post-irradiation) to monitor their survival rates, which will inform the appropriate sampling timepoints for subsequent gene sequencing and protein analysis.

3.3. Expected outcome

Irradiated groups are expected to exhibit dose-dependent survival (with no immediate mortality), and significant differences in gene and protein expression are anticipated compared to the non-irradiated control group. The grouping design and radiation treatment protocol are illustrated in Fig.3.

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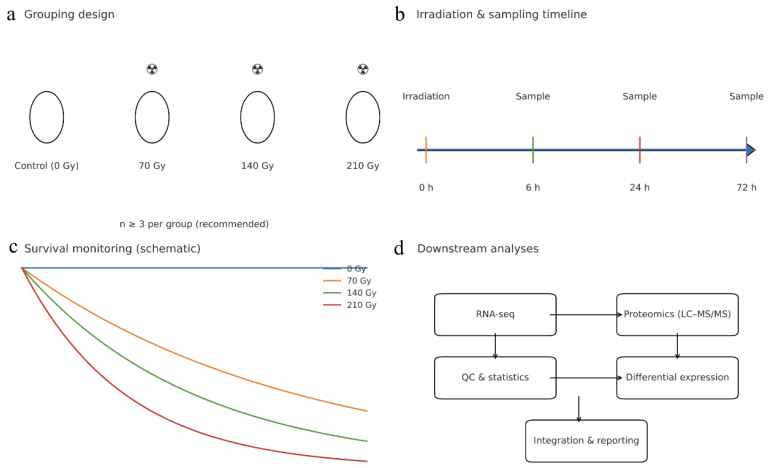

Figure 3. Graphical abstract of radiation treatment and grouping experiments (a). Grouping design (b). Irradiation & sampling timeline (c). Survival monitoring (d). Downstream analyses

4. Objective2: RNA sequencing for searching potential radiation resistance genes

4.1. Rationale

High-throughput RNA sequencing technology has become a mature and powerful tool for transcriptomic analysis. This approach will enable comprehensive comparison of gene expression profiles between M. lignano specimens exposed to different radiation doses (70 Gy, 140 Gy, and 210 Gy) and non-irradiated controls, thereby facilitating the identification of differentially expressed genes that may contribute to radiation resistance.

4.2. Method

4.2.1. Sequencing platform selection

Use Illumina which is one of the most widely used high-throughput RNA sequencing platform, based on sequencing-by-synthesis (SBS) technology. It features large data output and high accuracy, which provides a great way for comparative transcriptomics analysis [7]. High resolution transcriptional responses of M. lignano under different radiation doses can be captured by this platform, which enables us to identify novel radiation resistant genes.

4.2.2. Significant data screening analysis

RNA sequence data will be used to compare the expression differences (upregulated or downregulated) before and after radiation. Volcano plot provides a robust theoretical framework for analyzing RNA-seq data, allowing researchers to clearly identify genes with significant expression differences and statistical confidence by integrating fold variation with statistical significance [8]. This method effectively bridges the gap between large-scale sequencing data and biological significance, avoiding the missing of important signals in high-throughput experiments.

In this study, volcano plot will be used to quickly screen out the genes with the most significant changes in M. lignano under different radiation dose treatments. Focusing on these core candidate genes can make subsequent proteomic validation, RNA-FISH localization, and functional knockdown experiments more efficient and targeted.

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4.2.3. Data classification and database refseq comparison

Studies have shown that the resistance mechanism of extreme radiation-tolerant organisms usually includes two types of genes: one is a family of genes with known well-defined functions, such as repair enzymes, UV damage recognition proteins, or antioxidant factors; the other is a new type of sequence unique to the organism whose function is not yet clear, which may constitute its unique adaptation mechanism. For example, Yuan's group [9] showed that classical DNA repair genes such as phrB and recB are upregulated after UV radiation, while a large number of unknown functional genes may be involved in extreme UV tolerance and γ radiation adaptation.

This study will adopt a "two-pronged" data classification strategy: first, the differentially expressed genes identified from M. lignano will be functionally classified based on prior knowledge, such as UV resistance, DNA repair, and oxidative stress response. Those genes that are clearly annotated in the NCBI Refseq or UniProt database and belong to the functional groups described above will be retained as "prospective candidate genes". Second, those genes that lack homology or similarity in the database will be labelled as potential novel radiation resistance factors (similar to a large number of unknown genes in D. gobiensis [9] or TDR1/Dsup [6] in tardigrade) and will be prioritized for further research.

Methods can be referred to Anoud's study [6].

4.3. Expected outcome

The genes with fold differences and statistically significant differences found in M. lignano before and after radiation sequencing will be identified, and potential new M. lignano radiation resistance genes will be found through database comparison and classification. The method of gene sequencing and expression analysis before and after radiation is shown in Fig. 4

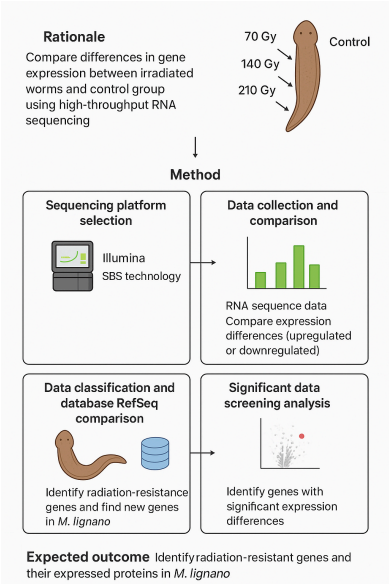

Figure 4. Graphical abstract of genome sequencing and data analysis

High-throughput sequencing of M. lignano genes before and after radiation will be performed to analyze statistically significant up-regulated and down-regulated genes, and to compare with the NCBI Refseq database to identify novel radiation-resistant genes in M. lignano.

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5. Objective3: proteomic analysis for corresponding proteins

5.1. Rationale

High throughput mass spectrometry proteomic analysis will be performed on M. lignano before and after radiation exposure, which aims to identify proteins significantly upregulated or downregulated under radiation stress. In combination with RNA sequencing in Objective 2, novel proteins associated with radio resistance unique to M. lignano may be identified.

5.2. Method

5.2.1. Extract proteins from M. lignano and compare

An efficient extraction method should lyse cells completely, solubilize proteins across different cellular compartments (nuclear, cytoplasmic, and membrane fractions), and minimize degradation or loss during sample handling.

Total proteins will be extracted from M. lignano samples before and after radiation exposure to identify radiation-responsive proteins.

Worms will be collected and homogenized in a lysis buffer containing detergents (e.g., SDS or urea) and protease/phosphatase inhibitors to preserve protein integrity. Mechanical disruption (e.g., sonication or bead beating) will be applied to ensure complete lysis, followed by centrifugation to remove cell debris. The supernatant containing soluble proteins will be quantified using a BCA assay, then subjected to reduction, alkylation, and trypsin digestion [10].

5.2.2. Tandem mass spectrometry analysis

The analytical method to be employed is Tandem Mass Spectrometry, which is composed of two stages.

Tandem mass spectrometry (MS/MS) is a high-precision proteomics technique based on two-stage mass analysis that works in two key stages. In the MS1 stage, the ionized peptides are accurately separated and detected in the first mass analyzer according to their mass-to-charge ratio (m/z), and the system automatically screens out peptide ions with high signal intensity and suitable for further analysis as precursor ions. Subsequently, in the MS2 phase, these selected precursor ions undergo controlled breakage by collision-induced dissociation (CID) or high-energy collision dissociation (HCD) techniques, resulting in a series of characteristic fragment ions (mainly b- and y-type ions) whose mass spectra contain complete peptide sequence information for precise identification and quantification of proteins. The theoretical foundation of this technology is based on ion physics and molecular dissociation kinetics, and by precisely controlling the collision energy and detection conditions, high-resolution, high-quality and accurate mass spectrometry data can be obtained [11].

5.2.3. Data analysis and comparison

Proteomic sequencing data will be analyzed by a computer, and the results will be presented using Venn diagrams. Data will also be compared with the database (NCBI protein FASTA reference sequences) to identify whether there will be novel proteins (potential anti-radiation proteins) which may help the M. lignano resist or recover from radiation.

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5.3. Expected outcome

The protein that is upregulated after exposure to radiation has been discovered, suggesting that this protein may have potential radioprotective abilities, and genes that might regulate its expression can be found in Objective 2. The Venn diagram analysis method of protein expression before and after radiation is shown in Fig.5.

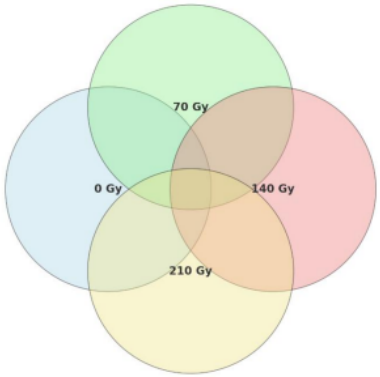

Figure 5. Protein group comparison Venn diagram

M. lignano exposed to 0, 70, 140, and 210 Gy radiation will undergo protein mass spectrometry, and the results will be presented using Venn diagrams for comparison

6. Objective 4: RNA-FISH combined with immune-fluorescence to detect the expression of target genes

6.1. Rationale

Fluorescence in situ hybridization (FISH), particularly RNA-FISH, offers an effective method for localizing mRNA, enabling the identification of tissues or cell populations in which radiation-resistant target genes are predominantly expressed.

It can be a very effective strategy to combine FISH with immune-fluorescence since it can simultaneously locate the special distribution of mRNA and protein. This can verify the expression consistency of RNA and protein and infer their functions.

6.2. Method

6.2.1. RNA-FISH and immunofluorescence co-localization to assess mRNA-protein spatial concordance

If the target gene maintains histological consistency at the transcriptional and translational levels (mRNA and protein spatial distribution are in the same direction), it suggests that its post-transcriptional regulation is weak, the protein stability is high, and the protein is more likely to be a "cell-autonomous" anti-radiation factor (such as directly involved in DNA/chromatin protection or repair).

Use control M. lignano or irradiated M. lignano 6–24 h after treatment (time points can be aligned with RNA-seq/proteomic peaks). Fix with 4% PFA (RNase-free PBS). FISH probes span exons, avoid repetitive sequences, have a GC content of 45–55%, and are 20–25nt in length.

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(a) RNA-FISH (annealing temperature optimized according to probe Tm with 30–50% formamide buffer.

(b) mild annealing/blocking.

(c) IF (with primary/secondary antibodies).

Confocal acquisition (Nyquist sampling) to quantify mRNA-protein colocalization using Pearson correlation coefficient (PCC) and Manders overlap coefficient (M1/M2).

Result interpretation: High PCC/Manders (>0.5 empirical threshold) + tissue-level overlap supports "transcription-translation equipotency" [12].

6.2.2. RNA-FISH and γH2AX immunofluorescence to evaluate target gene expression in DNA damage foci

γH2AX (phosphorylation of H2AX at Ser139) is a classic marker of ionizing radiation-induced DNA double-strand breaks (DSBs), forming foci of DNA damage and recruiting repair factors. The target mRNA spatially overlaps with γH2AX, suggesting that its transcription may be enhanced in damaged microenvironments or that its encoded product plays a role in the DNA damage response pathway [13].

Samples will be collected at multiple time points: 0.5/2/6/12/24 h after irradiation. To protect the epitope, γH2AX IF will be performed first followed by mild FISH. In addition to pixel overlap, the “nearest neighbor distance/proximity” will be calculated.

6.2.3. RNA-FISH and PIWI immunofluorescence to determine stem cell-specific expression

The PIWI family is a conserved marker of flatworm stem cells; in Macrostomum lignano, piwi is expressed in both germline and somatic stem cell populations. If the target mRNA strongly colocalizes with PIWI, it suggests that it may contribute to "stem cell-driven" radiation resistance by maintaining genomic homeostasis (e.g., transposon silencing) or enhancing repair; conversely, it is more likely to act on differentiated cells or the microenvironment.

Use a validated piwi probe/antibody; calculate the proportion of double-positive cells and combine Edu/Ki67 stratification; if strong overlap is observed, combine transgenic and RNAi/CRISPR for functional validation [14,15].

6.3. Expected outcome

Through FISH technology, the expression location of the target RNA discovered in Objective 2 can be inferred, and the cell types expressing it (such as neoblasts or other types of cells) can be hypothesized. In combination with immunofluorescence techniques, the expression sites of proteins identified in Objective 3 can be deduced, along with the continuity between target gene expression and target protein expression. Additionally, by integrating specific cellular markers (such as PIWI), hypothesize regarding the cell types that expressing the target protein can be formulated, thereby facilitating cross-system experiments and drug development. The experimental methods of RNA-FISH and immunofluorescence are illustrated in Fig.6.

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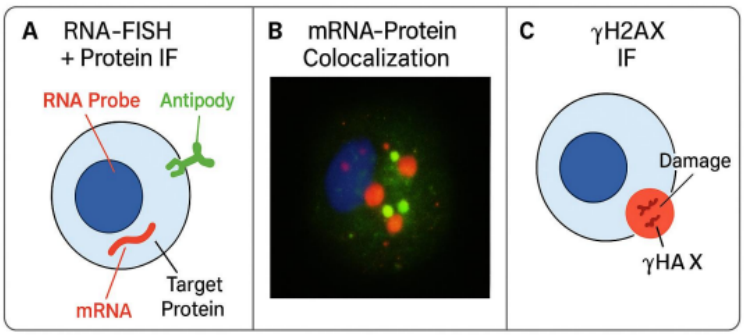

Figure 6. Graphical abstract of RNA-FISH combined with immune fluorescence experiment (a). RNA-FISH combined with immunofluorescence (b). mRNA- protein colocalization (c). immunofluorescence for gamma H2AX

7. Objective 5: gene knockdown experiment to confirm target gene's function

7.1. Rationale

Gene knockdown experiments use techniques such as RNAi (a common approach for M. lignano) to knock down previously identified radiation-resistant target genes. This method has been recently optimized to work with one soaking step maintained over several day. Afterward, they are subjected to different doses of radiation, and the role of the target genes in radiation resistance is assessed by comparison with a non-knockdown control group.

7.2. Method

7.2.1. Techniques for knocking down target gene expression

Knockdown the expression of the target gene in different group of M. lignano using RNA interference technology (RNAi). The RNA interference can be achieved by delivering dsRNA via a single immersion in artificial seawater, which enables the RISC complex to mediate sequence-specific mRNA degradation.

Double-stranded RNA (dsRNA) covering hundreds of base pairs of exons will be designed, and approximately 80 worms will be placed in 400 μL of f/2 artificial seawater per well at a ratio of approximately 2 μg of dsRNA. The worms will be cultured in this dsRNA medium for 10–14 days. The knockdown effect will be verified on day 4 by RT-qPCR (ΔΔCt) and immunostaining.

Negative controls (such as gfp) will also be set up to ensure that the experimental results are not non-specific effects caused by the dsRNA molecules themselves or the experimental manipulations. Positive controls (such as piwi) will also be set up to ensure that the RNAi system is working properly. If the phenotype is weak, another non-overlapping dsRNA will be used to enhance the silencing efficiency within the feasible range without introducing side effects [16].

7.2.2. Grouping method

M. lignano samples will be divided into four groups, which shows in the table below:

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Table 1. Grouping methods of objective 5

	Knockdown the gene	Radiation treatment
Group 1	-	-
Group 2	-	+
Group 3	+	-
Group 4	+	+

7.2.3. Method for assessing radiation resistance

To test whether gene knockdown causally impairs radiation resistance, Group 4 (RNAi + irradiation) will be compared with Group 2 (no RNAi + irradiation) after gamma irradiation. Kaplan–Meier survival curves, combined with log-rank tests and hazard ratios, a standard framework for handling censored survival data, will be used for analysis.

The dose design and interpretation will be anchored by the known tolerance of M. lignano: a cumulative dose of approximately 210 Gy is lethal [1], while ≤150 Gy is reversible. Based on this, a dose ladder of 0/70/140/210 Gy will be set, and survival will be recorded daily until the end of the study. At the same time, γ-H2AX and comet tail detection will be carried out to quantitatively detect DNA single-strand or double-strand breaks.

7.2.4. Experimental operation and data collection

Kaplan–Meier curves will visually demonstrate survival trends across treatment groups throughout the experiment and appropriately account for individuals who have not yet died (i.e., censored data), avoiding bias caused by simply averaging survival days. The log-rank test will compare the two curves for statistically significant differences. The hazard ratio (HR) will further quantify the risk difference, for example, "the risk of death in the knockdown group at any time point is several times greater than that in the control group." This combination of methods not only will ensure the scientific and reliable nature of the analysis but also will make the results more intuitive, easier to interpret, and easier to compare.

Each group will include ≥60 worms, with ≥3 biological replicates and blinded assessment. Results will be reported as median survival, log-rank P-value, and hazard ratio (95% CI) to ensure standardized and scientific statistical analysis of survival data. If the two survival curves intersect during follow-up, relying solely on the proportional hazards assumption of the Cox model could lead to bias. Therefore, a restricted mean survival time (RMST) will be calculated. The RMST directly measures the mean survival time within a predefined timeframe (e.g., 7 or 10 days after irradiation). This method not only intuitively reflects intergroup differences but also does not rely on the proportional hazard assumption. This will provide stable, reliable, and easily interpretable comparisons even when the curves intersect or the hazard ratio changes over time [17].

Whole-cell immunofluorescence targeting phosphorylated H2AX (Ser139) will be used to quantify ionizing radiation-induced DSB signals. Samples will be collected at 0.5, 2, 6, and 24 hours after irradiation; confocal z-scans will be acquired, and the number of foci per nucleus and the fraction of focus-positive cells will be counted using automated spot detection. γH2AX is a reliable surrogate for DSB induction and processing in vitro and in vivo. At the transcript level, H2AX mRNA will be detected by RT-qPCR or RNA-seq [18].

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An alkaline comet assay will be performed on dissociated M. lignano cells to capture single-strand breaks (SSBs)/alkali-labile sites (a neutral comet assay is also available to capture double-strand breaks (DSBs)). The percentage of tailed DNA, tail length, and Olive tail moment will be reported; samples will be taken between 0.5 and 24 hours to analyze damage and repair kinetics [19].

7.3. Expected outcome

M. lignano specimens with the target gene knocked down are expected to exhibit significantly increased radiation sensitivity compared to the control group under different radiation doses, specifically reflected in data such as post-radiation survival time. The gene knockdown (RNAi) and radiation treatment experimental procedures are illustrated in Fig. 7.

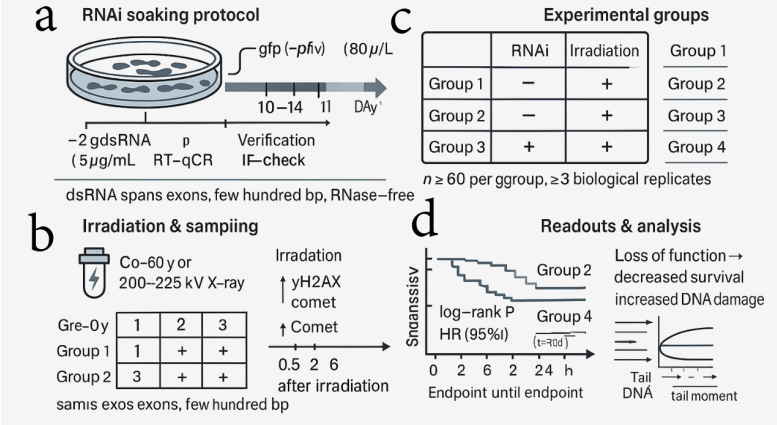

Figure 7. Graphical abstract of gene knockdown experiment (a). Soaking protocol (b). Irradiation and sampling (c). Experimental groups (d). Readouts and analysis

8. Objective 6: cross system expression experiment to confirm the broad-spectrum function of genes across multiple species

8.1. Rationale

Through cross-system expression, it can be demonstrated that the radiation resistance of the target gene found in M. lignano is broadly effective for other species. This serves as a premise for genetically engineering certain species to enhance their radiation resistance, while also laying a theoretical foundation for the development of radiation-resistant drugs.

8.2. Method

8.2.1. Target expression system

Considering factors such as animal welfare and the significance of the experiments, cross-system expression will focus on human cell lines (such as HEK293T and SH-SY5Y) [20,21].

8.2.2. Cross-system expression methods

AAV vectors have low immunogenicity, broad tissue tropism, and stable, long-term gene expression, making them ideal for cross-system expression in human cell lines. So that selected genes (objective

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2) will be induced into the target cell line using AAV virus [22]. After that, incubate the cells for 72-96 hours to ensure strong expression [23]. Use Co-60 g-ray or x-ray (200–225 kV) at dose of 4 Gy, 0.5-1 Gy/min. Cells will be Irradiated when expression peaks. Group separation as follows:

Table 2. Grouping method of objective 6

	Radiation	AAV Treatment
Group 1	-	-
Group 2	-	+
Group 3	+	-
Group 4	+	+

Results will be confirmed by GFP, qPCR, Western blot, etc. These methods provide great way to ensure the expression of candidate proteins [24,25].

8.2.3. Assessment methods for cell radiation resistance

Assess the anti-radiation capability of cells by using the proportion of dead cells after radiation; if the cells can still proliferate after radiation, it is considered that the cell group has complete resistance to that radiation intensity. The speed of growth after radiation can be used as a deeper indicator of radiation resistance [26]. The experimental idea of cross-system expression is shown in Fig.8.

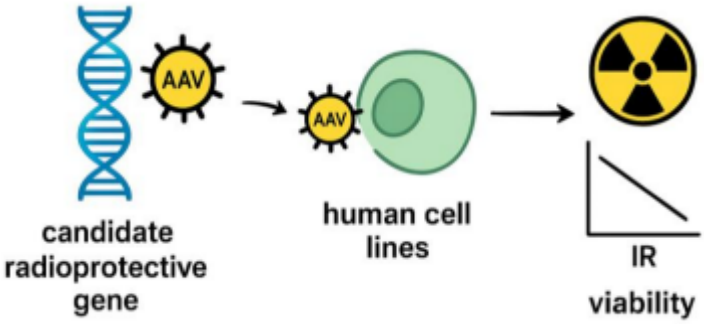

Figure 8. Cross system expression of candidate radioprotective genes

Introduce the candidate gene into the target cell line using AAV virus.

8.3. Expected outcome

After AAV-mediated gene transfer into human cell lines, cells expressing the target radioprotective gene are expected to exhibit significantly enhanced radiation resistance compared to non-transfected control cells. The expression of corresponding radioprotective proteins will be confirmed by Western blot and immunofluorescence.

9. Conclusion

This project proposes a comprehensive research pipeline for the discovery and validation of radio resistance molecules from Macrostomum lignano: including controlled irradiation treatment with doses of 0–210 Gy, RNA sequencing and MS/MS proteomics screening for candidate genes, RNA-

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FISH/immunofluorescence localization, causal validation via RNAi knockdown in worms, and cross-system expression in human cell lines using AAV to demonstrate translational potential. This pipeline aims to advance from observational observations to mechanistic elucidation and proof-of-concept applications.

9.1. Potential impact

If successful, this research has the potential to uncover previously undescribed DNA protection/repair factors or regulatory nodes that confer radio resistance. These factors could provide strategies for: (i) protecting normal tissues during radiotherapy; (ii) improving cell tolerance during deep space missions and nuclear radiation environments; and (iii) inspiring the development of biomimetic drugs or protein engineering for genome stability. By validating AAV-mediated expression in human cells, the project also establishes a practical path from planarian discovery to human application.

9.2. Limitations

Biological differences between M. lignano and human cells may limit the translatability of the findings; some candidate results may reflect physiological characteristics unique to planarians, or even some candidate genes and proteins may cause harm to mammals (previous studies have demonstrated the neurotoxicity of the tardigrade Dsup protein in rats [27]). Insufficient antibody supply may hinder protein-level validation, and RNAi efficiency can vary across different gene loci. Budget and time constraints will limit the breadth of omics replication and mechanistic experiments. Finally, in vitro radiation resistance may not directly translate to overall biological safety, and off-target effects (such as those affecting the cell cycle) are particularly important.

9.3. Future directions

Expand validation to organoid or small animal models; conduct loss-of-function and overexpression interaction experiments to locate candidate genes in DNA repair pathways; conduct biochemical experiments to identify binding partners and functions; explore protein minimization or peptide/small molecule mimetics; and assess long-term genomic stability and safety under chronic or fractionated radiation.

9.4. Summary

This research program is both challenging and feasible: it combines a systematic discovery process with causal validation and a clear translational bridge. Even if a single "effect factor" is not discovered, the resulting datasets, validated targets, and research pipeline will significantly advance our understanding and engineering capabilities for radiation resistance mechanisms.

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