

Identification of Differentially Expressed Genes responding to Ionizing Radiation in Planarians and Mammalian Stem Cells

Xiangyu Wong^{1,4,*†}, Minjie Li^{2,5,†}, Yiyang Liu^{3,6,†}

¹Shanghai High School International Division, Shanghai, 200231, China

²Hefei No.1 American High School, Hefei, 230092, China

³HT Nanjing Impact Academy, Nanjing, 211113, China

⁴xiangyu.wong@hotmail.com

⁵hfyzgjbzmb@126.com

⁶Violetta.Liu1646@outlook.com

*corresponding author

†These authors contributed equally to this work and should be considered co-first authors.

Abstract. Radiation therapy (RT) often damages healthy tissues, limiting the dosage level and tumor damage potential due to single-strand and double-strand breaks in the DNA, leading to apoptosis. Therefore, this study aims to investigate the transcriptomic differences between planarian Neoblasts (NBs) and mammalian bone marrow mesenchymal stem cells (bmMSCs) to identify key genes in the ionizing radiation (IR) response pathway. NBs are extracted from *Schmidtea mediterranea* and bmMSCs from C57BL/6 mice and human donors. RNA sequencing (RNA-seq) is performed on the cell post-IR to identify differentially expressed genes. Techniques such as BLAST, DESeq2, and OrthoFinder identify homologs and unique genes. Gene ontology (GO) analysis, RNA interference (RNAi), and Clustered Regularly Interspaced Short Palindromic Repeats-Cas9 (CRISPR-Cas9) study the impact of manipulating these genes on IR tolerance by indirectly validating the function of the candidate genes. This study expects to be able to isolate cell populations and identify differentially expressed genes (DEGs) related to IR tolerance across the three models. Meanwhile, phylogenetic analyses model the evolutionary relationships between conserved and unique genes, providing insight into fundamental cellular processes and alternative pathways for therapeutic intervention. Comparative genomic techniques facilitated the identification and functional validation of homologs and unique genes. However, the holistic identification of candidate genes is limited by the inability to uncover detailed signaling pathways and mode limitations.

Keywords: Planarian Neoblasts, Bone Marrow Mesenchymal Stem Cells, Ionizing Radiation, Differentially Expressed Genes.

1. Introduction

1.1. Current Demands behind Comprehending Mechanisms of Irradiation Tolerance

One of the greatest challenges currently faced by doctors during cancer treatment is managing the tradeoffs when using radiotherapy (RT). Enhancing radiation damage to tumor tissue while reducing

side effects to healthy tissue remains a formidable challenge [1]. The radiation dosage administered is often limited by the sensitivity of the tissues surrounding the tumor [2]. In addition, cancer stem cells (CSCs) drive further tumorigenesis and contribute to the inefficacy of RT treatments [3]. Consequently, a significant biomedical demand exists for novel mechanisms that differentially sensitize tumor cells and protect healthy tissues [1]. Ionizing radiation (IR) during RT causes single-strand breaks (SSB) and double-strand breaks (DSB) in DNA, disrupting critical regulatory mechanisms such as mitosis and, in severe cases, leading to apoptosis and cell necrosis [1]. However, certain naturally occurring cells exhibit relatively high levels of IR tolerance. For example, planarian neoblasts (NBs) and human mesenchymal stem cells (MSCs) demonstrate resistance to DNA damage [4,5]. Understanding the survival mechanisms after IR exposure in stem cells will provide valuable biomedical insights [2,3].

1.2. Radiation Tolerance & Planarian Neoblasts (NBs)

Schmidtea mediterranea planarians were selected as the model organism and neoblast source (NBs). Neoblasts are the "only proliferative cell type in asexual planarians" and "can be specifically eliminated by irradiation" [6,7]. Therefore, they are a fundamental model to investigate the conservation of animal pluripotency and self-renewal [7]. These cells are distributed throughout the body and can migrate to restore homeostatic balance during injuries [8]. Additionally, chromatoid bodies (CBs) in the cytoplasm morphologically distinguish them from other cells [7]. For functional genomic studies, planarians offer the advantage of accessible transcriptomic manipulation via RNA interference (RNAi). They are essential for comparative studies between organisms to gain insights into conserved gene functions due to the ease of handling large amounts of transcriptome data [7-9].

In the context of ionizing radiation (IR) tolerance, NBs exhibit a comparatively high level of resistance: their regenerative capacity and proliferative function are maintained at up to 15 Gy of irradiation, with lesions being completely repaired after sufficient recovery time. Some stem cells can survive at higher doses but fail to retain their pluripotent characteristics [9,10]. This evidence supports the viability of planarians as a model for studying gene conservation and suggests that "nonregenerative scenarios will require the correct redeployment of well-known genetic networks" [11].

Currently, the underlying mechanisms for the conspicuous radio-tolerance of neoblasts are still shrouded in secrecy. In spite of this, neoblasts do show some unique transcriptomic responses to IR. For example, EGFR-3 expression is up-regulated in neoblasts after sublethal IR, benefiting neoblast clonal expansion to repopulate the animal [12]. Meanwhile, although the role of EGFR in IR damage of human stem cells remains unclear, EGF expression in human epithelial cell line Het-1a is, in contrast to neoblast, reduced by IR. Exogenous EGF treatment effectively protected Het-1a from IR-induced apoptosis via EGFR signaling [13]. Based on this, the hypothesis is that under IR exposure, the spectrum and regulation patterns of differentially expressed genes (DEGs) are different between neoblasts and human stem cells. This will partially explain the stronger radio-tolerance of neoblasts. The DEGs most differentially regulated between species will contain key genetic regulators of radio-tolerance.

1.3. Radiation Tolerance & Human/Mice Bone Marrow MSCs (bmMSCs)

Bone marrow mesenchymal stem cells (bmMSCs) are multipotent adult stem cells that can migrate and differentiate into various tissue types depending on their microenvironment [14]. These long-lived MSCs can differentiate into multiple cell types, including adipocytes, chondrocytes, osteoblasts, and hepatocytes, and were first isolated from bone marrow [15,16]. A defining characteristic of MSCs is their ability to adhere to plastic surfaces and differentiate along the adipogenic, osteogenic, and chondrogenic lineages [17]. Regarding radiation tolerance, bmMSCs exhibit significant resistance to high doses of ionizing radiation (IR). Even at doses up to 10 Gy, bmMSCs maintain their differentiative potential, notably higher than that of primary fibroblast and differentiated cells. This exceptional IR resistance has led to their application in combined cancer therapy. Additionally, high doses of IR do not result in increased irradiation-induced apoptosis in bmMSCs [15-17]. The extent of these mechanisms is hence accessible by Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated protein, which enables precise gene knockdown and knock-in [18,19].

1.4. Radiation Tolerance & Human/Mice Bone Marrow MSCs (bmMSCs)

This study plans to find out the neoblast-specific transcriptomic changes to IR with functional genomics. A myriad of studies have demonstrated the feasibility of performing functional genomic analyses on planarians and many other mammalian cells [6,7,14,19,20]. Planarian neoblasts can be separated from other tissue cells by FACS for RNA-seq [6,7,19]. BLAST and OrthoFinder can help establish homologous relations for IR-related differentially expressed genes (DEGs) between species [21,22], which enables inter-species comparison of their expression pattern under IR. For functional validation of candidate genes extracted from RNA-seq and bioinformatic analysis, experiments including RNAi, CRISPR technologies, in situ hybridization and many others are available in planarians and/or mammalian cells [6,7,9-13,18,19,23–25]. However, this study aims to incentivize trans-genetics research and insights into the biomedical applications of IR tolerance (see Figure 1).

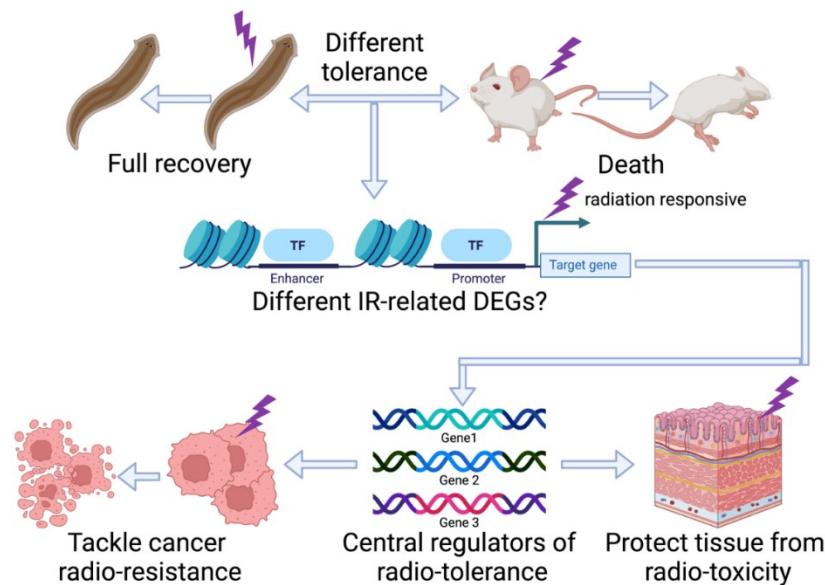


Figure 1. Graphical abstract created with BioRender.com.

2. Project Description

2.1. Research Question

Which genetic orthologs exhibit differential regulation in response to IR between planarian NBs and mammalian bmMSCs? Moreover, how do these transcriptomic differences contribute to their varying radiation tolerances?

2.2. Hypothesis

Investigating the homologous and unique genes will yield candidates that signify the differential responses and tolerance to IR by utilizing comparative functional genomic techniques and selecting comparable stem cells in terms of tolerance to IR.

3. Objective 1: Identify & Compare Genetic Homologs in NBs & Mammalian Stem Cells induced by High-dose IR

3.1. Rationale

Identifying homologs and unique transcripts in IR response enables comparative genomic techniques to identify key genes that regulate IR tolerance for further study and functional validation.

3.2. *Methods (see Figure 2)*

3.2.1. *NB Fluorescence Activated Cell Sorting (FACS)*

FACS is used to obtain NB-enriched cell populations to separate NBs from the planarian cell population. Neoblasts are relatively more irradiation-sensitive, so selective irradiation identifies NBs by their unique cytoplasmic volume to DNA content. Thus, the X1 cell population mainly comprises NBs with a few differentiating (X2) and differentiated cells (Xins).

3.2.2. *RNA-seq on Differential IR-Exposure*

Human and mouse bmMSCs are harvested from the bone marrow of healthy donors and cultured in an MSC basal medium. While NBs are stored within the planarians. Cells and planarians were plated and exposed to 2, 4, 6, 8, and 10 Gy of IR at room temperature. Post-IR exposure, cells were sequenced at 0, 2, 3, and 7 days to gain perspective into differential gene expression and variations over time. The control group was non-irradiated cell populations sequenced at the same periods. RNA-seq is conducted by converting fractionated samples to cDNA and conducting pair-end sequencing with Illumina. The reads were then mapped to a reference transcriptome, and significantly different expression values were identified [7,15,26].

3.2.3. *BLAST Analysis*

From differential change in gene expression, homologs are identified through BLAST by searching for sequence similarities between the genes that are differentially expressed in all three models using the National Center for Biotechnology Information's (NCBI's) BLAST server. Converting RNA-seq data to Fast All (FASTA) format and removing low-quality reads aligns sequences with human, mouse, and planarian genome databases. By starting alignments from short matches between sequences, BLAST provides significant matches with high similarity scores as future candidates for analysis [27].

3.2.4. *DESeq2*

However, uniquely expressed genes could also play a key role in IR tolerance. Thus, DESeq2 enables alternative analysis of these sequences. Its utilization of count data and shrinkage estimations enables the quantitative analysis of the presence and abundance of unique gene expressions. By creating and normalizing a dataset of DESeq2 and then estimating the dispersion for each gene, we obtain differential expression data based on fold changes and significance values. By testing the null hypothesis that logarithmic fold change between IR-exposed and non-IR samples is exactly 0, a list of genes marked as uniquely expressed are identified and ranked by p-values [28].

3.2.5. *OrthoFinder*

With similar and different genes identified, OrthoFinder aims to map the homologs between NBs and bmMSCs. "Orthologs are the set of genes in a species pair descended from a single gene in the last common ancestor of those two species. An orthogroup is the set of genes from multiple species descended from a single gene in the last common ancestor (LCA) of that set of species" [22]. Through orthogroup inference and the analysis of gene trees, phylogenetic information identifies the complete set of orthologs between the three models and extensive comparative genomic statistics [22].

3.2.6. *Gene Ontology (GO) Analysis*

Therefore, verified homologs alongside uniquely expressed genes are selected as main candidates for GO analysis. Genes are annotated to GO terms using the EasyGO software. GO enrichment analysis is then conducted to identify GO terms overrepresented in the differentially expressed genes (DEGs) compared to the expected results. Lastly, Fisher's exact test determines the significance of the overrepresented GO terms, followed by multiple testing corrections [19,29].

3.3. Expected Outcome

Possible interpretations that could be made based on phylogenetic data are as follows: Homologs of the same function indicate fundamental cellular processes crucial in the IR response, such as DNA repair mechanisms, cell cycle regulators, and apoptosis. The conservation of these functions underscores their importance in maintaining cellular integrity across organisms. Alternatively, unique genes with a shared function indicate the occurrence of convergent evolution whereby certain cellular functions are so critical that multiple evolutionary pathways lead to the same solution. Identifying these genes could reveal alternative pathways that can be targeted for therapeutic interventions. However, unique genes with unique functions indicate specialized IR response mechanisms in bmMSCs and NBs.

The IR-related DEGs in all the groups are expected to be most related to DNA damage repair, cell death and survival, mitosis checkpoints and stress responses. The DEGs can be categorized into 3 groups regarding their inter-species regulation differences under IR:

1) DEGs whose regulation is similar between groups: These genes are likely irrelevant to the different radio-tolerance between neoblasts, human and mouse. They may be related to stress responses, which will conceivably happen in all cell types.

2) DEGs whose regulation is significantly different between groups: These genes likely account for different radio-tolerance between species. The expected results are that DEGs significantly more expressed in neoblasts are related to DNA damage repair, ROS elimination, cell survival, and proliferation. In contrast, those more expressed in bmMSCs are likely related to cell death.

3) DEGs unique to certain species: These genes might contribute to the radio-tolerance or -sensitivity of the cell type it belongs to. They may also participate in some unique cellular processes in that particular cell type under homeostatic state.

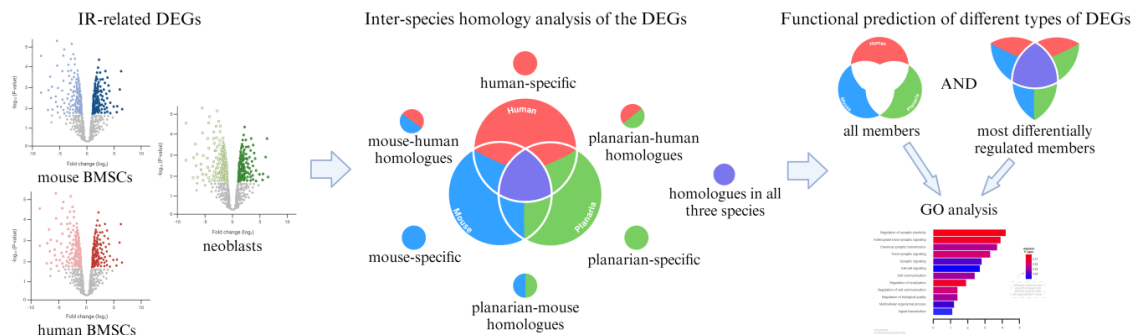


Figure 2. Overall workflow of the bioinformatic analysis of RNA-seq data created with BioRender.com (human BMSCs refer to bmMSCs).

4. Objective 2: Elucidate & Validate Functional Roles of Conserved & Differentially Expressed Genes

4.1. Rationale

Despite seeing differential expression post-exposure, further validation is required to investigate whether candidate genes play a role in increasing IR tolerance through means such as DDR or DNA protection.

4.2. Methods

4.2.1. Candidate Genes & Bilateral Validation

A shortlist of 10 IR-related homologues whose expression pattern is most different between neoblasts and bmMSCs is created alongside 5 unique IR-related genes in neoblasts whose expressions are most significantly altered after IR, and 5 such genes in human bmMSCs.

Gene knockdown in planarians and overexpression in bmMSCs enables functional validation of candidate genes significantly up-regulated in neoblasts but not in bmMSCs.

Sole knockdown in bmMSCs is performed on candidate genes that are significantly downregulated in neoblasts but not in bmMSCs

Gene knock-in in bmMSCs and knockdown in NBs are performed for candidate genes unique to NBs and upregulated genes in NBs respectively.

Lastly, knockdown in bmMSCs is completed for candidate genes unique to bmMSCs.

Then, irradiation experiments at same doses to the modified animals and cells are repeated, and samples collected at the same time points, to assess their recovery from IR damage. Controls will be wild type cells and planarians.

4.2.2. *RNAi*

Double strand RNAs (dsRNAs) are designed and synthesized for candidate genes, and use GFP dsRNA as control. The dsRNAs will be injected into planarians [19], or be loaded into lipids for BMSCs transfection. With the candidate genes, RNAi in planarians and bmMSCs can indirectly determine the functional role of IR response. The control group would be Green Fluorescent Protein (GFP) double-stranded RNA (dsRNA). This process is mediated by the RNA-induced silencing complex (RISC) via dsRNA molecules. The animals are injected with dsRNA and then subjected to the same intervals of irradiation whilst being monitored at the same time intervals post-IR [7,30].

4.2.3. *bmMSCs CRISPR-Cas9 Activation*

For bmMSCs, modified CRISPR-Cas9 can activate a gene upon interaction with a protospacer adjacent motif (PAM). At the same time, controls treat cells by activating an insignificant gene. Mutations are introduced to the RuvC and HNH domains of Cas9 to prepare for CRISPRa and create a deactivated Cas9 (dCas9). Then, with programmed dCas9-effector fusions, a target-specific transcription factor is created. The bmMSCs are infected with a lentiviral vector, causing candidate gene overexpression with single guide RNAs targeting 1000 bp upstream. Similarly, overexpression is performed, and the cell is subject to its irradiation regime.

4.2.4. *bmMSCs CRISPR-Cas9 Knock-in*

sgRNAs are designed and synthesized for target cleavage sites, and single strand DNA (ssDNA) templates for candidate genes to be knocked in. The sgRNA-Cas9 complex and ssDNA templates will be transfected into bmMSCs via co-electroporation [31]. The knock-in efficacy will be examined by western blotting of the candidate genes.

4.2.5. *DNA Damage & Stem Cell Viability Assays*

COMET assays are performed on irradiated planarians and bmMSCs [32]. This will reflect the resistance of their genome to IR damage, as well as the speed of their DNA damage repair. Then FISH is performed on Smedwi-1 and α H3P in planarians to quantify their neoblast presence and proliferation rate. The recovery rates of the animals' homeostasis, as shown by their appearance, will also be assessed to reflect neoblast differentiation ability [19,33]. bmMSCs viability after IR will be assessed with CCK-8, and their proliferation rate will be assessed by performing immunofluorescence of BrdU. Their multipotency will be assessed by inducing their osteogenic and lipogenic differentiation, and measuring their expression of MSC markers such as CD106, CD44 and CD90 [23,24].

4.3. *Expected Outcome*

Both DEGs and functional validation will yield a myriad of scenarios. Figure 3 elaborates upon the possible interpretations made when comparing the DEGs between NBs and bmMSCs and provides possible mechanistic regulators for those genes to control. Figure 4 details knockout and overexpression's impact on the organisms' IR tolerance, thereby validating their role. These genes hold the potential to become therapeutic targets for desensitizing stem cells, or sensitizing cancer cells to IR.

However, when interpreting the results of knock-in experiments, efforts should be made to discriminate the real reason for decreased radio-tolerance, as some heterogenic gene products might be toxic to the recipient cells in themselves and will surely prime them to cell death even in the absence of IR.

These key genes may affect in different aspects of radio-tolerance, which can be partially discriminated by our experimental results. For example, if the overexpression of a candidate gene doesn't affect the results of COMET assay, but still results in higher cell viability as shown by CCK-8, then the gene probably doesn't participate in DNA damage repair but is rather a cell survival signal. Despite this, the genes' precise functions still need to be elaborated by more detailed study.

NBs Expression	bmMSCs Expression	Interpretation
		Conserved response (DDR, Cell Cycle, Stress)
		Conserved, suppressed pathway (Nonessential metabolism)
		Differential role of homolog (DDR suppression due to tumor-suppressor genes)
		Crucial mammalian response genes (apoptosis)
		Evolutionary divergence with unique strategies to cope with similar stress.
		Identify these genes could uncover new pathways in mammals.
		Genes involved in specific functions only relevant in context of mammalian physiology
		Potential therapeutic targets for enhancing resistance in humans.

Figure 3. Possible Interpretative Scenarios of DEGs.

Procedure	Radio-tolerance	Interpretation of target gene function
Knock-down	↑	Related to radio-sensitivity, possibly promotes cell death under IR and other stimuli.
	--	1) Downstream products of IR damage, don't have an regulatory role (i.e. markers of stress response). 2) Redundant regulator whose functional repression can be readily compensated.
	↓	Related to radio-tolerance, possibly promotes cell survival, DNA damage repair etc.
Overexpression/ Knock-in	↑	Related to radio-tolerance.
	--	1) Overexpression: Irrelevant genes whose expression is suppressed as a result of IR damage. 2) Knock-in: Only participate in unique cellular processes of the donor species, don't have any roles in recipient cells.
	↓	1) Overexpression: Related to radio-sensitivity. 2) Knock-in: Related to radio-sensitivity, or gene products are toxic in recipient cells.

Figure 4. Summary of possible experiment results and the corresponding roles of the candidate genes of interest in cell radio-tolerance.

5. Conclusion

Comparative genomic techniques facilitated the identification and functional validation of homologs and unique genes. However, the inability to decipher detailed pathways of identified IR-response DEGs and the technological limitations faced by planarians as a model hinder the potential of this study. Furthermore, in vitro, bmMSCs may exhibit different IR responses than in vivo cells. Future studies could investigate the mechanisms of key DNA repair mechanisms and the role of epigenetic regulators on gene expression. T study paves the way towards trans-genetics research into transferring IR-tolerance mechanisms and biomedical applications of selective sensitization of cancer cells or the desensitization of healthy tissue during radiotherapy.

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