Research of transcriptome responses after gamma-ray irradiation exposure in Japanese medaka (*Oryzias latipes*)

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[Introduction]

Radiation (IR) is widely utilized in modern technology for medical testing, radiotherapy, and nuclear power generation. Its impact on cells can occur through direct DNA damage or the formation of reactive oxygen species (ROS) and free radicals via water radiolysis (indirect effect). While high-dose radiation is known to cause cell death, chromosomal aberrations, DNA damage, mutagenesis, and carcinogenesis, the effects of low-dose and low-dose-rate radiation remain less understood due to limited research in this area.

The Japanese medaka (*Oryzias latipes*), a small freshwater fish, has long been a valuable model vertebrate in radiation biology research due to its manageable size and ease of laboratory breeding and experimentation. Its suitability for gene expression analyses at the systemic level makes it particularly conducive to study the biological effects of irradiation.

A goal of this study is to comprehensively elucidate the systemic impacts of low-dose/low-dose-rate chronic irradiation on the whole-body transcriptome of inbred adult Medaka fish (Hd-rR strain). Tissues with varying sensitivity to radiation, such as the testes and intestine (high sensitivity) and muscles (low sensitivity), will undergo transcriptome analysis after exposure to approximately 100 mGy of low-dose/low-dose-rate chronic irradiation. The goal is to provide detailed insights into the molecular mechanisms underlying the biological responses induced by chronic low-dose irradiatio, contributing to a better understanding of impacts of irradiation on biological systems and informing radiation protection strategies and risk assessments against low dose rate chronic irradiation.

As mentioned before, medaka spermatogenesis in testis has a high sensitivity to irradiation. Gamma-ray irradiation induces the cessation of type B spermatogonia proliferation, promoting their transition to meiosis and spermiogenesis. Among medaka spermatogonia, only type A spermatogonia has DNA repair capabilities, whereas type B spermatogonia lack such abilities. Therefore, it is believed that in cases of genome damage due to irradiation, type B spermatogonia promptly convert into sperm and discardedinstead of repairing the damaged genome.

Moreover, it has been reported that in *p53*-deficient medaka testis, ova-like cells (testis-ova) were induced by gamma-ray irradiation. *p53* is the most established tumor suppressor gene, widely involved in cellular functions including cell cycle regulation and apoptosis induction. Studies have shown that male zebrafish lacking *p53* gene function develop and grow as females. On the conversely, male *p53*-deficient medaka produce testis-ova under radiation exposure, strongly suggests that *p53* works differently in germ cell sexual determination even inside teleost. Administering female hormone to male medaka resulting in differentiation from type B spermatogonia into testis-ova, meanwhile, it suggests a possibility that irradiation induced testis-ova from type A spermatogonia. By uncovering the molecular mechanism underlying the production of testis-ova induced by irradiation, we will gain insights into the molecular mechanisms that govern spermatogonia and determine their fate as male germ cells. Furthermore, there is also a possibility to reveal the mechanisms which support spermatogonia A differentiating to type B spermatogonia instead of oogonia. In this study, I preformed single-cell RNA sequencing (scRNA-seq) on medaka testis one week after gamma-ray irradiation, to elucidate the molecular mechanism underlying the induction of testis-ova differentiation.

[Methods and Results]

Part One. Single cell RNA Sequencing of medaka testis

Clustering of spermatogenesis process

Single cell RNA sequencing analysis were performed at 4 types of medaka testis, which were Hd-rR and *p53*-deficient Hd-rR adult medaka non-irradiated and irradiated with low-dose acute irradiation (Cs-137 gamma-rays, dose rate of 7.5 Gy/min and total dose of 0.5 Gy), and their RNA were extracted 7 days after the irradiation.

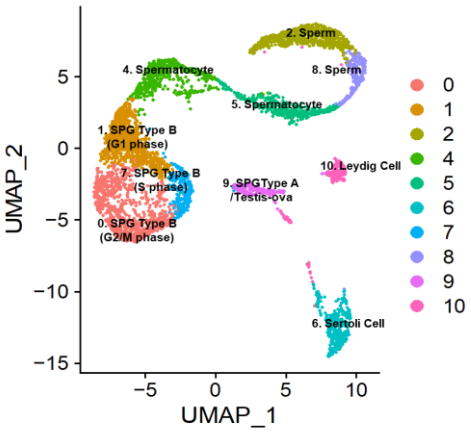

Figure 1. UMAP of Hd-rR medaka testis. Each cluster was assigned a unique colour. SPG is short for spermatogonia.

By conducting single-cell RNA sequencing on Hd-rR male medaka testis, I successfully clustered specific spermatogenetic stages: clusters representing spermatogonia (type A and B), differentiated spermatogonia, spermatocytes in meiosis, spermatids, and sperm were identified based on their expression of germ cell marker genes such as *ddx4* (*vasa*) and *dazl*. Additionally, distinct clusters corresponding to Sertoli cells (expressing *rgs13*) and Leydig cells (expressing *cyp17*) were also identified. Cluster 9, expressing *dnd1* in addition with germ cell markers, likely represents undifferentiated spermatogonia (type A). The expression of *nanos2* further supports the classification of cluster 9 as type A spermatogonia. These findings were consistent in *p53* knock-out medaka testis, indicating a similar spermatogenic process irrespective of *p53* status.

Gamma-ray accelerated meiosis with a *p53* gene dependency.

Clustering was also succussed in gamma-ray irradiated Hd-rR strain medaka testis. Compared to non-irradiated group, expression of S phase marker (*pcna*, *pclaf*) and G2/M phase marker (*ccnb1*, *ccnb3*) reduced in type B spermatogonia, which clearly shows that a cessation of mitotic cell division of type B spermatogonia after the gamma-ray irradiation. In addition, a cluster absent in non-irradiated dataset was developed form S phase type B spermatogonia and showed a connection to spermatocytes before meiosis. Cells in that cluster have a strong expression of meiosis marker (*sycp1*, *scp3*), as well as the expression of late stage spermiogenesis genes like *plcz1*, *ccnb2*, *spatcl1*(ENSORLG00000021836).

It is reported histologically that spermatogonia stop proliferation and rush into meiosis and spermiogenesis after gamma-ray irradiation. The cluster induced by gamma-ray irradiation can be believed to be spermatocyte entering spermiogenesis by skipping normal process of proliferation due to the irradiation. On the other hand, irradiation did not induce proliferation cessation and acceleration of meiosis in the gamma-ray irradiated *p53*-deficient medaka testis.

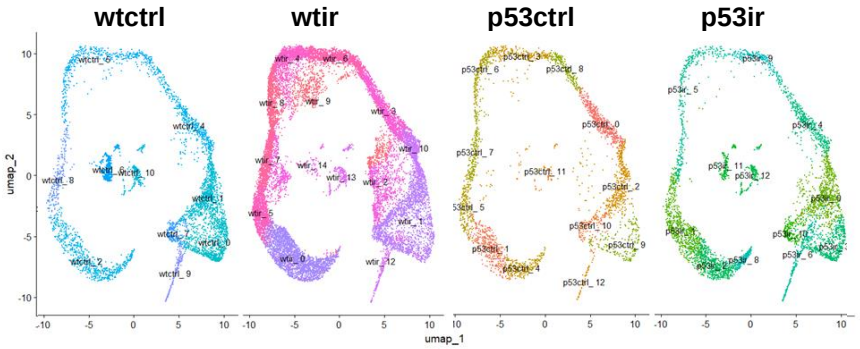

Figure 2. UMAP of 4 datasets integrated, wild-type control group, wild-type irradiated group, p53 deficient control group and *p53* deficient irradiated group. Cells were grouped by the original cluster numbers. Wtctrl: Hd-rR wild-type non irradiated group; p53ctrl: *p53*-deficient Hd-rR non irradiated group; Wtirl: Hd-rR wild-type irradiated group; p53ir: *p53*-deficient Hd-rR irradiated group.

Expression profile of type A spermatogonia

Type A spermatogonia in medaka testis express specific markers such as *dnd1* and *nanos2*, while showing low or no expression of pluripotency markers like *sox2*, *oct4*, and *klf4*, as well as *ckit* (*kitb*). They exhibit high expression levels of *sox19b* and *fabp11a*, along with *myca* and *c-myc17*. Type A spermatogonia also uniquely express *Orla-DCA* and *cd74a*, MHC class II molecules. Additionally, all male germ cells, except for type A spermatogonia, express *mif* (macrophage migration inhibitory factor). Furthermore, the immunoproteasome *psmb8*, responsible for generating antigen peptides presented by MHC class I molecules, is also exclusively expressed in type A spermatogonia. These findings highlight unique molecular signatures and interactions within male germ cells during spermatogenesis in medaka testis.

Type A spermatogonia partially differentiate into testis-ova

I performed single-cell RNA sequence analysis on *p53*-deficient medaka testis to understand the molecular mechanism under the formation of irradiation induced testis-ova in testis. Clusters were similarly arranged and identified in the testes of the non-irradiated Hd-rR medaka and the gamma-ray irradiated *p53*-deficient medaka. Cluster with expression of *nanos2* and the topology neighboring to type B spermatogonia were identified as type A spermatogonia. Expression of oogenesis specific genes (*nanog*, *mos*, *nobox*, ENGORLG00000028896, *nanos3*, *h1f8*(*h1foo*, ENSORLG00000024434)) was found in cells belonging to this cluster, which strongly speculated as early-stage testis-ova. On the other hand, oogenesis specific genes were also partially expressed in type A spermatogonia cell cluster of all 4 medaka testis datasets, regardless the existence of irradiation or *p53* gene. In the scRNA-seq dataset obtained in this study, there was no apparent enhancement of testis-ova induction by deficiency of *p53* gene, nor by gamma-ray irradiation. Instead, this result strongly suggests that there is a constant abnormal differentiation into testis-ova in Hd-rR strain medaka type A spermatogonia.

Part Two. Total RNA sequence of Hd-rR medaka after chronical low-dose gamma-ray irradiation

To elucidate the physiological impacts of low doses/dose rate gamma-ray irradiation, during subsequent recovery phases, RNA transcriptome sequencing was performed on adult medaka 7 days after the low dose/dose rate irradiation (7 days with a total dose of 100mGy). To get a comprehensively picture of the effects of irradiation on the whole body, RNA sequencing analyses at a whole-body level were conducted. Furthermore, organ responses against irradiation vary due to differences in their physiological functions, histological structures and tolerance levels, they are largely expressed by the rates of cellular division. Consequently, I selected the intestines and reproductive organs (ovary and testis) in this study, which are known to be more radiation-sensitive, for RNA-seq analyses.

RNA-seq analysis of medaka whole-body

Differential expression genes (DEGs) were extracted between transcriptome profiles of irradiated group and control group. Gene Ontology (GO) enrichment analysis and KEGG pathway analysis were conducted to annotate the up-regulated and down-regulated DEGs separately. In the up-regulated DEGs, the GO and KEGG terms related to lipid were enriched, including “fatty acid metabolism”, “biosynthesis of unsaturated fatty acid”, “steroid biosynthesis” and so on. It is known that low dose irradiation will increase reaction oxygen species (ROS) in cytoplasm, which can modify biomolecules like proteins and lipids, and lead to oxidative damage, especially unsaturated fatty acids and plasma membrane. The up regulation of lipid metabolism strongly suggests that the medaka were under deficiency and repair of damaged lipids in a whole-body scale after the low dose/dose-rate irradiation. The low dose/dose-rate irradiation also disputed the signal transduction activity, leading to down-regulation of calcium related signaling pathway such as “Wnt signaling pathway”, “Calcium signaling pathway” and “MAPK signaling pathway”, which are crucial for cell activation for cell growth, proliferation and differentiation.

RNA-seq analysis of medaka tissues

Unlike whole-body analysis, the irradiation did not induce apparent modification of lipid metabolism in selected irradiated intestine and gonads (ovary and testis), instead, they expressed tissue specific responses after the low dose/dose-rate irradiation.

Intestine exhibits an up-regulation of cells in G1 and S phase but down-regulation in G2 and M phase, suggesting that the intestine might be under repairing and regeneration of intestinal epithelium ells including enterocytes, to maintain barrier function and absorptive capacity, and cell division of stem cells in crypt was delayed repairing irradiation induced-damages in their genome.

In ovary, glycosaminoglycan synthesis and binding were up-regulated, and metabolism, steroid hormone biosynthesis, estrone related and transportation were down-regulated after the irradiation, suggesting a reduction in reproductive activity in ovary maybe due to oxidative stress and/or nutrition deficiency caused by low dose/dose rate irradiation. In contrast in testis, structural constituent of ribosome （MF）, translation （BP）, ribosome (CC) and mitochondrion(CC) exhibited the extremely low p values in down-regulated GO list, which suggests that ribosome biogenesis and translation were significantly down-regulated after the irradiation in the testis. This finding also suggests that type B spermatogonia stopped proliferation, and that meiosis and spermiogenesis was promoted after the irradiation, which has been reported by the previous histological studies and single-cell RNA-seq analysis in this

study, presumably to discharge damaged spermatogenetic cells from the testis.

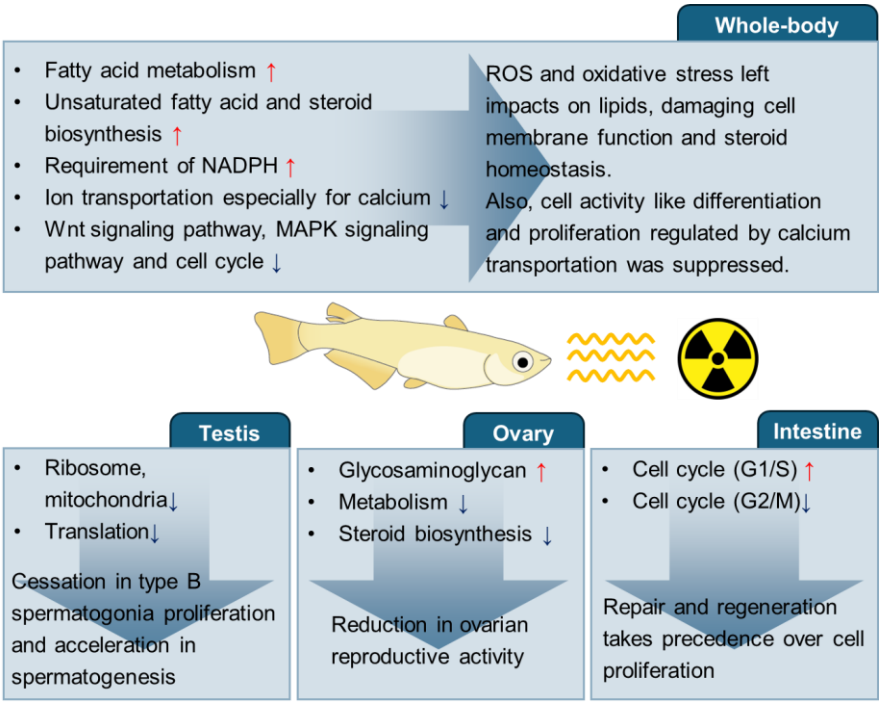

Figure 3. Schematic summary of whole-body and tissue-specific responses after low dose / dose rate irradiation.

[Conclusions]

1. In the first this study, Single cell RNA-seq was introduced to investigate medaka spermatogenesis and testis-ova induced by low dose gamma-ray irradiation. A constant abnormal differentiation toward testis-ova in Hd-rR strain medaka type A spermatogonia was found, which might be difficult to notice by traditional histological investigation.
2. Expression profiles in single-cell resolution provides us a broader perspective for studying the fate determination of spermatogonia, as well as the molecular switches between mitosis and meiosis.

3. More comprehensive investigation of transcriptional responses to low dose irradiation has been conducted on the tissues highly sensitive against irradiation and whole-body scale level. I made clear the tissue specific responses and confirmed functional disruption in intestine and gonads, however, in whole-body level, typical irradiation responses like apoptosis or DNA damage repair are not significantly observed after one week recovering, instead, a disturbance on lipid metabolism remains as a long-term irradiation impact.

4. In this study, I deeply appreciated the powerful capabilities of NGS, particularly single-cell RNA sequencing, in revealing subtle biological changes. These changes, such as those induced by low-dose gamma-ray irradiation, are often too intricate for traditional physiological methods to detect. By analyzing the expression profiles of individual cells, we can not only understand their current state but also infer their past and predict their future. Additionally, the sequencing data contains vast amounts of information, and with the advanced databases available today, it provides limitless possibilities for future biological research.

[Publications]

Wang, D., **Li, D**., Liang, F., Cao, Q., & Yin, S. (2017). Cloning and effects of different salinities on aquaporin 3 (AQP3) in juvenile marbled eel, Anguilla marmorata. *Marine Fisheries*, *39*(5), 518-528.

Yasuda, T., Funayama, T., Nagata, K., **Li, D**., Endo, T., Jia, Q., ... & Oda, S. (2020). Collimated Microbeam Reveals that the Proportion of Non-Damaged Cells in Irradiated Blastoderm Determines the Success of Development in Medaka (Oryzias latipes) Embryos. *Biology*, *9*(12), 447.

Yasuda, T.*, **Li, D**.*, Sha, E.*, Kakimoto, F., Mitani, H., Yamamoto, H., ... & Oda, S. (2022). 3D reconstructed brain images reveal the possibility of the ogg1 gene to suppress the irradiation-induced apoptosis in embryonic brain in medaka (Oryzias latipes). *Journal of Radiation Research*, *63*(3), 319-330. * Equally contributed authors.