

Mission and Specific Objectives

The Department of Molecular Biosciences (MBS) contributes to the mission of RERF by conducting mechanistic studies to elucidate and interpret radiation effects observed in clinical and epidemiological studies of RERF cohorts, including A-bomb survivors and their offspring. These interdisciplinary studies are carried out in collaboration with other RERF departments and external research institutions. The MBS comprises four specialized laboratories: Molecular Genetics, Cellular Genomics, Molecular Pathology, and Immunology. Additionally, some MBS researchers hold dual appointments with the Biosample Research Center (BRC) to facilitate cross-departmental collaboration. The primary goals of the MBS are:

- 1) to elucidate the molecular mechanisms underlying neoplasm development and non-cancer diseases associated with radiation exposure, and
- 2) to investigate the potential transgenerational effects of radiation exposure.

To achieve these goals, the current MBS research program is organized into the following five categories:

1. Analysis of the mutagenic effects of ionizing radiation on the human genome from the macro (chromosomal aberrations) to the micro level (DNA sequence alterations) in both somatic cells and germlines
2. Analysis of the dynamics and functions of cells after radiation exposure, focusing on the relationships between radiation exposure, clonal cell proliferation during the process of post-exposure recovery, and persistent inflammation, which could play a role in disease development
3. Elucidation of the molecular mechanisms of the development of radiation-induced neoplasms and non-cancer diseases and identification of biomarkers of radiation exposure and disease development, in particular through -omics approaches such as genomics and proteomics using blood and pathological samples provided by A-bomb survivors
4. Elucidation of the molecular basis for individual differences in radiation sensitivity and risks for neoplasms and non-cancer diseases due to radiation exposure by identifying genetic, environmental, and lifestyle factors that determine radiation sensitivity and risk
5. *In vitro* and *in vivo* model experiments using volunteer samples, cultured cells, and mouse models to validate hypotheses on molecular mechanisms and develop research methodologies

Research projects conducted by the MBS are prioritized to elucidate the molecular mechanisms of late effects of ionizing radiation in A-bomb survivors and their children, and to develop biomarkers of past radiation exposure and related disease risks, all of which further the mission of RERF. The MBS is central to RERF's strategic goals as follows:

Create: The MBS uses state-of-the-art analytical technologies to study genetic, epigenetic, transcriptional, biochemical, and immunological characteristics to gain mechanistic insights into the long-term health effects of radiation exposure in A-bomb survivors and their children in the RERF cohorts. Currently, such studies include the Trio Genome Study to perform whole-genome sequencing (WGS) of exposed parents and their children, and the exome sequencing study of clonal hematopoiesis in A-bomb survivors decades after exposure.

Collate: Biosamples and data generated by the MBS are being collated and organized for future research use.

Collaborate: The MBS conducts these studies in collaboration with all internal departments at RERF, as well as with external scientists, as described below.

Communicate: The sensitive nature of research on the biosamples of A-bomb survivors and their offspring requires ongoing communication with research participants, A-bomb survivor groups, local communities, and the media. The importance of this continued communication to the successful conduct of the research cannot be overstated.

Department Resources

The majority of the MBS collections of blood samples from A-bomb survivors and their children have been transferred to the Biosample Research Center (BRC) for centralized management. The MBS has a large volume of data spanning many decades from chromosomal analysis of A-bomb survivors and their children, as well as lymphocyte subset analysis and measurement of circulating protein factors such as cytokines in blood samples of Adult Health Study (AHS) participants.

The main equipment and instrument resources of the MBS include a secure human genome analysis room equipped with an on-premise server, a cloud computing system for genome data analysis, an animal experiment facility, a P2 (physical containment level 2) laboratory, an animal X-ray irradiator, a chromosome analysis laboratory, fluorescence-activated cell sorters (FACS), and a pathological specimen preparation laboratory. With the relocation of RERF to the Kasumi Campus of Hiroshima University, the animal experiment facility will be discontinued and merged with the research program at the Joint Usage Facility of Hiroshima University.

Internal and External Collaborations

The MBS has many years of experience in the analysis of chromosomal aberrations and dental enamel electron spin resonance (ESR), and has contributed to the development of international biodosimetry through the Biodosimetry and Emergency Exposure Network (WHO REMPAN and IAEA-HICARE Cytogenetics Program) and the Genetic Effects Committee of the International Commission on Radiation Protection (ICRP).

Using a combination of recently developed WGS technology and our own high-quality methods, we have fostered collaborative research not only within RERF but also with external organizations, such as the Institute for Environmental Sciences (Aomori, Japan), the Central Research Institute of Electric Power Industry (Chiba, Japan), Hiroshima University, Osaka University, and Nagasaki University to investigate transgenerational effects in laboratory animals and somatic mutations. We are also working to improve AI-based mutation detection methods and collaborating with experts from Tokushima University, Osaka University, and other institutions.

The Trio Genome Study (RP 3-23) includes investigators from all departments of RERF, RIKEN, the University of Tokyo, and the U.S. National Cancer Institute (NCI). Hiroshima University and Nagasaki University provide further support for genetic counseling and clinical care for secondary genetic findings from WGS.

The clonal hematopoiesis study (RP 1-23-1) to investigate the relationships between radiation exposure, clonal hematopoiesis, and inflammatory diseases by exome sequencing is being conducted in collaboration with Nagasaki University, Kyoto University, and the University of Tokyo. The preliminary study (RP-P2-22) for single-nucleotide polymorphism (SNP) array analysis is being conducted in collaboration with the National Center for Global Health and Medicine (NCGM), Hiroshima University, and Nagasaki University. A preliminary study using stored pathological tissue samples to search for biomarkers of radiation exposure is being conducted in collaboration with Kyoto University (RPs in preparation).

Summary of Response to 2024 SAC Recommendations

Recommendation: Researchers for experimental science in RERF are also expected to contribute to the health and welfare of A-bomb survivors. They are always required to show how their research contributes to the benefit of A-bomb survivors and humanity as a whole. The current mission of the MBS is to provide the molecular and cellular evidence (mechanisms) that support the mechanisms of radiogenic diseases and precise risk estimation of cancer, non-cancer, and genetic effects in A-bomb survivors. Discussion and interaction with researchers in other RERF departments, in particular, the Departments of Epidemiology (Epi), Statistics (Stat), and Clinical Sciences (CS), can be of significant benefit to the MBS by helping to prioritize their research agenda(s) around human research issues. We believe that hypotheses that arise from studies in the Epi and CS can provide novel ideas for MBS research that focus on the mission of RERF and the unique resource of biosamples and data from A-bomb survivors, rather than on more general topics of radiation biology that might be suggested by studies from other institutions. Advice and criticism from the Epi, Stat, and CS are important and valuable. Mutual interaction, at least, periodic sharing of results among related departments, is highly recommended to organize what kind of research is undertaken and how the research is being pursued in each department.

Response: We agree that discussions and interactions with researchers in other departments are crucial, particularly for studies involving human populations. We have maintained frequent contact with other departments to discuss study designs and related issues, and we have established regular team meetings for the Trio Genome Study. Additionally, the MBS tests hypotheses derived from epidemiologic and clinical data using experimental mouse models and human cell lines.

Recommendation: The SAC was favorably impressed with the performance of the individual researchers in the MBS and the overall high quality of the work produced and presented. With the increasing availability of new genomic technologies, it is an auspicious time to refocus these new approaches on the important data and biosamples available from A-bomb survivors. Within the MBS, research proposals should first be evaluated based on whether the hypothesis can be tested using biosamples stored at RERF. If biosamples are limited or analytical methods applicable to human samples are lacking, mouse experiments may be useful. An example of a positive application of this approach is the identification of multi-site mutations in the offspring of irradiated parental mice in experiments carried out by the MBS. These findings facilitated the subsequent re-analysis of data of the offspring in the Chernobyl trio study. This approach of using mouse studies to augment and enhance human-focused research is encouraged. However, there is concern that some hypotheses worthy of evaluation may focus on mouse models because of ease of execution and experimental design

rather than relevance to humans. If a means of extension to human studies is not considered at the time these hypotheses are first considered, subsequent studies may fall short of the RERF mission. The SAC recommends that each research protocol include a clear rationale, feasibility, and plan for extension to human samples.

Response: We agree that the primary goal of animal studies is to inform research using samples from A-bomb survivors. In fact, we have developed research protocols that prioritize what samples are available and the research RERF should conduct. However, we believe that this effort can be further enriched by incorporating basic mechanistic studies. In the field of genome analysis, developing appropriate methodologies that align with RERF's objectives is essential. Additionally, it is particularly important for basic researchers to conduct cutting-edge studies using the latest methods. Such studies not only provide significant insights and directions for A-bomb survivor research but also serve as valuable research topics, fostering the training of the next generation of scientists. This is particularly crucial for early-career researchers, who must balance long-term projects involving A-bomb survivor biosamples with short-term projects that utilize more readily accessible samples.

Recommendation: The MBS presented a large number of projects with a range of goals and findings. Overall, the shift in the department's research portfolio toward genetic and genomic approaches using data and biosamples from A-bomb survivors was viewed by the SAC as a particular strength, and further efforts in this direction are encouraged. Specific strengths noted by the SAC were the Trio Study, the GWAS, and the studies of clonal hematopoiesis in A-bomb survivors. Some concerns were raised about the number of studies using mouse models without clear connections to human studies. The SAC recognizes that some studies cannot be easily performed in humans or are better tested in mouse models before precious human biosamples are devoting to them. However, in these cases, it would be helpful for the research protocol summary to include a specific section addressing the relevance of the proposed studies to humans and the plans to translating the work to human biosamples.

Response: Studies utilizing A-bomb survivor samples, which are a cornerstone of the MBS, have been well-established and prioritized. However, we believe it remains crucial to conduct studies aimed at elucidating the underlying mechanisms of radiation effects. Without allowing a degree of free scientific thinking, young researchers might not be interested in working at RERF, potentially leading to stagnation within the institute. Encouraging research projects funded through external grants or postdoctoral fellowships for short-term researchers is also essential, as we strive to develop a well-balanced and dynamic research department. Framing these projects in terms of their relevance to translational work in humans will be important.

FY2024 Departmental Highlights:

● **Molecular Genetics Laboratory**

Transgenerational Effects “Trio Genome Study” (Human Studies)

- We began obtaining consent forms for the Trio Genome Study from participants in January 2024. By the end of December 2024, consent had been obtained from 314 trios representing about 55% of the total target samples. In February 2024, we launched a pilot study to determine DNA quality and establish an analysis pipeline using samples from three trios for which consent had been obtained early. WGS with 60x coverage was performed using short-read next-generation sequencing (NGS). Data analysis using the supercomputer SHIROKANE proceeded smoothly, and *de novo* germline mutations were successfully identified. Data analysis for mutation detection was performed independently using both our RERF pipeline and a pipeline developed by research collaborators at the U.S. National Cancer Institute, with almost identical results. These promising results confirmed the technical feasibility of the Trio Genome Study.
- Given the particularly sensitive nature of research on biospecimens of A-bomb survivors and their offspring, ongoing communication with research participants, local communities, and other stakeholders is considered essential to the success of the Trio Genome Study. In FY2024, a series of information sessions and public lectures were held to inform research participants and the public at large about the Trio Genome Study. Many people who attended the information sessions and public lectures expressed their desire for this study to be carried out. These activities were also widely covered in newspapers and on television.
- The issue of returning secondary genetic findings has also been carefully considered, and we plan to return any information from the genome analysis that is considered beneficial to an individual’s health. To address this issue, we have established partnerships with Hiroshima University Hospital and Nagasaki University Hospital to prepare specific procedures for the return of genetic results. In December 2024, we invited several national and international experts, including the co-chair of the American College of Medical Genetics (ACMG) working group on secondary findings, to an international symposium to discuss the ELSI (Ethical, Legal, Social Issues) related to return of results for the Trio Genome Study. Based on the discussions that were had in the symposium, we are finalizing a list of genes to guide the return of findings. We plan to submit a report on this symposium to a journal in 2025. (Uchimura, *et al.* RP 3-23)

Transgenerational Effects (Animal Studies)

- The main objectives of the animal model experiments are (1) to create a high-precision pipeline for capturing genome (epigenome) DNA changes that occur at the whole-genome level, (2) to understand the frequency and characteristics of naturally occurring mutations, and (3) to clarify the characteristics of radiation-induced mutations. We have developed a new technology to detect large-scale chromosomal reorganization possibly

related to human diseases. Combining short-read and long-read sequencing technologies in exposed mouse families as a model experiment has allowed the capture of structural variants (SVs) with unprecedented precision, enabling the estimation of SV frequency in successive mouse generations and the effects of parental exposure. We are currently preparing the first paper in the world to clarify the frequency and characteristics of spontaneous *de novo* structural variants in the mouse germline (Satoh *et al.*, manuscript in preparation). In addition, through joint research with Osaka University, we used our high-precision mutation detection system to analyze mutations occurring in a single fetal neuron, and we elucidated that abnormal demethylation causes specific *de novo* mutations in neurons (Sugo, Uchimura *et al.*, manuscript submitted). (Satoh *et al.* RP 2-13, RP S3-11). Dr. Uchimura was awarded the 2024 Research Encouragement Prize by the Japanese Environmental Mutagen and Genome Society in recognition of a series of studies contributing to the understanding of *de novo* germline and somatic mutations. (The Research Encouragement Prize was established to grant recognition to young researchers who have accomplished outstanding research in the fields of environmental mutagenesis and genome science.)

● Cellular Genomics Laboratory

Radiation and Molecular Mechanisms of Disease (Animal Studies)

- For chromosomal aberrations, the effects of fetal exposure to A-bomb radiation have been found to be significantly lower than those of adult exposure. Fetuses could have an intrinsic mechanism for eliminating abnormal cells. In mice, we found that the frequency of translocations in long-term hematopoietic stem cells (LT-HSCs) in mice at 17 to 66 days of age, following *in utero* exposure to 2 Gy of X-rays, was 6.9% (2/29), which was lower than the frequency of 21% (9/43) observed in prenatal analysis one day after exposure, suggesting that chromosomal aberrations that occur in fetal LT-HSCs disappear as mice develop. (Hamasaki, *et al.* RP P4-17)
- To identify genetic and environmental factors that might modulate radiation-induced somatic mutagenesis, the potential effects of the oxidative stress response on mutations in LT-HSC were investigated using mutant mice with loss or constitutive activation of NRF2. In wild-type control mice, WGS analysis of clonal cell populations grown *ex vivo* from LT-HSCs isolated from bone marrow showed that SNVs and small indels were the most common types of somatic mutations and were increased up to 2- to 3-fold by whole-body 3.8-Gy X-irradiation at 8 weeks of age (Matsuda Y, *et al. Proc Natl Acad Sci USA*, 2023). The same experiments have been performed with mutant mice in which WGS analysis of somatic mutations is ongoing. (Tanabe, Matsuda, *et al.* RP P3-19)
- Epidemiological studies at RERF have identified age at exposure as an important modifier of radiation risk for oncogenesis. However, the molecular basis of age effects has not been elucidated. To address this, wild-type C57BL/6J mice were exposed to 3.8-Gy X-rays at 1 and 2 weeks of age (in infancy) to identify somatic mutations by WGS of LT-HSC-derived clones and to compare with the above results from mice exposed at 8 weeks of age (in adulthood). For the mice exposed at 2 weeks of age, the LT-HSC isolation and clonal expansion experiments were successfully completed, but most of

the mice exposed at 1 week of age died within 5 weeks prior to LT-HSC isolation. (Tanabe, Matsuda, *et al.* RP P3-19)

Radiation Biodosimetry

- Biological dosimetry using the frequencies of chromosomal aberrations (mainly stable translocations) in A-bomb survivors as markers of radiation exposure could support and improve individual physical dose calculations. The project, which began in 1966 and incorporated fluorescent in situ hybridization (FISH) technology in 1989, has resulted in measurements from a total of 1,868 A-bomb survivors (1,179 in Hiroshima and 689 in Nagasaki). In the most recent analysis using the dosimetry DS02R1, over 95% of the individual cytogenetic gamma-ray doses were found to be within the expected range of plus/minus approximately 1 Gy from the DS02R1 dose, and the mean slope for linear regression was 0.98, reassuring us of the validity of the DS02R1 study, and the results were published as a summary report (Kodama Y, *et al.*, *Int J Radiat Biol*, 2024). (Kodama Y, *et al.*, RP8-93; completed with paper publication)

● **Molecular Pathology Laboratory**

Platforms, Infrastructure, and Methods

- We are investigating the possibility of using archived autopsy samples stored at RERF to elucidate the pathological mechanisms of radiation carcinogenesis and to explore new biomarkers. As a first step towards this goal using mouse models, we conducted a preliminary analysis of mouse liver formalin-fixed, paraffin-embedded (FFPE) tissue specimens using liquid chromatography-mass spectrometry (LC-MS). We identified HNRNPA2/B1 and STT3B as potential quality markers for predicting the duration of formalin fixation (Kobayashi G, Ito R, Taga M *et al.* *J Proteomics*, 2024). The results demonstrated the feasibility of biomarker identification by integrating mass spectrometry with FFPE tissue analysis. (Ito, Taga, *et al.* no RP; paper published)

● **Immunology Laboratory**

Radiation and Molecular Mechanisms of Disease (Human Studies)

- To investigate the occurrence of clonal hematopoiesis (CH) in A-bomb survivors and its possible relevance to disease, a study of CH was initiated using stored blood samples obtained from AHS participants. Deep whole-exome sequencing (WES) and T-cell receptor (TCR) sequencing have been completed, and the data are currently being validated at RERF to identify CH somatic mutations and T-cell clonal expansion. A further study using stored blood samples collected over time is planned to clarify the dynamics of hematopoietic cell proliferation and expansion during the process of health recovery following the atomic bombing. (Yoshida K, *et al.* RP 1-23-1)

Radiation and Molecular Mechanisms of Disease (Animal Studies)

- In the mouse, radiation induces CH with high-frequency somatic mutations in blood cells, but the effects of radiation on the clonal expansion of hematopoietic progenitors

and lymphocytes have remained elusive. We examined CH mutations and TCR and B cell receptor (BCR) sequences in mice 18 months after 3-Gy irradiation and found that radiation preferentially induces clonal expansion of hematopoietic progenitors over mature lymphocytes in the bone marrow (Yoshida K, *et al. Stem Cell Reports*). (Yoshida K, Kusunoki, *et al.* RP 1-23-3)

Platforms, Infrastructure, and Methods

- In preparation for planning genome-wide association studies (GWAS) in A-bomb survivors to better understand individual susceptibility to radiation risks, this feasibility study evaluates the suitability of using DNA extracted from aged blood smears, Giemsa-stained chromosome preparations, and blood infiltrated paper discs of A-bomb survivors for SNP-array genotyping with 720,000 probes. DNA extracted from 20-year-old paper discs and amplified with REPLI-g was successfully genotyped using the SNP array. Using standard methods, the quality of DNA extracted from smears stored for over 10 years was too poor to produce acceptable genotyping data even after REPLI-g amplification. However, we developed a novel multicomination system to successfully amplify DNA from 10- to 50-year-old blood smears to produce acceptable call rates and precision in SNP array typing. DNA extracted from 30-year-old Giemsa-stained samples, on the other hand, could not be analyzed even after amplification using the multicomination system. (Hayashi, *et al.* RP P2-22)