Departmental Overview

The Department of Molecular Biosciences (MBS) supports the mission of RERF by conducting mechanistic studies on radiation effects uncovered by clinical and epidemiological studies on the cohort of A-bomb survivors and children of survivors in collaboration with the other RERF departments. The department's studies include those on 1) genetic effects; 2) radiation carcinogenesis; and 3) non-cancer diseases associated with radiation exposure. The Department consists of 4 (Molecular Genetics, Cytogenetics, Cell Biology, and Immunology) Laboratories. Currently the department is in a transition phase to a large extent toward implementation of the RERF strategic plan. This year, Dr. Tsuruyama, formerly a pathology professor at Kyoto University, joined our department as the chief of Cell Biology laboratory. He will be expected to engage pathology-based molecular imaging approaches using FFPE biospecimen from the A-bomb survivors. This area of research, based on molecular imaging and multiplex analytic approaches, is a major research initiative in RERF's strategic plan.

Because of concerns about heritable effects following exposure to radiation from the atomic bomb (based on results from animal model systems conducted in several laboratories world-wide) the relationship between radiation exposure and heritable genetic effects has been a major concern and was among the first series of studies to be initiated at ABCC, the predecessor to RERF. Studies were performed using many different approaches. These studies suggested no substantial heritable effects in the children of survivors. There are, however, limitations in the endpoints used and the statistical power of these studies and research on such effects continues today. As a result a major effort in MBS is to conduct a WGS study in trios comprised of mothers, fathers and children. In these trios one of the other of the parents (in some cases both) were exposed to radiation from the atomic bombings. These studies are being supplemented with studies using model systems to examine mechanisms. All of these studies are being linked and integrated with studies of children of survivors in epidemiological and clinical cohorts, as an F_1 umbrella multidisciplinary program project.

Studies of processes involved in radiation carcinogenesis and immunological effects were also begun relatively soon after the formation of ABCC and such studies continue as we move forward with our strategic plans. These studies were originally housed in the former Department of Radiobiology/Molecular Epidemiology and are now being conducted primarily in the Cell Biology Laboratory and the Immunology Laboratory.

MBS is currently developing new programs examining processes involved in radiation carcinogenesis designed to interrogate radiation effects at the molecular, cellular, and tissue levels using state-of-the-art imaging, genomic, proteomic, and immunological approaches using tissue based biosamples such as FFPE samples from survivors and their children. Our new team led by Dr. Tsuruyama will tackle these cutting-edge studies. In addition, model systems will be used to provide additional insight into these effects and to critically test hypotheses that emerge from the biosample studies. These studies will be integrated with data derived from RERF cohort studies as part of an institutional wide molecular epidemiology approach.

Other studies in molecular epidemiology involve institutional wide collaborative efforts examining genetic susceptibility and gene-environment interactions and studies of potential biomarkers. Our immunology and immunogenetics laboratories will be involved in these

tissue-based studies as well as continuing current efforts examining mechanisms of non-cancer associated disease with an emphasis on cardiovascular disease.

These areas of emphasis rely on collaborative studies within RERF and with outside experts to perform integrated analysis using genomic, transcriptomic, metabolomic, proteomic and immunologic approaches. These data will be linked with epidemiological and clinical data that will provide new insights into radiation related disease processes that are not currently possible at other institutions.

FY2021 Molecular Biosciences Achievements

Radiation and Genetics Effects

The most important research initiative in the MBS genetics program and for RERF in general is the whole genome sequencing (WGS) study focusing on human trios consisting of atomic bomb survivors and their offspring. This is a major part of the institutional-wide F1 umbrella program. In FY2021, we have completed statistical calculations regarding the power estimates of detection of radiation induced mutations in F1s. We had meetings with external collaborators to prepare a new RP and submitted it to the Genetics Research Cluster. Now, the RP is under review. In addition, we have prepared a human genome data analysis system consisting of an external cloud server and an on-premises server. One of the most important issues of the human WGS study is getting social agreement and treating with ethical problems. In this regard, last year (Dec. 2020), we held an international workshop entitled "ELSI workshop toward RERF future genome studies on atomic bomb survivors and their children". In this year, we published the summaries of the discussions and their advice (Noda et al, 2021). Also, we have initiated a stakeholder meeting and discussed the aim of the study and related ethical issues. By the proceedings with the ethical examination of the research and the detailed examination of the scientific research plan in parallel, we plan to carry out appropriate research at an early stage. Uchimura, Satoh, Noda (MB) and Sposto (S) A part of CR162, PI; Uchimura.

- Feasibility study of the Trio-WGS using human cells. The purpose of this study is to prepare the pipelines which are required for the human trio WGS study. To date, we have established a WGS data analysis pipeline for detecting base substitutions and small size indels (~ less than 30bp). In FY2021, we have tested the feasibility of additional whole-genome analysis pipelines; (i) for returning the genome data to individual participants for clinical benefits and (ii) for data analysis using nanopore WGS data to detect structural variants (SVs) and to assess the epigenetic status. Methods: In this study, we used lymphoblastoid cell lines derived from Japanese male (total 4 clones: 2 pairs of radiation-exposed [2-Gy of X-ray] clones and unirradiated controls). Results: (i) As a practice for that, we added clinical annotation data to the genetic polymorphisms identified in the WGS data of GM18943 cell clone. We found 11 variants as 'pathogenic' or 'likely pathogenic' by ClinVar database. The genome data returning policy will be planned in collaboration with experts in genomic medicine at Hiroshima University and Nagasaki University. The criteria for the mutations returned will be determined in consultation with the collaborators.
  (ii) In FY2021, we used WGS data of Nanopore for three types of analysis. First, we used the data to detect de novo SVs. We detected several de novo SVs including three inversions and two translocation in 4 cell clones (2 irradiated and 2 control). Inversions and translocation were observed only in the irradiated clones. Second, we used the Nanopore data in whole-genome assembly to search for structural variations including outside the

reference sequence. We assembled entire genome using Flye software; N50 length of the assembled contig was 15 Mb and the largest contig was 97 Mb. Third, we used the same Nanopore data to assess the methylation status at CpG sites. We compared the results from Illumina methylation-chip and the Nanopore whole methylome-WGS. It showed a very high correlation (r2 = 0.95). Conclusion: We have prepared the pipeline for returning the genome data to the participants. In addition, we have prepared several pipelines for analysis of Nanopore WGS data. In particular, it was clarified that by using nanopore sequencing, changes in DNA sequences and CpG methylation states can be analyzed at the same time with high accuracy. (Uchimura, Satoh), PI; Satoh

- Characterization of radiation-induced mutations in F1 mice born to exposed spermatogonia or mature oocytes. Purpose: In 2020, we reported de novo germline mutations (mainly base substitution and small size deletions) in the mouse offspring born to irradiated parents (Satoh et al, 2020). In FY 2021, we have investigated de novo SVs. As a first step, we worked on elucidating the incident rate and characteristics of spontaneous de novo SVs in mouse germline. Methods: We conducted WGS of long-read NGS (Pacbio and Nanopore) and short-read NGS (illumina), and optical mapping (Bionano). We analyzed four lines of the mutation accumulation (MA) mouse lines (passing more than 50 generations during 15 years of successive inbreeding). Results: We developed original pipelines to detect SVs and identified total 68 validated SVs, including 36 retrotransposon insertions (LINE, LTR, SINE, etc.), five other insertions, 26 deletions and one complex variant (combination of 200 kb segmental duplication and its partial inversion). This indicate that 1.22 SVs (including 0.48 LINE transpositions) occur per generation in mice. In addition, to characterize the retro-transposon insertions, we developed a new method to identify the donor sites of the transpositions by noting sequence similarity and identified the donor sites. Conclusion: By using long-read sequencing methods, we have succeeded in estimating per generation de novo SV rate for the first time in the world. Our estimated rate (1.22 SVs and 0.48 LINE transpositions in C57BL strain) was much higher than the previous estimates of 0.16 SVs in human and 0.13 LINE transpositions in mice. Our new knowledge about mammalian de novo germline mutations should be helpful for the future human trio study at RERF. (Uchimura, Satoh). PI; Satoh. Satoh, 2020a, 2020b, and 2021. A summary manuscript is in preparation.

- Development of a method to reconstruct phylogenetic trees of ontogeny using mosaic mutations. The purpose of this study is to develop a method to reconstruct phylogenetic trees of cell lineages during mouse embryogenesis using mosaic mutations. Purpose: The purpose of this study is to develop a method to reconstruct phylogenetic trees of cell lineages during mouse embryogenesis using mosaic mutations. This method is useful in many areas of life science, including radiation biology. For instance, this method is planned to be used in “Clonal hematopoiesis project (PI, Yoshida)”. Methods: We detect de novo mutations existing in the mosaic state in a somatic tissue with deep coverage whole genome sequencing. Then, we accurately measure their variant allele frequencies (VAFs) in several tissue samples. Finally, we use a mathematical model and the VAF data to reconstruct phylogenetic tree during early embryogenesis. Results: We succeeded to establish a novel method to reconstruct phylogenetic trees (patent has been submitted in 2020). We have also conducted validation experiment and several additional experiments required for completion of a full paper. In 2021, we submitted the manuscript, entitled “Early embryonic mutations reveal dynamics of somatic and germ cell lineages in mice"; the paper is now in revision status. The summary of the paper as below: De novo mutations

accumulate with cell divisions from zygote. However, how these mutations occur and are inherited by somatic cells or germ cells are not fully understood. Here, we present a novel method to reconstruct the cell lineages. We identified mosaic mutations in mouse tissues by deep whole-genome sequencing and reconstructed embryonic cell lineages based on the variant allele frequencies of the mutations. The reconstructed trees were confirmed by nuclear transfer experiments and genotyping of ~50 offspring of each tree. The most detailed tree, which had 32 terminal nodes, showed cell divisions from the fertilized egg to germ and somatic cell-specific lineages, indicating at least five independent cell lineages that would be selected as founders of the primordial germ cells. Contributions of each lineage to germ cells and offspring varied widely. At the emergence of the germ cell-specific lineages, 10–15 embryonic mutations accumulated, suggesting that the pre-gastrulation mutation rate would be 1.0 mutations per mitosis. Subsequent mutation rates (per cell division) were estimated to be 0.7 for germ cells and 13.2 for tail fibroblasts. Our results demonstrate a new framework to assess embryonic lineages; further, we suggest an evolutionary strategy for preserving heterogeneity due to postzygotic mutations in offspring. (Uchimura, new RP)

 Radiation-induced mutations in mouse spermatogonia cells in culture. To mechanistically understand how radiation exposure induces mutations in spermatogonia stem cells and how their mutations transmit to the next generation, we have initiated an in vitro culture approach to examine mouse spermatogonia cells (hereinafter GS, germline stem cells). The cultured GS cells were X-ray irradiated and surviving cell colonies were recovered. Structural changes of the genome were analyzed by aCGH (Macrogen/Agilent standard methods) for each 5 clones of control (unirradiated), 2-Gy-irradiated and 4-Gy-irradiated GS cells. For the entire genomic sequencing, short-read WGS was conducted in control and X-irradiated GS cell clones to detect radiation-induced SNVs and small InDels and multi-site mutations. aCGH analysis revealed only one deletion in irradiated clones which was supposed to be mediated by NHEJ. In the WGS, 4-Gy exposed clones showed apparent 2.5 and 4 hold increase of multi-site and deletion mutations, while SNVs and insertions showed only slight increase. Interestingly, while these InDels detected in the unexposed controls largely derived from repeat sequence, radiation-associated changes were largely occurred in the unique sequence, indicating the role of NHEJ in the radiation-associated mutagenesis in GS cells. These results are summarized for publication. Since large scale structural changes were scarcely detected in irradiated GS clones, we have initiated to introduce such changes by using gene editing technology. We plan transplantation of these GS cells into male mice testes to examine the transmissibility of the individual mutations next year (Noda, Hamasaki, Satoh and Uchimura). PI: Noda, partially supported by MEXT grant No. 20K12179

Radiation Biodosimetry

 In order to evaluate the dose dependent effects of A-bomb radiation on humans, a cytogenetic biological dosimetry study was conducted for a subset of A-bomb survivors in the AHS cohort. A total of 1,868 survivors (1,179 in Hiroshima and 689 in Nagasaki) were examined using the 2-color-FISH method for detecting the frequency of stable translocations involving chromosomes 1, 2, and 4. The dose response from FISH data showed a wide scattering of individual translocation frequencies in both cities as we observed in the previous solid Giemsa staining study. The difference between the two cities remained significant but much reduced, suggesting the large city difference in the past study was mainly due to different aberration detection rates between Hiroshima and Nagasaki laboratories. The city difference was not significant when Nagasaki factory workers were

excluded from the analysis. The results suggest a shielding-related bias in physical dose estimates in some survivors. This FISH study also reconfirmed that our previous Giemsa staining had successfully detected about 70% of translocations. A manuscript will be submitted in 2022 (Kodama, Hamasaki, Cordova, Cullings). PI; Kodama.

Radiation and Cancer

- Epidemiological studies have demonstrated that radiation exposure has led to an excess relative risk of hepatocellular carcinoma (HCC) among A-bomb survivors. Certain mouse strains are also susceptible to radiation-induced HCC. In 2021, a new isolation method of hepatic stellate cells from 1-week-old mice was established to evaluate the direct effects of irradiation on expression of inflammatory cytokines and senescence-related molecules in the cells using in vitro irradiation experiments. As a result, real-time PCR and ELISA analyses of isolated stellate cells indicated the elevated levels of inflammation marker, CCL5, in a dose-dependent manner after irradiation. We now plan to prepare a new RP to investigate the morphological changes of inflammatory hepatic stellate cells and macrophages in liver steatosis and fibrosis using mouse model systems. (Taga, a manuscript in preparation).

- Preliminary studies of chromosome aberration frequencies in hematopoietic stem cells (HSCs) following fetal irradiation of mice. This project is to test the hypothesis that radiation effects on the induction of persistent chromosome translocations may change in the stages of fetal development, namely, whether or not the stem cells have already settled into their niche. As a first step, we examined whether reciprocal translocations are induced in fetal HSCs soon after irradiation. Pregnant mice (E13- 15d) were exposed to 2Gy of X rays and sacrificed 1 day later to collect fetal livers. The isolated HSCs were aliquoted into the wells of a 96-well plate (1 cell/well) to obtain single-cell derived colonies. Proliferated colonies were collected, and the cells were prepared for karyotype analyses using mFISH to determine translocation frequencies. mFISH results showed that 9 out of 43 (21%) fetal clones contained translocations. Our previous studies have shown that translocations in hematopoietic cells are rarely observed in adult after in utero exposure, but this result showed that translocations are observed in fetal hematopoietic stem cells soon after exposure. However, their frequency tended to be lower than that of mothers (37%). In addition, it is expected that abnormal cells bearing translocations start to be cleared at once, resulting in a state in which almost no cells with translocations are observed in the adult, except for cases of clonal expansion with abnormality. A summary report based on the current data is in preparation for publication (Hamasaki). PI; Hamasaki, a manuscript in preparation.

- Preliminary study to determine the applicability of Wright-stained blood smears in GWAS. A preliminary study to determine whether amplifying whole genomes using very small amounts of DNAs obtained from Wright-stained smears would make it possible to perform GWAS studies using the Toshiba Japonica SNP arrays. Genome analysis is considered important from the viewpoint of elucidating and evaluating genetic susceptibility to radiation-induced cancer. RERF holds several kinds of biological samples from the AHS cohort, including blood smears, which have been preserved since 1958. A potential large-scale genome study for all AHS subjects, consisting of approximately 21,000 A-bomb survivors, using old samples preserved after 1958, may enable us to examine genetic and gene-environmental interaction related to radiation exposure susceptibility. In order to conduct the genome analysis, it is necessary to determine whether SNP analysis that uses DNA samples extracted from smear samples is feasible. For initial studies using a pool of RERF-employee volunteers, six subjects were randomly selected. DNAs were extracted

from whole blood samples (W-DNAs). Wright-stained smears were prepared from whole blood samples. Then the DNAs extracted from Wright-stained smears were amplified using the QIAGEN REPLI-g DNA amplification kit (amplified-DNAs). W-DNAs and amplified-DNAs were genotyped using SNP arrays (Toshiba Japonica array). The standard protocol of Thermo Fisher Scientific (Best Practices Workflow) recommends the use of SNPs that fall into the Poly High Resolution, Mono High Resolution, and No Minor Homozygous categories of the six conversion types classified by SNP QC. The number of recommended SNPs was 622,822 (92.8%) out of 671,119, and we analyzed the call rates and concordances. As a result of the analysis using the recommended method, the average call rates were very high, 99.84 and 98.01% for W-DNA and amplified DNA samples, respectively and the average of genotype concordances in W-DNA and amplified DNA samples was 95.51%. These results suggest that a whole-genome amplified DNA prepared from the stained smears represents a similar copy of the genomic DNA template and there are comparable call rates when used in high-throughput SNP genotyping assays, making it possible to use the stained smears for GWAS. (Hayashi, Yoshida K, Ohishi, Yoshida N, Kato, Sposto, Tokunaga, Ueki, and Ozasa). PI: Hayashi.

- Preliminary study (RP-P) on possible roles of oxidative stress response in protection against radiation-induced mutagenesis and oncogenesis. The aim of this study is to identify roles of an oxidative stress response pathway controlled by a transcription factor NRF2 in protection against radiation-induced mutagenesis using mouse models. Identification of factors that can protect against radiation mutagenesis could lead to the elucidation of molecular mechanisms of the radiation oncogenesis and the inter-individual variability in radiation risks and could lead to the development of protective measures to reduce the risks. In case of - and X-irradiation, the primary mechanism of the radiation oncogenesis is believed to be the somatic mutation caused by the DNA damage induced by reactive oxygen species (ROS) derived from ionized water. Reportedly, activation of NRF2, the master transcriptional activator of antioxidant genes, significantly reduces the acute radiation toxicity. We examine the mutagenic effects of X-irradiation on wild-type control mice and two mutant mouse lines, one with the NRF2 null mutation and the other with diminished expression of a Keap1 protein, an inhibitor of NRF2, exhibiting constitutive NRF2 activation, and thereby determine whether or not modulation of NRF2 activity in mouse can affect the mutagenic effects of X-irradiation. The two mutant mouse lines and wild-type mice are exposed to whole-body X-irradiation, and whole-genome sequencing (WGS) will be conducted with DNA samples extracted from clonal cell populations expanded in vitro from isolated hematopoietic stem cells (HSCs). From these analyses, the mutagenic effects of X-irradiation are determined, and the effects of NRF2 loss or activation will be evaluated. From these analyses, we should be able to test whether or not the oxidative stress response pathway controlled by NRF2 play any role in protection against radiation mutagenesis. We have established experimental methods to isolate HSCs from mice with or without prior whole-body X-irradiation, to culture single HSCs for in vitro clonal expansion, to extract high- quality DNA from HSC-derived clones, and then to conduct WGS with the extracted DNA. We have also established computational methods to identify, characterize, and quantify somatic mutations such as single nucleotide variants, insertions, deletions, and structural mutations based on WGS data from HSC-derived clones and matched tails as bulk reference samples for germline variants. So far, we have successfully obtained preliminary results of frequencies and characteristics of various somatic mutations from WGS data of wild-type mice with or without prior whole-body X-irradiation. This study will provide a model system for future studies to characterize radiation-induced somatic mutations in human by WGS analysis with preserved biosamples donated by A-bomb survivors. (Tanabe

O, Matsuda Y, Kajimura J, Yoshida N, Sposto R, Kato N). PIs: Tanabe and Matsuda. Partially supported by MEXT grant No. 19K12338.

Radiation and Immunologic Effects

- Relationship between intracellular reactive oxygen species (ROS) in blood cells and inflammatory biomarkers among atomic-bomb survivors. This study is to investigate the relationship between intracellular ROS (H2O2 and O2•-) levels in blood cells or T-cell subsets and serum C-reactive protein (CRP) levels, as well as how these variables are affected by age and radiation exposure in A-bomb survivors. Intracellular ROS and CRP levels were measured among 2,495 healthy Hiroshima A-bomb survivors. As a result, after adjusting for the effects of gender, age at examination, smoking and drinking habits, body mass index, and blood sampling time, intracellular O2•- levels in monocytes, granulocytes, and T cells tended to be higher in survivors exposed to higher radiation doses. In addition, the intracellular O2•- levels in blood cells were higher in survivors exposed to higher radiation doses, and this was most prominently observed in the group with the highest CRP levels split into three groups according to blood CRP level. The results suggest that increased intracellular ROS levels might be associated with inflammatory conditions such as increased CRP levels, especially after radiation exposure. In this study, we observed a tendency toward higher levels of oxidative stress — a situation marked by decreased immune function and increased inflammation — in the blood cells of older A-bomb survivors who had been exposed to higher doses of radiation. Publications; Hayashi et al. Free Radical Biol Med, 171:126-34, 2021. (Hayashi, Furukawa, Kato, Yoshida, Kusunoki, Kyoizumi, and Ohishi, terminated in 2018.). PI: Hayashi.

Radiation and Other Noncancer Conditions

- Clonal hematopoiesis (CH), potentially associated with radiation exposure and increased risks of inflammatory diseases, has not been evaluated in animal model studies. To develop strategies for assessments of CH linking to radiation-associated noncancer diseases, specifically arteriosclerosis as a part of CH program project, we conducted preliminary experiments to establish one or more mouse models that can test the hypothesis that CH in irradiated mice is involved in pro-inflammatory phenotypes and can promote atherosclerosis formation. Preliminary mouse experiments using deep whole-exome sequencing (WES) and targeted amplicon sequencing ensured an extremely high prevalence of CH in 3-Gy whole-body irradiated mice, i.e., 16 months after irradiation recurrent somatic mutations with variant frequency exceeding 2% (a definition of CH in humans) were observed in 11 of 12 irradiated mice but in none of 6 controls. The mutations appeared almost specifically in hematopoietic tissues (bone marrow and spleen) and hematopoietic stem/progenitor single cell-derived colonies but not in non-hematopoietic organ (tail, brain, testis etc.) cells. Moreover, CH in each of the irradiated mice contained multiple clones that expanded to collectively comprise 60-80% of a whole population of bone marrow nuclear cells, suggesting that high-dose radiation can induce massive hematopoietic cell generation and proliferation from a tiny number of stem/progenitor cells, which is somewhat concordant with our previous observation of clonal chromosome aberrations in blood cells among heavily exposed A-bomb survivors. The blood of irradiated mice exhibited elevated levels of both pro-inflammatory myeloid cells and red blood cell distribution width (RDW), which is often observed in human populations having CH. These results validated our experimental system involving WES-based CH detection and CH-related blood cell profiling for assessment of radiation-induced CH and associated proinflammatory phenotypes in mice as well as in humans. (Yoshida, Kusunoki et al., CR155). PI; Yoshida, a manuscript in preparation.