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. The departments' studies include those on 1) genetic effects; 2) radiation carcinogenesis; and 3) non-cancer diseases associated with radiation exposure. The department was formed by merging the Departments of Genetics and Radiobiology/Molecular Epidemiology in 2015. 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.

Because of concerns about heritable effects following exposure to radiation from the atomic bomb (based on results in animal model systems conducted in several laboratories world-wide) the relationship between radiation exposure and heritable genetic effects was 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 measurable 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.

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 which involves all of the departments at RERF.

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. 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 approaches 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.

1. FY2020 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 FY2020, statistical calculations regarding the power estimates of detection of radiation induced SNVs, small InDels, and multi-site mutations in F1s were conducted in collaboration with Department of Statistics. These were based on the results from our previous animal experiments and the data published by others. The statistical results provided the numbers of the trios needed for this project. In addition, we constructed two security-controlled computer rooms for the human genome analysis studies at RERF. One of the most important issues of the human WGS study is related to ELSI. In this regard, as suggested by SAC last year, we have held an international workshop entitled "ELSI workshop toward RERF future genome studies on atomic bomb survivors and their children" in Dec. 10-11 via Zoon conference. Nine genome ethicists from Japan and US met to discuss genome research ethics at RERF. Summaries of the discussions and their advice will be published in an appropriate journal in 2021. Uchimura, Satoh, Noda (MB) and Sposto (S), 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 an entire WGS analysis pipeline for detecting base substitutions and small size indels (~ less than 30bp). In FY2020, we have tested the feasibility of additional whole genome analysis pipelines; (i) for detecting more complex mutations using latest software for short-read NGS data and using long-read NGS (Nanopore), (ii) for assessing epigenetic effects using methylation chip and the data of long-read NGS and (iii) for scanning entire genome using gene-chip. In this study, we used human lymphoblastoid cell lines derived from Japanese individual (total 4 clones: 2 pairs of radiation-exposed [2-Gy of X-ray] clone and unirradiated control). (i) We installed several software packages, such as SvABA and Delly, in house cloud server and called candidate variants. The candidates were validated by using the WGS data obtained from long-read NGS (Nanopore). So far, all of the candidates provided by SvABA have been finished to be validated and 4~8 de novo structural variants (including 10 Mb deletion) were found per cell clone. For long-read NGS analysis using Nanopore, we prepared DNA samples from the lymphocyte cells and obtained 70~90 GB sequence data (equivalent to 23-30× coverage) per a sample. N50 length of a single read was ~22 Kb. We identified several structural variants, some of which had not been detected by short-read NGS data, with Sniffles software from the read data of Nanopore. (ii) We analyzed the methylation status in 4 cell clones (2 irradiated, 2 control) by using Illumina 800K methylation array. We could not find any apparent features specific to irradiated clones but found a relationship along the cell lineage. Now, we are setting up the pipeline to assess the methylation status by using the WGS data of Nanopore. After this, we will compare the results and verify the data. (iii) We tested the feasibility of Illumina 600K SNP array. Out of 666K SNP sites,

~96K sites showed heterozygotes in this sample. In addition, we could identify 2 structural variants (5 Mb deletion on chromosome 3 and duplication of the long arm of chromosome 16) in the two irradiated cell clones, respectively. We have successfully prepared some additional analysis pipelines needed for the human trio WGS study. Due to budgetary constraint etc., the methods used in the trio study have not yet been decided, but by using the pipelines prepared here, it will be easier to proceed with the analysis in
cooperation with the outside collaborators. (Uchimura, Satoh), PI; Satoh

- Characterization of radiation-induced mutations in F1 mice born to exposed spermatogonia or mature oocytes. To understand the effects of parental exposure on offspring, it is important to analyze the more complex types of mutations. Until FY2019, we had investigated de novo germline mutations (mainly base substitution and small size deletions) in the offspring born to irradiated parents. The results were reported as a paper (Satoh et al, 2020). The purpose of our new study is to uncover the incidence rate of the de novo mutations including complex structural variants in mouse germline and to establish a methodology for that purpose. For detection of the de novo mutations, we used the following methodologies: short-read NGS (illumina), long-read NGS (Pacbio and Nanopore) and optical mapping methods (Bionano Saphyr). To characterize de novo spontaneous germline mutations efficiently, by using these methods, we analyzed the mutation accumulation (MA) mouse lines, which has been established by 14 years of breeding (passing more than 40 generations). We obtained sufficient WGS data both qualitatively and quantitatively in all sequencing platforms and got several new findings about de novo germline mutations. For instance, it has become possible to accurately detect mutations on short tandem repeat regions, and we found that the spontaneous incidence rate of indels is 40 times higher than previously reported our estimate (Uchimura et al. 2015). In addition, we identified 11 de novo retrotransposon mutations (mainly ~7 Kb LINE insertions) and at least 41 large size structural variants including a very complex mutation consisting of a combination of a segmental duplication (200 kb region) and its partial inversion. This incidence frequency was much higher than the previous estimates based on the conventional methods. Using state-of-the-art technologies, we have successfully developed several pipelines to detect various types of mutations. At the same time, the characteristics of spontaneous mutations in mice have been becoming clear. Our results are important for the future human trio WGS study. (Uchimura, Satoh). PI; Satoh. Satoh, 2020a, 2020b, and in press.

- Development of a method to reconstruct phylogenetic trees 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. 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)”. We detect de novo mutations existing in 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. 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. The manuscript, entitled “Early embryonic mutations reveal in vivo dynamics of somatic and germ cells", will be submitted soon. The summary of the paper as below: Postzygotic mutations lead to genetic disease syndromes and

predisposition to cancer, and lead to genetic diseases in the offspring. However, it has been difficult to know in what order these mutations occurred, our knowledge about how they occur and how they are inherited to somatic cells and germ cells remains limited. Here, we detected mosaic mutations existing in a bulk tissue sample with deep whole genome sequencing and reconstructed an embryonic mutation accumulation history by using arithmetical relationship between the variant allele frequencies of each mosaic mutation. The reconstructed phylogenic trees have at most 32 branches, demonstrating the process from a fertilized egg to the germ cell specific lineages or the somatic cell specific lineages. The reconstructed trees showed that the two daughter cells of a postzygotic cell division would asymmetrically contribute to adult tissues and to the offspring. In addition, the tree provided an estimate of mutation rate, ~1.0 mutations per postzygotic cell division. The results show that our new method would make it possible to trace overall embryonic lineages easily in a whole-body scale. (Uchimura)

- 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). This year in vitro GS cell mutagenesis experiments were conducted for in vivo transplantation studies in next year. 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. 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 investigate the 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. Following the completion of data collection, we have recently completed an analysis of the FISH data compared to DS02R1-estimated bone marrow doses, and now we are currently drafting a manuscript presenting the results. An overview of some of the results are as follows: 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. Difference between two cities was 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. When we took people who were exposed in Japanese houses as a reference, the people in other shielding categories including Nagasaki factory had significantly lower dose responses. 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. (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 2020, an experimental protocol was established to evaluate expression of inflammatory cytokines and senescence-related molecules in hepatic stellate cells and macrophages isolated from X-irradiated mice. Preliminary results obtained using this protocol showed that hepatic stellate cells in mice 3 months after irradiation tended to express higher levels of Ifnb1 and Ccl5 genes but lower levels of the Lmnb1 gene than those in non-irradiated controls. The results suggest a possibility of radiation-associated activation of the cGAS-STING pathway, which may be ascribed to reduced nuclear integrity through Lmnb1 insufficiency. This may enhance production of inflammatory cytokines through a type I IFN response, in hepatic stellate cells.

- Preliminary studies of chromosome aberration frequencies in hematopoietic stem cells (HSCs) following fetal irradiation of mice. To test the hypothesis that radiation effects on the induction of persistent chromosome translocations may depend on the stage 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 liver. The isolated HSCs were distributed into 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. We had already obtained the mFISH data that the translocation frequencies were 21% (9 out of 43 clones) and 51% (21 out of 41 clones) in single presumable LT-HSCs from both the fetuses and the mothers, respectively. However, there was a problem whether sorted cells are true LT-HSCs or not. This year we tried to confirm the reliability of these results. Our LT-HSCs culture method were partly improved by introducing an epoch-making method that HSCs are maintained in primitive state *in vitro* for a month (Wilkinson AC, 2019, Nature 571:117). Specifically, presumed LT-HSCs were isolated by FACS sorting, and then they were further cultured with this newly method. A total additional 24 clones were obtained. Among these, translocations were found in 2 clones out of 7 derived from irradiated fetuses and in 5 out of 17 clones from irradiated mothers. Although the number of clones examined is small, clones with translocations were observed in both fetal and adult HSCs immediately following 2 Gy of X-irradiation by using the improved culture method. Therefore, original data were

probably expected to reflect the result of LT-HSCs clones.
(Hamasaki). PI; Hamasaki.

- 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 theToshiba 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 20,000 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 for a pool of RERF-employee volunteers, six subjects were randomly selected. DNAs (W-DNAs) were extracted from whole blood samples. Wright-stained smears were prepared from whole blood samples. 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). Result demonstrated that the amplified-DNA obtained from the smears was suitable for SNP analysis, with a high call rate of 97.0% for the amplified-DNA and a high concordance rate of 93.70% between the W-DNA and amplified-DNA. 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. To identify roles of an oxidative stress response pathway controlled by a transcription factor NRF2 in protection against radiation-induced mutagenesis and oncogenesis using mouse models. Identification of factors that can protect against radiation oncogenesis could lead to the elucidation of molecular mechanisms of the oncogenesis and the inter-individual variability in the radiation risk and could further lead to the development of protective measures to reduce the risk. In case of gamma- 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 will examine the mutagenic and oncogenic 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 or oncogenic effects of X-irradiation. For analysis of mutagenic effects, the two mutant mouse lines and wild-

type mice will be 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 (HSC). From these analyses, the mutagenic effects of X-irradiation will be determined, and the effects of NRF2 loss or activation will be evaluated. Then, for analysis of the oncogenic effects, the same mutant and wild-type mice on the C57BL/6J strain background, in which thymic lymphomas will frequently develop after radiation exposure, will be exposed to X-ray and then monitored for lymphoma development. 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 or oncogenesis. Initially, using wild-type mice, experimental protocols were established for HSC isolation from bone marrow after whole-body X-irradiation, *in vitro* colony formation, and extraction of DNA from the colonies. From each HSC-derived colony, we could extract an enough amount of DNA to conduct WGS analysis. We have obtained preliminary sequencing results of HSC-derived colonies from wild-type mice with or without 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 of cloned or single cells derived from biosamples donated by A-bomb survivors. (Tanabe and Matsuda).
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Radiation and Immunologic Effects

- Relationship between intracellular reactive oxygen species (ROS) in blood cells and inflammatory biomarkers among atomic-bomb survivors. To investigate the relationship between intracellular ROS (H_2O_2 and $O_2^{\bullet-}$) levels in immune cells including T cell subsets and serum iron, ferritin, and C-reactive protein (CRP) levels, and to determine how these variables are affected by age and radiation exposure in A-bomb survivors. Intracellular ROS ($O_2^{\bullet-}$) levels in immune cells and T-cell subsets among 2,495 Hiroshima healthy A-bomb survivors were measured using a combination of fluorescence-labeled antibodies and a fluorescent reagent, hydroethidine. *Results and* The results indicated that intracellular $O_2^{\bullet-}$ levels in immune cells and certain $CD8^+$ T cells including effector memory and terminally differentiated effector memory $CD8^+$ T cells, increased with radiation dose. As observed in our previous studies, serum CRP levels increased significantly with increasing age and radiation dose. When divided into three groups according to serum CRP levels, dose-dependent increase of the intracellular $O_2^{\bullet-}$ levels of immune cells and certain $CD8^+$ T cell subsets were most prominently observed in the high CRP group. These results suggest that an increase in intracellular $O_2^{\bullet-}$ levels, particularly after radiation exposure, may link to enhance inflammatory status, including elevated serum CRP levels, and reduced serum iron levels. This study reveals that aging and radiation exposure increase oxidative stress in blood cells that are involved in impairments of immune function and acceleration of pre-clinically persistent inflammation in radiation-exposed individuals (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, we conducted researches 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. Mouse models to be developed in this research may be useful for assessing the effects of a variety of environmental factors on somatic mutagenesis and CH development. Preliminary experiments conducted in FY2020 indicated that, deep whole-exome sequencing (WES) fully detected somatic mutations with variant frequency exceeding 2% (a definition of CH in humans) in mouse bone marrow cells, and that the prevalence of CH was higher in 3-Gy whole-body irradiated mice than controls when examined 18 months after irradiation. Both pro-inflammatory monocytes and red blood cell distribution width (RDW) increased in the blood of irradiated mice, which is consistent with our recent findings in the AHS. Because these blood cell phenotypes are often observed in human populations exhibiting CH, these results validated the feasibilities of using WES-based CH detection and determining CH-related blood cell phenotypes for evaluating radiation-induced CH in mice as well as in humans. We have also planned clonality assessment of monocytes accumulating in atherosclerotic plaques that are formed in LDLR-KO mice irradiated and fed with a high-fat diet, which would be able to test the hypothesis that CH following radiation exposure promotes atherosclerosis through clonal accumulation of pro-inflammatory monocytes into atherosclerotic lesions. Publications; Yoshida et al, *British Journal of Haematology* (2021), in press.

- To test the possible involvement of radiation exposure in circulatory diseases (CD), experiments were conducted using the spontaneous hypertensive rat (SHR), stroke prone–SHR rat (SHRSP) and Wistar Kyoto rat (WKY) irradiated with moderate/low dose and low dose rate gamma-ray. The results indicated that systolic blood pressure of SHR and the number of SHR having cystic degeneration in liver increased with increasing dose, but they were not in WKY. By contrast, retardation of body weight gain with increasing dose was observed in both strains. These results suggest that the SHR and WKY animal models may be useful for studying radiation effects on non-cancer diseases including circulatory diseases, chronic liver disease and developmental retardation. The onset time of stroke in SHRSP was used as an endpoint to evaluate the radiation effects of low dose and low dose-rate exposures. With respect to acute exposure, the results showed the presence of a threshold in the radiation dose around 0.1Gy. For low dose-rate exposure, chronically irradiated SHRSP did not show any significant radiation effects. These findings are novel and demonstrate that the SHRSP system can be used to determine the association between the risk of stroke and radiation exposure with high sensitivity. Moreover, these studies provide important information regarding the association between the low dose and low dose-rate radiation exposure and circulatory diseases, especially stroke. (Takahashi). PI; Takahashi. Publications; Takahashi et al., Radiat Res 193:552-559, 2020; J Radiat Res 61:666-673, 2020.