Departmental Overview

The Department of Molecular Biosciences supports the mission of RERF by studying radiation effects uncovered by clinical and epidemiological studies at cellular and molecular levels. The department's studies include (1) Studies of genetic effects, (2) Studies of radiation carcinogenesis and (3) Studies of noncancer diseases among A-bomb survivors.

In the studies of genetic effects, we aim to determine the frequency and nature of heritable mutations in members of survivor families (mother, father, and offspring). RERF genetic studies have indicated that the survivor families did not show any significant genetic effects of parental exposure to radiation, and previous animal studies conducted by ours and other group indicated the average mutation rate of 2×10^{-2}/Gy per genome of relatively large size deletion/amplification. Now a genomic era, we are planning whole genome sequencing-based genetic studies using next-generation sequencing (NGS) technology that will provide the capability to detect the entire spectrum of mutations in survivor families. Software improvements and new technologies for Long-read NGS to assess complex types of mutations i.e., large deletions and translocations, are in progress. We are also developing a mouse model for NGS-based measurement of de novo germline mutations to support the human data and to elucidate the intrinsic molecular mechanisms.

In the studies of radiation carcinogenesis, we aim to clarify mechanistic relationships between radiation exposure and cancer development. Previous studies of thyroid cancers in survivors indicated that *RET* or *ALK* gene rearrangements frequently occurred in papillary thyroid cancers exposed at young ages to high radiation doses. The carcinogenic potential of these rearrangements is currently being assessed using experimental animal models. Based on potential involvement of liver inflammation and fibrosis in radiation-associated liver cancer, we hypothesize that chronic inflammation due to radiation exposure may be involved in the development of liver cancer through liver metabolic abnormality and fibrosis. New research plans to explore the role of radiation-associated liver steatogenesis and fibrosis in liver cancer development is being prepared. We are also examining genetic factors in breast and thyroid cancers developed in survivors. Cytogenetic studies indicated that lymphocyte chromosomal translocations did not dose-dependently increase in survivors exposed in utero whereas their mother did. The underlying mechanism of negative selection of damaged cells in fetal development is being explored in animal experiments.

We are also making efforts to identify and evaluate biomarkers linking radiation exposure to diseases among A-bomb survivors. Biomarkers currently being assessed involve immunological endpoints and metabolic indicators potentially related to enhanced risks of chronic diseases including cancer. We are developing longitudinal study designs to test the hypothesis that hematopoietic and immune-cell homeostasis perturbed following radiation exposure may affect the development of inflammation-associated diseases such as cardiovascular diseases and liver fibrosis/cancer. Potential biological pathways linked to such diseases involve clonal hematopoiesis, pro-inflammatory immune cells, endogenous danger signals, and arteriosclerosis. Studies investigating the role of these pathways in AHS subjects are initiated, and comparable animal model system is being developed.

For better understanding of biological mechanisms of radiation-related diseases, we are also planning collaborative studies with outside experts to perform integrated analyses of multiple molecular (omic) endpoints such as genomics, transcriptomics, metabolomics, and proteomics. The biodosimetry data for the frequency of chromosome aberrations in blood T cells as well as the intensity of electron spin resonance (ESR) signals in tooth enamel are anticipated to provide information on possible random and systematic dose uncertainties in individual doses calculated by DS02R1 and prove to be valuable for use in cancer risk estimation.

Now a genomic era, genome analysis would play most important part of our study designs. Detection of mutations carrying radiation signature in soma (cancer and noncancer), and germ cells (genetic effects) is becoming a major target of our study. Results of clinical and epidemiological studies need to be revisited through whole genome sequencing.

FY2019 Molecular Biosciences Achievements

Radiation and Genetics Effects

- Most important issue of "radiation and genetic effects" is whole genome study focusing on human trio study including atomic bomb survivors and their offspring. In FY2019, we made research plan and received approval of ICP (initial concept proposal) including whole genome sequencing study of the trios in RERF. By the end of FY2019, we will submit full scale RP of the human trio study to the genetic research cluster for evaluation of the research plan. In addition, we constructed security-controlled computer room for the human genome analysis in RERF. One of the most important issues of the human WGS study is getting social agreement and treating with ethical problem. So, we started a series of communication and education programs for citizens.

- Feasibility study of the Trio-WGS using human cells. *Purpose:* To evaluate genetic effects of atomic bomb radiation exposure on survivors using WGS, we develop a system to detect mutations in human samples. This time we used standard Japanese normal cells (GM18943) available from public cell bank. *Methods:* We isolated single cell derived colonies from the original cell populations and then conducted short-read (illumina) and long-read WGS (nanopore) using their genome DNA. The raw WGS data (illumina) were mapped to human reference sequence (GRCh38) using BWA-MEM. Then, duplicate-reads and reads showing low quality values were removed by using Picard and our customized SAMtools based program. Candidate variants were called by HaplotypeCaller (GATK). We identified the candidate de novo variants by comparing the called variants in each cell clone. To validate the de novo variants, we checked the variants using Sanger sequencing and IGV inspection and obtained the list of de novo mutations in each cell clone. To identify the parental origins of each de novo mutation, we checked haplotype around the mutation. All of the data processing is performed at a RERF genome analysis server. *Results*: We obtained 100~140Gb raw read data (illumina) per a sample and 80~111Gb high-quality mapping read data per sample after the removal of duplicate reads and low-quality mapped reads. Our customized pipeline provided highly credible ~2,000 base substitutions among individual clones (false positive rate: 0.0%), while analysis using conventional mutation detection pipeline provided ~4,500 base substitutions (false positive rate: 67%). In addition, we could detect ~90 small insertion/deletions (indels) and 6~9 multisite mutations among the clones and could discriminate the allele origins by using haplotype information. For long-read WGS, we could prepare a high-quality, high-molecular-weight DNA samples. Now, the samples are being conducted for WGS using PromethION (Oxford Nanopore). *Conclusion*: By using knowledge from our mouse study and Chernobyl study (conducted at NCI) and a new RERF cloud computing resource, we successfully developed high quality mutation detection pipeline, which is available for the human WGS study including atomic bomb survivor's families. (Satoh, RP S3-11)

- Characterization of radiation-induced small-size deletions in F1 mice born to exposed spermatogonia or mature oocytes. *Purpose:* In an earlier study we have detected SNVs and small-size deletions in F1 mice. This study aimed to clarify characteristics of radiation-induced indels identified in F1 mice born to exposed spermatogonia or mature

oocytes. In addition, we improved statistic calculation by considering aging effect of parental mice on mutation induction. *Methods:* We examined further details of neighboring sequences of *de novo* mutations. Also, we examined the number of *de novo* mutations of unexposed parental alleles to estimate the aging effect on *de novo* induction of SNVs. We conducted Quasi-long-read assay (10X Chromium by Illumina short read and stLFR assay by DNBseq) to improve phased sequencing for determining parental origin of mutations. *Results:* Increases of the numbers of SNVs in the offspring could be explained by aging-related effects in both spermatogonia and mature oocytes. In contrast, the number of indels observed in F1 mice born to exposed parent was significantly larger than those in control group even when it was assumed that aging effect is three times higher than predicted. We found two types of remarkable characteristics of small deletions in exposed group; one is small deletions (mainly 1~12 nucleotides) in non-repeat sequences, many of which showed microhomology at the breakpoint junction, and the other is single-nucleotide deletions in mononucleotide repeat sequences. These features were also observed in irradiated human iPS cells as recently published by Kucab *et al.* (Cell, 2019) *Conclusion:* Deletions having abovementioned features and multisite mutations could be a typical signature of mutations induced by parental irradiation in mammals. These results will provide useful information for planning WGS analysis in A-bomb survivor families. Satoh,Scientific Reports 10:37, 2020. (Satoh, RP 2-13).

- It is quite important to develop high-quality mutation detection methodologies using whole genome sequencing data for radiation biology, especially in genome-wide study of the genetic effects. To prepare WGS studies in the human trios and animal model study, we have been working on an improvement of mutation detection pipeline. In FY2019, we successfully established the following two outstanding mutation detection systems using our original mouse model (mutation accumulation mouse lines).

  (1) Develop a new methodology for detecting various types of mutations from WGS data. *Purpose:* Develop a new methodology for detecting various types of mutations, mainly focusing on large structural variants in this year. *Methods:* We compared the whole genome sequencing data derived from 4 different sequencing methods (short-read [illumine], Long-read [PacBio], Long-read [Nanopore] and Quasi-long-read [Chromium, 10X]) and developed the pipeline which can detect large size variants (size > 1kb). *Results:* Our new pipeline could detect total 15 de novo structural variants (including 11 retrotransposon insertions) as a result of Long-read WGS data of 2 mice from the above mentioned mutation accumulation study. Though this is not related to radiation effects, the result indicated that spontaneous mutation occurrence rate of such large size mutations in mice is much higher than the previous estimates. Now, we are working on improving the accuracy and efficiency of the detection pipeline. *Conclusion:* This method is very useful to investigate the induction effects on large size mutations by radiation exposures (Uchimura and Satoh, new RP).

  (2) Develop a new methodology for detecting mosaic mutations and cell lineage reconstruction. *Purpose:* Develop a new methodology for detecting mosaic mutations and a novel method for a reconstruction of cell-lineage based mosaic mutations at a single-cell-division scale. *Methods:* In our previous study, we have established very

accurate de novo mutation detection pipeline (SNVs and small indels). By using this pipeline, from 100x coverage WGS data, we could detect most of mosaic mutations, of which VAF (variant allele frequency) is more than 5%. In FY2019, we could establish a new method to measure the VAF of each mosaic mutation very accurately by using amplicon-seq and develop a brand new mathematical method to reconstruct the cell lineages based on the mosaic mutations. *Results:* We successfully detected ~30 mosaic mutations in 100x coverage WGS data per individual and reconstructed early embryonic cell lineages in 5 mice, all of which could depict 8~10 cell lineages per an individual. We found the early embryonic mutation occurrence features and their asymmetrical contributions to adult somatic tissues and germ cells. *Conclusion:* We could establish an innovative method for cell-lineage analysis. This method is principally applicable for human samples. This is useful to uncover induction of the mosaic mutations by radiation exposures and also useful to investigate the dynamics of cell population change (e.g. clonal expansion). (Uchimura, new RP)

- Radiation-induced mutations in mouse spermatogonia cells in culture. Purpose: 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). Materials and Methods: 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 10 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. Conclusion: aCGH analysis revealed a few candidates of deletions in irradiated clones. Then we have initiated WGS of the clones. (Noda, RP-P3-17).

Radiation Dosimetry

- 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've 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: (1) the overall dose-response for translocation frequency was nonlinear, wherein the slope leveled off in the higher dose range (above around 1.25 Gy); (2) a wide scatter of individual translocation frequencies against DS02R1 dose was observed in both cities, as seen in the previous Giemsa staining study; (3) the Nagasaki factory workers had a significantly lower dose-response than those exposed in Japanese houses; (4) the city difference was no longer significant after excluding the Nagasaki factory workers; (5) observed dose responses were also significantly lower among survivors whose shielding categories were outside but shielded, in open without shielding, and in other house; (6) Such differences in dose response among different shielding categories suggests a systematic, shielding-related bias in physical dose estimates. (Kodama, RP8-93)

Radiation and Cancer

- To explore the role of *EML-ALK* gene fusions in radiation-induced papillary thyroid cancer (PTC), we have created *EML4-ALK* transgenic mice that can significantly express the fusion gene in thyroid tissues of the mice a few years ago. The mice did not show any histological features of the development of cancer in any organs including the thyroid, until at least one year of age. To test a hypothesis that cells highly expressing the *EML4-ALK* fusion gene selectively proliferate following radiation exposure and develop cancer, we were examining *EML4-ALK* expression, histological features in the transgenic mice following 0- or 3-Gy whole-body irradiation. We also examined the fusion generation in immortalized human thyroid epithelial cells (Nthy-ori-3 cells) following *in vitro* X-irradiation. However, a real-time or a droplet digital RT-PCR could not reproducibly detect the fusion gene in the cells irradiated with 0, 0.1, 0.2, 1.0, or 5.0 Gy, which could not well test another hypothesis that low-to-middle dose radiation can induce the *EML4-ALK* fusion. (Kusunoki and Taga, RP1-14)
- Epidemiological studies at RERF demonstrated that radiation exposure has led to the excess relative risk of hepatocellular carcinoma (HCC) among atomic bomb (A-bomb) survivors, and mouse model studies well verified that radiation could induce HCC. To better understand mechanisms for radiation-associated HCC, we plan to evaluate hepatic steatosis and fibrosis following radiation exposure in mice (Project 3 in the Liver Cancer Program Project). With special attention to stellate cells and inflammatory macrophages in the liver, we hypothesize that radiation exposure accelerates hepatic steatosis and fibrosis through stellate cells and inflammatory macrophages, which may lead to the development of HCC. We test this hypothesis with the following specific aims: 1) to morphologically evaluate involvements of hepatic stellate cells and macrophages in steatosis and fibrosis using liver tissues obtained from irradiated and unirradiated mice, 2) to evaluate radiation-related changes in expressions of inflammatory cytokines and chemokines in hepatic stellate cells and macrophages obtained from irradiated and unirradiated mice, 3) to evaluate direct effects of irradiation on the functions of stellate cells and macrophages using in vitro irradiation experiments, and 4) to identify possible biomarkers of radiation-associated HCC development in mice, which can then be utilized in a future study using biosamples from A-bomb survivors. In this project, we use B6C3F1 mice (F1 mice from female C57BL/6 and male C3H mice) that are known to frequently develop HCC by whole-body irradiation, especially in infant (e.g. 1 week old) irradiation, and evaluate expressions of genes associated with DNA damage, cellular senescence and inflammation. Biosamples such as serum and intestine tissues are utilized for narrowing down candidate markers to identify novel biomarkers by transcriptome approach. We also plan to isolate macrophages from the liver and analyze gene expressions in a similar way.

  Our preliminary experiments conducted in FY2019 showed that hepatic stellate cells can be isolated with a purity of more than 90% by a combination with pronase-collagenase perfusion of the 3-month-old liver and density gradient centrifugation, suggesting the feasibility of the studies for specific aims 2) and 3). The purity of hepatic stellate cells was found to be easily assessed in our improved immunofluorescent staining method using desmin as a hepatic stellate cell marker and a

gel reagent kit for cell fixation. As one of the preliminary results using this system, an tendency of increased expressions of Ccl5, a chemokine known be involved in liver steatosis and fibrosis, was observed in hepatic stellate cells isolated from mice (n = 7) at 3 months after X-ray irradiation compared with non-irradiated control mice (n = 7). Based on the preliminary data, we are conducting power calculation for the sample size determination to complete an RP for the full-scale study. (Taga, a new RP in liver cancer program project)

- Preliminary study of chromosome aberration frequency in hematopoietic stem cells (HSCs) following fetal irradiation of mice. *Purpose:* To test a hypothesis that radiation effects on the induction of persistent chromosome translocations may depend on the stage of fetal development, namely on whether or not the stem cells have already settled into their niche. As the first step, we examined whether reciprocal translocations are induced in fetal HSCs soon after irradiation. *Materials and Methods:* Pregnant mice (E13-15d) were exposed to 2Gy of X-ray, 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 the mFISH method to determine translocation frequencies. *Results and Conclusion:* The mFISH data revealed that the translocation frequency was 21% (9 out of 43 colonies) for colonies derived from the fetuses, and 51% (21 out of 41 colonies) for clonies derived from the mothers. This result suggest that translocations might be induced in fetal HSCs immediately after the 2Gy of X-irradiation but, the frequency was about 50 % compared to mother's frequency 1 day after the exposure ($p=0.026$). If we take the results at face value, they might indicate that fetal HSCs bearing radiation-induced translocations are at least partially eliminated during the 1 day period after the exposure in fetal life. Furthermore, we encountered two difficult problems to solve: one is the reliability of isolated LT-HSC, and the other is much higher frequency of translocation of colonies derived from 2Gy irradiated mothers LT-HSC (51%) than expected. (Hamasaki, RP-P4-17)

- Preliminary Study to determine the applicability of Wright-stained blood smears in GWAS. *Purpose:* To confirm whether amplifying whole genomes using very small amounts of DNAs obtained from Wright-stained smears would make it possible to use Toshiba Japonica arrays for SNP analyses. *Materials and methods:* From among employees, six subjects were randomly selected. Wright-stained smears were prepared from blood samples. DNA obtained from Wright-stained smears were amplified using the QIAGEN REPLI-g DNA amplification kit. DNA samples obtained from blood samples and amplified DNA-samples were genotyped using SNP arrays (Toshiba Japonica array). (Hayashi, RP-P1-19, new study initiated in October 2019).

- Preliminary study (RP-P) on possible roles of oxidative stress response in protection against radiation-induced mutagenesis and oncogenesis. *Purpose:* To identify possible roles of an oxidative stress response pathway controlled by a transcription factor NRF2 in protection against radiation-induced mutagenesis and oncogenesis. *Background:* Identification of a factor that protects against radiation oncogenesis could lead to the elucidation of molecular mechanisms for oncogenesis and for inter-individual variability in the radiation risk for oncogenesis, and could also lead to the development

of methods to reduce the risk. In case of gamma- and X-irradiation, the primary mechanism of the radiation oncogenesis is the somatic mutations caused by DNA damages by reactive oxygen species (ROS) derived from ionized water molecules. Reportedly, activation of NRF2, a master transcriptional activator of antioxidant genes, significantly reduces the acute radiation toxicity. *Methods:* We will examine the mutagenic and oncogenic effects of X-irradiation on genetically modified mice with either loss or activation of NRF2 as well as on wild-type controls, 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 the oncogenic effects, mice will be monitored after radiation exposure for the development of myeloid leukemia. For analysis of mutagenic effects, those mouse lines will be bred with a transgenic reporter mouse line named "*gpt* delta" for an *in vivo* mutagenicity assay. Compound mutant mice thus obtained will be subjected to radiation exposure, and then later the mutagenicity assay will be carried out using bone marrow, spleen, and liver tissues. Further, for direct analysis of mutagenic effects of X-irradiation on the mouse genome, we will conduct whole-genome sequencing of DNA extracted from normal clonal cell populations expanded *in vitro* from single hematopoietic stem cells isolated from bone marrow. From these studies, 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 and oncogenesis. *Results:* We have tested conditions for hematopoietic stem cell isolation, culture for colony formation, and extraction of DNA from the colonies, and established protocols for these procedures (Tanabe, RP-P3-19, new study initiated in Dec. 2019).

Radiation and Immunologic Effects

- Relationship between intracellular reactive oxygen species (ROS) in blood cells and inflammatory biomarkers among atomic-bomb survivors. *Purpose*: To investigate the effects of age and radiation exposure on intracellular ROS levels in blood cells. *Methods:* We examined 2,789 Hiroshima A-bomb survivors who underwent health examinations from 2007 to 2012. Intracellular ROS ($O_2^{\cdot-}$) levels in T-cell subsets were measured using a combination of fluorescence-labeled antibodies and a fluorescent reagent, hydroethidine. *Results and Conclusion*: Intracellular ROS ($O_2^{\cdot-}$) levels in lymphocytes (especially memory $CD8^+$ T cells) increased as age and radiation dose increased. In addition, the intracellular $O_2^{\cdot-}$ levels of memory $CD8^+$ T cells in the high-plasma CRP level group increased significantly with increasing radiation dose when divided into three groups according to plasma CRP levels. These results suggest that increases in intracellular $O_2^{\cdot-}$ levels of memory $CD8^+$T cells, particularly by radiation exposure, may have been induced by enhanced inflammatory status such as an increase in plasma CRP levels. The manuscript will be submitted in FY2020. (RPs 3-09 and 4-02, terminated in 2018.)

Radiation and Other Noncancer Conditions

- Clonal hematopoiesis (CH), 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 potentially linking to radiation-associated noncancer diseases, specifically arteriosclerosis, we have proposed a research plan 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 FY2019 indicated 1) that it is difficult to identify CH with viable mutant cells in the HPRT-dup-GFP model (Noda, PLos One 2015) since none of 42 irradiated mice had a large amount of mutant GFP-positive blood cells; 2) that longitudinal analyses of hematologic indicators are feasible since RDW (Red cell Distribution Width) and monocyte counts, which are known to be associated with CH in human, increased in some of the 3-Gy irradiated mice; and 3) that using digital-droplet RT-PCR and flow cytometry, CH-related pro-inflammatory phenotypes can be evaluated with expression levels of inflammatory genes in BM-derived macrophages. Based on these results, we decided to use whole-exome sequencing (WES) but not the HPRT-dup-GFP system for CH identification. Currently, BM DNA samples from 8 irradiated and 4 control mice are being assessed for WES to identify CH with recurrent somatic mutations, which we expect to investigate the feasibility of mouse BM WES for CH assessment. 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 formation through clonal accumulation of pro-inflammatory monocyte into atherosclerotic lesions. This proposal has been fully discussed and approved in the Noncancer Research Cluster. (Kusunoki, Yoshida, Taga, Hamasaki, Satoh, Uchimura, Misumi and Noda, a new RP in the Clonal Hematopoiesis Program Project).

- 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. The association of radiation with those phenotypes may be affected by genetic background as evident from strain difference. 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 significant radiation effects. Thus the dose-rate effectiveness factor seems to be expected to be infinity. In addition, metabolome analyses and measurement of cytokines showed that chronic dose-dependent alteration was observed in various markers. Those data may provide useful tool for elucidating molecular mechanisms of association between CD and radiation exposures. The studies will be terminated after the summary papers are published. (Takahashi, RPs 1-11, 2-12, S1-15).

2. FY2020 Molecular Biosciences Plans

Radiation and Genetic Effects

- For WGS study of the human trios including atomic bomb survivors and their offspring in FY2020, we will make a complete research plan under the review of Genetics Research Cluster and external advisors. After the reviews, we will submit the research proposal to IRB and get approval. When research environment (including financial, ethical and social issues) will have been completely prepared, we will start to access to the participants and get informed consent from them for the WGS study. After getting informed consent, we will start experiment using WGS.
- By using cultured human cell lines we will examine what method is suitable for the analysis of Atomic bomb survivors' samples. We also develop improved methodologies to detect large size mutations and structural variations in analysis of human samples using knowledge obtained from mouse genome long-read sequencing. In addition, we will develop detection method to explore epigenetic status from long read sequencing data. (Satoh, RP S3-11)
- Previous animal model studies identified more small deletions and multisite mutations in F1 mice of 4 Gy-irradiated spermatogonia and mature oocyte than in those from unexposed parents. However, larger size mutations including structural variants are not fully understood. Currently, we are developing new pipeline to detect larger size mutations using long-read NGS technique and mouse mutation accumulation line. Our new pipeline will uncover spontaneous occurrence rate and characteristics of such variants (especially in transposon mutations) in mice. After this, we will submit the paper demonstrating new pipeline for detecting germline de novo large-size mutations in experimental animals. (Satoh, RP 2-13 addition).
- For a study developing detection system of embryonic mosaic mutation and reconstruction of early cell-lineage, we will publish the paper "Early embryonic mutations reveal in vivo cellular dynamics of mouse somatic and germ cells". In addition, we will start to use this cell-lineage reconstruction method for clonal hematopoiesis project (Uchimura, a new RP in F1 umbrella program project).
- For a GS cell study, mutations in irradiated GS cell clones will be characterized by aCGH and WGS analyses, and then the cell clones will be transplanted into Busulfan-treated testes of adult male mice. We will then examine the offspring derived from the GS cells for heritable genetic changes. Along with this experiment, efficiency of spermatogenesis from the transplanted GS cells will be analyzed by testis tissue cross sections. As a control, GFP positive GS cells and testicular cells from GFP positive mice will be used. We plan to make artificial large scale deletions or inversions in GS cells via newly developed gene editing systems (Noda, RP-P3-17).

Radiation and Cancer

- We will initiate a full-scale study (Project 3 in the Liver Cancer Program Project) with approvals from the Cancer Research Cluster and RERF's relevant committees. Morphological and molecular changes in hepatic stellate cells and macrophages will be evaluated in B6C3F1 mice receiving infant (e.g. 1 week old) whole-body irradiation and

control mice. Following mouse breeding sufficient for the full-scale study, liver tissue samples will be collected from the mice of three, six, and nine months after irradiation with 0 or 3.8 Gy. As for the specific aim 2 (evaluation of radiation-related changes in expressions of inflammatory cytokines and chemokines), we will isolate hepatic stellate cells from irradiated and unirradiated mice. In addition to liver morphological assessments in collaboration with Nagasaki and Hiroshima Universities, we will analyze isolated hepatic stellate cells and macrophages for their gene expressions associated with DNA damage, cellular senescence and inflammatory biomarkers by real time PCR. We will also conduct in vitro irradiation (0, 3.8, or 7.6 Gy) experiments in primary culture systems using stellate cells and macrophages isolated from liver tissues of unirradiated mice (1 to two weeks old). Through transcriptome approach using serum samples obtained from irradiated and unirradiated mice, candidate biomarkers of hepatic steatosis and fibrosis will be selected, which may contribute to planning of a human liver cancer study (Project 1 in the Liver Cancer Program Project) that will provide information on novel biomarkers related to detection, mechanisms, and radiation sensitivity of hepatocellular carcinoma (Taga, a new RP).

- As Project 1 in the Liver Cancer Program Project to investigate risks and mechanisms of radiation-associated hepatocellular carcinoma (HCC), this research proposal is under development for initiation in FY2020, which aims to identify biomarkers related to detection, mechanisms, and radiation sensitivity of HCC among A-bomb survivors through proteomic and metabolomic analysis of blood and urine. Among A-bomb survivors, HCC is the third most common cause of cancer mortality, and has demonstrated an elevated risk for radiation; the relative risk of HCC not associated with hepatitis virus (non-B, non-C HCC) is as high as 2.74 for radiation at 1 Gy (Ohishi *et al.*, 2011). It has been suggested that metabolic stress as well as immune responses play crucial roles for HCC development. We hypothesize that hepatic inflammation and consequent steatofibrosis are involved in HCC development among A-bomb survivors, and thus cause alterations in molecular profiles of blood and urine, which could be exploited as predictive or diagnostic biomarkers, and could also serve as clues to elucidate the pathogenesis of radiation-associated HCC. The availability of well-defined populations, *i.e.,* AHS subjects, and access to longitudinal biosamples therefrom provide us a unique opportunity to apply the omics approaches to the study of radiation-associated HCC. We plan to identify the alterations in molecular profiles during the progression of hepatic inflammation and consequent steatofibrosis associated with HCC development through proteomic and metabolomic analysis by mass spectrometry of blood plasma, serum, and urine samples longitudinally provided from AHS subjects with and without HCC (Tanabe, a new RP).

- We are considering designing a nested case-control study of breast cancer as a part of a potential program project. We would use the Toshiba Japonica Array developed for analyzing gene polymorphisms potentially involved in the development of radiation-associated breast cancer in the Japanese. DNA samples extracted from old smears must be used to conduct a genome study using a cohort of all AHS subjects. For this reason, it is necessary to determine whether an SNP analysis using the Japonica Array is possible by amplifying a whole genome using a very small amount of DNA

obtained from a blood smear stored for many years. In FY 2020, DNA extraction from preserved, old blood smears of AHS subjects will be carried out to examine a suitable preparation method for REPLI-g amplified DNA. Then, we will evaluate DNA availability by analyzing those DNA samples with the Japonica Array (Hayashi, RP P1-19 and a new RP).

- As described in *Achievements* on "Preliminary study (RP-P) on possible roles of oxidative stress response in protection against radiation-induced mutagenesis and oncogenesis", we will conduct analysis of mutagenic effects of X-irradiation using the *gpt* delta transgenic reporter mouse with or without either null mutation or constitutive activation of a transcription factor NRF2. The constitutive NRF2 activation will be achieved by reducing Keap1, a protein inhibitor of NRF2, by genetic manipulation. The *gpt* delta mouse line carries a λEG10 phage as a transgene, which can be retrieved as live phages from DNA extracted from the mouse, and then be subjected to two different selection cultures for detection of both base-substitution and deletion mutations based on emergence of mutant phages. The λEG10 transgene will be introduced to the mutant mouse line with either null mutation or activation of NRF2 by breeding with the *gpt* delta mouse. Subsequently, the mutant and wild-type control mice will be exposed to 3-Gy X-irradiation, and then later DNA will be extracted from liver, spleen, and bone marrow for the analysis. We will conduct the mutation frequency analysis by the selection cultures as well as the sequence analysis of the retrieved phage clones. Further, when collecting bone marrow tissues from those mice, we will isolate hematopoietic stem cells and culture them on semi-solid media for colony formation to obtain normal clonal cell populations. We will then extract DNA from the cell populations and subject it to whole-genome sequencing by the Illumina sequencer to identify somatic mutations in the mouse genome. By comparing the mutation frequencies and the sequences between the exposed and the non-exposed, mutagenic effects of X-irradiation on each genotype will be determined, and the effects of NRF2 loss and activation will be evaluated (Tanabe, RP-P3-19, initiated in Dec. 2019).

- Regarding the issue related to the cancer risk following fetal exposure to radiation, we will change the direction of the study; namely, try to focus on pre-B cells. This is because around 40% of childhood cancers consist of leukemia, and about 70 % of them are acute lymphoblastic leukemia (ALL). Further, pre-B cells are thought to be the target of childhood ALL. Therefore, if fetal exposures to radiation really caused an increased risk of ALL after birth as indicated by the Oxford Survey of Childhood Cancer, it is anticipated that the effect of radiation exposures should be recorded in pre-B cells, which is testable using mice. (Hamasaki, RP P4-17 and a new RP).

Radiation and Immunologic Effects

- Previous immunological studies suggested that A-bomb radiation may have accelerated aging-related changes in both adaptive and innate immunity, which play key roles in hepatocellular carcinoma (HCC) development. To test the hypothesis that radiation exposure accelerates the progression to HCC through dysregulation of T-cell immunity and innate inflammatory response, we will analyze existing immunological/ hematological data in AHS subjects (e.g., longitudinal data of naïve T-cell, NK-cell,

monocyte counts, and red blood cell distribution width [RDW]), in relation to radiation dose, hepatitis virus infection, inflammation, liver steatosis, fibrosis, and HCC (Yoshida and Kusunoki, a new RP in preparation).

Radiation and Other Noncancer Conditions

- As the Clonal Hematopoiesis Program Project that has been reviewed in the Non-cancer Research Cluster, we are developing strategies for assessments of clonal expansion of HSCs (i.e., clonal hematopoiesis) and inflammatory changes in the hematopoietic system potentially contributing to the radiation-associated noncancer diseases, specifically atherosclerosis, in A-bomb survivors. Project 1 of this program aims to test the hypothesis that, in AHS subjects who exposed to high-dose (> 1Gy) radiation several decades ago, clonal hematopoiesis is promoted with recurrent somatic mutations in epigenetic modifier genes (*TET2, DNMT3A, ASXL1,* etc) and/or DNA damage response genes (*TP53*, *PPM1D*, etc). Clonal hematopoiesis with somatic mutations will be evaluated by performing NGS using cryopreserved blood cells, in collaboration with Dr. Miyazaki in Nagasaki University and Dr. Ogawa in Kyoto University. Statistical power calculation shows that the proposed study should have adequate power to demonstrate a difference in prevalence of clonal hematopoiesis between the heavily-exposed and negligibly-exposed dose groups (about 100 subjects in total). Plasma levels of endogenous danger signals (alarmins), which may promote clonal expansion of HSCs, will also be assessed in relation to the development of clonal hematopoiesis following radiation exposure. This project has been approved by the Non-cancer Research Cluster, outside scientific experts, and the RERF Committee on Biological Samples (Yoshida and Kusunoki, a new RP of the Clonal Hematopoiesis Project 1).

- The proposal of mouse CH study currently relies on very limited preliminary data that can support the feasibility of the study and that enable power calculations to determine the sample size. Thus, we will obtain preliminary WES data in 8 and 4 mice irradiated with 3 and 0 Gy, respectively, and evaluate the frequency of recurrent mutations in each mouse to detect CH with the frequency >0.02, which would allow to conduct power calculation for the sample size in a full-scale study to investigate the frequency of radiation-induced CH in wild-type B6C3F1 mice. We will also conduct preliminary experiments using 15 irradiated and 5 unirradiated LDLR-KO mice, for 1) frequency evaluation of early embryonic spontaneous somatic mutations (SPMs) in blood cells, 2) CH identification in BM through WES, 3) evaluation of atherosclerosis induction by a high fat diet and whole-body irradiation, and 4) SPM-based evaluation of clonal monocyte accumulation in atherosclerotic plaques. These preliminary experiments will provide data to support the feasibility of this study proposal and to enable power calculation to adequately determine the sample size for another full-scale study investigating involvement of radiation-induced CH in atherosclerosis formation. (Kusunoki, Yoshida, Taga, Hamasaki, Satoh, Uchimura, Misumi and Noda, a new RP in the Clonal Hematopoiesis Program Project, Project 3).

3. Molecular Biosciences Five-Year Plans

Five-Year Plans

The Department of Molecular Biosciences (MBS) aims to provide RERF with a strong basic science program for achieving a robust assessment of radiation health risks. Important tasks that this department should pursue over the coming five years involve 1) contributions to new program project type research initiatives being developed in Research Clusters, 2) development of cutting-edge research plans of genomics and omics studies for identifying new biomarkers of radiation exposure (radiation signature) and for predicting radiation-associated diseases long before the onset, and 3) recruitment of young and senior scientists to maintain and strengthen, and expand the current research activities to enable future collaborations with outside experts. Below is a brief summary of MBS strategic plans.

The department seeks to strengthen fundamental research capabilities emphasizing these three specific areas: 1) Genetics; 2) Cancer genomics and Omics; 3) Studies of noncancer diseases. In each area, characterization of radiation-induced mutations, in either somatic or germ cells, will be the major target in the next 5 years. Currently the number of full time scientists continues to decline. In 2019 we have started recruiting several of young and senior scientists, and recently one scientist, Dr. Matsuda joined our cancer research program. We plan studies on radiation-associated cancer genomics with collaborations with domestic and international researchers. Thus, the most urgent recruit is a leadership position for future cancer studies. Furthermore, we are planning recruitments of young scientists for developing WGS technologies, germ line stem cell research, and immunological studies. Through these recruitments, MBS will have 10-12 scientists with required expertise to develop studies in our proposed areas of research excellence.

A. Expansion of studies of genetic effects:

RERF possess a unique population of parents and children where either one or both parents were survivors of the atomic bomb. The MBS studies will be a part of an overall F1 program project involving Clinical Studies and Epidemiology as well. MBS studies need to identify transgenerational mutagenic effects both quantitatively and qualitatively. In conducting survivor studies, future studies in this area will further define these changes and examine potential mechanisms.

1. **Quantification and characterization of radiation-induced germ cell mutations in F1 populations of A-bomb survivors**

   Following the establishment of applicable analysis pipelines with our standard Japanese cell genome, we will expand the study to a large scale ensuring a statistical power enough to draw a genetically acceptable and publicly imaginable conclusion. By accomplishing this in the next 5 years, we will stand at a starting point for new studies of radiation genetics, such as those of the difference between germline and somatic cell mutations, effect of somatic mosaicism on the individual phenotype development, and epigenetic effects on heredity. Integration of multi-omics approaches into the genetics study also needs to be considered, with evidence that epi-mutations, other than germ cell mutations, could result in metabolite changes in the offspring. Investigators involved include Drs. Satoh, Noda, and Uchimura and newly hired scientist.

2. **Quantification and characterization of radiation-induced germ cell mutations in mouse models**

   Using similar WGS technologies to the survivor family study, we will investigate dose response relationship and mechanisms of germline mutagenesis in mouse models, especially focusing on comparisons of mutation frequencies in premeiotic diploid cells and F1, and on mechanisms underlying negative selection of mutated cells during gametegenesis. (RPs 2-13, P3-17 and new RPs). We will also explore new experimental systems to detect somatic mosaicism which is caused by somatic mutations acquired in early embryo development and which greatly affects individual phenotypes and subsequent germ cell mutations. Investigators involved include Drs. Satoh, Noda, and Uchimura and newly hired scientist.

3. **Comparison of radiation-induced chromosome translocations between mitosis and meiosis in mouse models**

   We will also test the hypothesis that chromosomally aberrant germ cells were negatively selected (meiotic checkpoint), by comparing translocation frequencies between mitosis and meiosis following irradiation of mouse spermatogonia using FISH (new RPs). Determination of junction sequence of radiation-induced translocations is also required to characterize the radiation signature. Investigators involved include Drs. Hamasaki and Noda.

B. Expansion of Cancer and Omics Studies:

Another major set of projects within the program are radiation carcinogenesis studies that will examine molecular events in the pathogenesis of radiation associated cancer, as well as potential predictive and diagnostic biomarkers. With regard to cancer study, program projects for tissue/organ specific cancer study will be developed in collaboration with other departments, Hiroshima-Nagasaki local pathologists and tumor registries. The targets are breast, thyroid, colon, and liver, etc., and the discussions toward the organization of Breast and Liver program projects are already initiated in Cancer Research Cluster. As for the omics studies, final goal is to find radiation-associated biomarkers from the past-exposed cells/tissues or from their metabolites, and correlate them with the radiation-induced carcinogenesis. This strategy can also be applied to noncancer diseases. With omics technologies rapidly evolving, in the near future we will foster collaborative interactions with domestic and world's most advanced research groups outside RERF. This will begin in practice with dispatches of young scientists to the research groups. As for outside use of RERF biosample, understandings and supports from Hiroshima-Nagasaki local residents will be needed, with which we are tackling as issues of all RERF.

Below are areas in carcinogenesis study projects that, at present, we consider to be the strongest and likely to go forward over the next several years.

1. **Integrative "omics" and radiation associated cancer**

   The availability of well-defined populations and access to longitudinal samples (particularly blood and urine samples) provide a unique opportunity to apply omics approaches to the study of the pathogenesis of radiation associated cancer. A study based on the Liver Cancer Program Proposal is aiming at identification of novel biomarkers

related to detection, mechanisms, and radiation sensitivity of hepatocellular carcinoma in humans, potentially through the omics approaches. Before exploring biomarkers using an amount of precious A-bomb survivors' biosamples, we will develop a comprehensive analytic approach involving transcriptomics, methylomics, proteomics, lipidomics, and metabolomics as well as other endpoints (e.g., miRNA) in mouse radiation liver carcinogenesis models, so that we can provide insight into mechanisms and potentially lead to the identification of biomarkers and development of diagnostics of hepatocellular carcinoma developed in survivors. Investigators involved will include Drs. Taga, Yoshida, Cologne, Misumi, Tanabe, Ohishi, Ozasa, and Hayashi, and a young scientist to be newly recruited.

2. **Mechanisms in radiation carcinogenesis**

To address questions with respect to cancer-site specificity and age dependency in radiation carcinogenesis, we are testing the hypothesis that radiation-induced tumors may originate from the self-renewing or transient amplification of stem cells established during embryogenesis and neonatal development. Using mouse experimental systems to monitor radiation-induced mutations and chromosome aberrations in tissue stem cells, for example, stem cell markers such as GFP reporter trans-genetically tagged or somatic mutations spontaneously acquired in early embryogenesis, will allow fate-mapping of tissue stem cells toward radiation-carcinogenesis. Furthermore, reprogramming of somatic cells needs to be considered. Investigators to be involved include Drs. Hamasaki, Satoh, Uchimura, Noda, Taga, Itoh, Yoshida, and Kusunoki, and a young scientist to be newly recruited.

3. **Biomarkers for past exposure and radiation disease risks**

We will develop new assay systems for detecting persistent types of damage in the exposed cells and tissues, potentially applicable to estimate of the exposed dose in RERF archival tissue specimens of A-bomb survivors. These biomarkers for past exposure as well as those discovered in aforementioned integrative omics studies will be used for multi-disciplinary collaborative studies to understand mechanisms underlying enhanced risks of cancers and non-cancer diseases among A-bomb survivors (new RPs). Investigators to be involved will include Drs. Yoshida, Satoh, Hamasaki, Uchimura, Taga, Hayashi, Noda, Kusunoki, Cologne, Misumi, Ohishi, and Ozasa.

C. Expansion of studies of noncancer diseases:

The Non-cancer Research Cluster is primarily aimed at examining the association between radiation exposure and non-cancer disease morbidity and mortality. Animal model studies are inevitable to understand biological mechanisms underlying increased risks of non-cancer diseases such as CVD in A-bomb survivors. In these five years, we will focus on the following research plan:

1. **Clonal hematopoiesis and inflammatory phenotypes in radiation-associated CVD**

To test the hypothesis that radiation may promote arteriosclerosis through clonal hematopoiesis and pro-inflammatory phenotypes, we will examine somatic mutations, immune cell phenotypes and inflammatory markers, using blood samples from AHS participants. Additionally, these molecular and cellular endpoints will be jointly analyzed

in relation to inflammation and arteriosclerosis indices evaluated in the AHS. The effects of radiation exposure on clonal hematopoiesis and chronic inflammatory markers including atherosclerosis development will also be evaluated in mouse and mathematical simulation models. Investigators to be involved will include Drs. K. Yoshida, N. Yoshida, Taga, Satoh, Hamasaki, Uchimura, Hayashi, Noda, Misumi, Cordova, Ohishi, Kusunoki, Cologne, Imaizumi, and Hida.

The other set of future prospective studies are:

- **Molecular Cytogenetics for determining junction sequencing of radiation-induced translocations**

  As a legacy of long lasting cytogenetics studies of survivors, we have huge amount of lymphocyte chromosome preparations bearing radiation-induced translocations and corresponding blood samples. In the near future, once the technique of single cell WGS matures and to be practical, we may be able to determine the precise junction sequences of translocations in individual cells, which will provide an opportunity to elucidate the mechanisms of radiation-induced translocations more than ever before.

- **Where is the persistent inflammation coming from?**

  Immunological studies have accumulated evidence for inflammation manifesting as a consequence of radiation-associated immunological changes decades after the A-bombing. We will examine radiobiological endpoints potentially related to inflammation in various tissue cells (e.g., bone marrow or white adipose tissue in some organ?), in order to deepen understandings of the mechanisms by which the late effects of radiation exposure can manifest as inflammatory disease development and to find the way to prevent the disease development in radiation-exposed individuals.