PROGRESS REPORT
Division of Cell Fate Regulation

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Division of Cell Fate Regulation

Chairman: Ryo Goitsuka, Ph.D., D.V.M.

Infancy and childhood are critical periods for the development and maturation of adaptive immunity. During this time, the immune system establishes protective immunity to pathogens and tolerance to innocuous microbial and environmental antigens. The tissue-resident immune cells, such as macrophages and innate lymphoid cells, which are derived from both embryonic and postnatal precursors, show differences in distribution as well as in functions. Their tissue-specific signatures appear to be acquired during this narrow perinatal window of time, thus implicating any defects in these processes in the risk for immune-associated diseases. As a key perinatal organ that connects fetal liver to bone marrow hematopoiesis, the spleen acts as a transient site both for hematopoiesis and lymphopoiesis, as well as for destruction of fetal-type erythrocytes. Fetal-type B cells, called B-1 cells, have unique autoreactive B cell antigen receptor specificities and first appear in the neonatal spleen. Later, they move into the peritoneal and pleural cavities, where they are clonally maintained as long-lived B cells by self-renewal. Their physiological functions still remain mysterious, but they serve as a source of IL-10-producing regulatory B cells under inflammatory conditions as well as precursors of plasma cells that produce natural antibodies. To understand the function of fetal-derived lymphoid cells in the adulthood in detail, we have recently established an experimental system that enables genetic manipulation of fetal lymphoid cells in the adulthood, thus being ready to expand our knowledge on early life immunity that might affect susceptibility and severity of immune-mediated disorders.

The origin of natural IgM-producing cells assessed by the RAG2-based cell fate tracking system

By using the tetracycline-controlled cell fate tracing system that can track lymphoid cells with a transcriptional history of the RAG2 gene, we assessed the contribution of embryo-derived B-lineage cells on the adult natural IgM-producing cells. This reveals that about 40% of $CD138^{+}IgM^{+}$ plasmablasts in the adult bone marrow (BM) had a history of RAG2 expression in the embryonic period, suggesting two possibilities that these plasmablasts persist to survive for a long period of time after birth or are continuously supplied from the self-renewed embryo-derive B-1 cell pool. Clonal assignment, based on utilization of the same V and J regions, identical CDR3 length, and at least 85% sequence similarity throughout CDR3 sequences of the Ig heavy chain, showed the clonotypic relationship of BM plasmablasts with peritoneal and splenic B-1 cells with a history of embryonic RAG2 expression. The most abundant clonotypes sharing these three embryo-derived B-lineage cells are IGHV11.2/D2.5/J1 and IGHV11.2/D1.1/J1. Furthermore, neonatal depletion of B cells by *in vivo* anti-CD20 antibody treatment reduced embryo-derived BM plasmablasts as well as B-1 cells generated during the embryonic period, while recovery of BM plasmablasts was observed after the transient depletion of plasma cells by bortezomib treatment. In addition, recently reported B-1 plasma cells, which have a short HCDR3 with a frequent lack of the D region sequence, such as CARGPYW or CARGAYW, were not detected in embryo-derived BM

plasmablasts but in adult-derived BM plasmablasts. Taken together, these findings suggest that a profound compartment of natural IgM-producing cells in the BM is continuously supplied from the B-1 cell precursor that is generated during the embryonic period.

Collaborators:

Keiko Fujisaki, Shogo Okazaki

Expression of the J chain in natural antibody-secreting cells

The J chain is essential for IgA dimer formation and poly-Ig receptor (pIgR) binding. Given that it shows no homology to any known protein structure and there are no family molecules, the function of the J chain is incompletely understood, regardless of its immunological importance. We thus sought to examine the expression of the J chain in B-lineage cells to utilize the reporter allele, in which a splice acceptor, EGFP-2A-CreERT2 cassette, and a poly-A sequence are knocked into the first intron of the J chain gene. In the bone marrow (BM) and the spleen of adult $^{J\ chain EGFP\text{-}CreERT2/+}$ mice, EGFP was detected in almost all the plasmablasts expressing IgA and IgM, while B cells, including follicular B cells and B-1 cells, lack EGFP signals. Furthermore, IgA^+ plasmablasts in the small intestinal lamina propria (LP) and Peyer's patches (PPs) expressed EGFP; however, B cells in LP and PPs were EGFP-negative. EGFP expression was also undetectable in B-1 cells in the peritoneal cavity. To assess whether these $EGFP^+$ plasmablasts express IgM pentamers or IgA dimers associated with the J chain inside the cells, we carried out intracellular staining utilizing the His-tagged N-terminal recombinant protein of pIgR, corresponding to the secretory component (SC), with the fluorescent-labeled anti-His-tag antibody This revealed that $EGFP^+$ plasmablasts expressing IgA in the BM, spleen and LP are all reactive to the SC. In contrast, IgM^+ plasmablasts in the BM, spleen and PPs contained the major SC-reactive population and the minor nonreactive population. The IgA^-IgM^- plasmablasts, probably the IgG-producing population, failed to react with the SC. Thus, all the IgA^+ plasmablasts appear to contain dimers linked with the J chain, whereas two distinct IgM^+ plasmablast populations likely exist, containing IgM hexamers or pentamers with or without the J chain.

Collaborators:

Nanako Okamoto, Keiko Fujisaki, Chiharu Nishiyama

Lack of HVEM signals in thymocytes impairs conventional CD8 T cell selection and promotes memory-like CD8 T cell development

Thymocytes having diverse antigen specificities are selected in response to self-MHC-peptide expressed in thymic epithelial cells, which contributes to the formation of a T cell repertoire. However, it is not well understood whether additional signals from epithelial cells are required to drive positive selection. In this study, we found that one of the TNF receptor (TNFR) superfamily members, herpes virus entry mediator (HVEM), when expressed on thymocytes provides signals for positive selection. HVEM deficiency in double-positive (DP) thymocytes impaired the positive selection of CD8 thymocytes. HVEM-deficient thymocytes in OT-1 TCR transgenic mice exhibited significant defects in positive selection and impaired CD69 up-regulation of selected thymocytes. HVEM ligands, LIGHT and BTLA, were expressed in cortical thymic epithelial cells (cTECs). Weak TCR ligation combined with HVEM signals enhanced ERK activation in DP thymocytes developed *in vitro*. Insufficient signals for positive selection in HVEM-deficient DP thymocytes led to the development of CD8 T

cells in an altered T cell lineage, which expressed high levels of CD122 and expanded highly in T cell-deficient mice. These results suggest that thymocytes receive activation signals through HVEM during positive selection. Thus, our findings provide evidence that the threshold of thymocyte positive selection is set by signals from TCR in association with HVEM.

Collaborators:

Yuki Kugii, Yui Kuroki, Yasushi Hara, Naoko Nakano

The spleen is the definitive site for the development and expansion of inhibitor producing-cells in hemophilia A mice upon FVIII infusion

Hemophilia A (HA) is a hereditary bleeding disorder caused by defects in endogenous FVIII. Approximately 20–30% of patients with severe HA who are treated with replacement therapy using recombinant factor VIII (rFVIII) develop neutralizing antibodies (inhibitors) against FVIII, which render the therapy ineffective. A previous report demonstrated that anti-FVIII IgG plasma cells (FVIII-PCs) produced in FVIII-KO mice after rFVIII administration can be detected in both the spleen and bone marrow (BM). However, the extent to which FVIII-PCs produce inhibitors in the lymphoid organs, including the spleen and BM, remains unclear. To identify lymphoid organs that are the definitive sites for the development and expansion of FVIII-PCs in FVIII-treated FVIII-KO mice. When FVIII-KO mice were intravenously injected with rFVIII or lipopolysaccharide (LPS)+rFVIII, in both the treatments, the kinetics of inhibitor titers in serum was consistent with the appearance of FVIII-PCs in their spleens. When splenectomized FVIII-KO mice were treated with rFVIII or LPS+rFVIII, the inhibitor levels in the serum decreased by approximately 80%. Furthermore, when splenocytes or BM cells from inhibitor+ FVIII-KO mice that were treated with LPS and rFVIII were grafted into immune-deficient mice, anti-FVIII IgG was detected only in the serum of splenocyte-administered mice and FVIII-PCs were detected in the spleen but not in the BM. In addition, when splenocytes from inhibitor+ FVIII-KO mice were grafted into splenectomized immune-deficient mice, inhibitor levels were significantly reduced in the serum. The spleen is the major site responsible for the initial production of inhibitors and the enhancement of FVIII-PCs.

Collaborators:

Akihisa Oda, Shoko Furukawa, Masahiro Kitabatake, Noriko Ouji, Shota Sonobe, Kaoru Horiuchi, Takuji Yoshimura, Yuto Nakajima, Kenichi Ogiwara, Midori Shima, Toshihiro Ito, Keiji Nogami

Publications

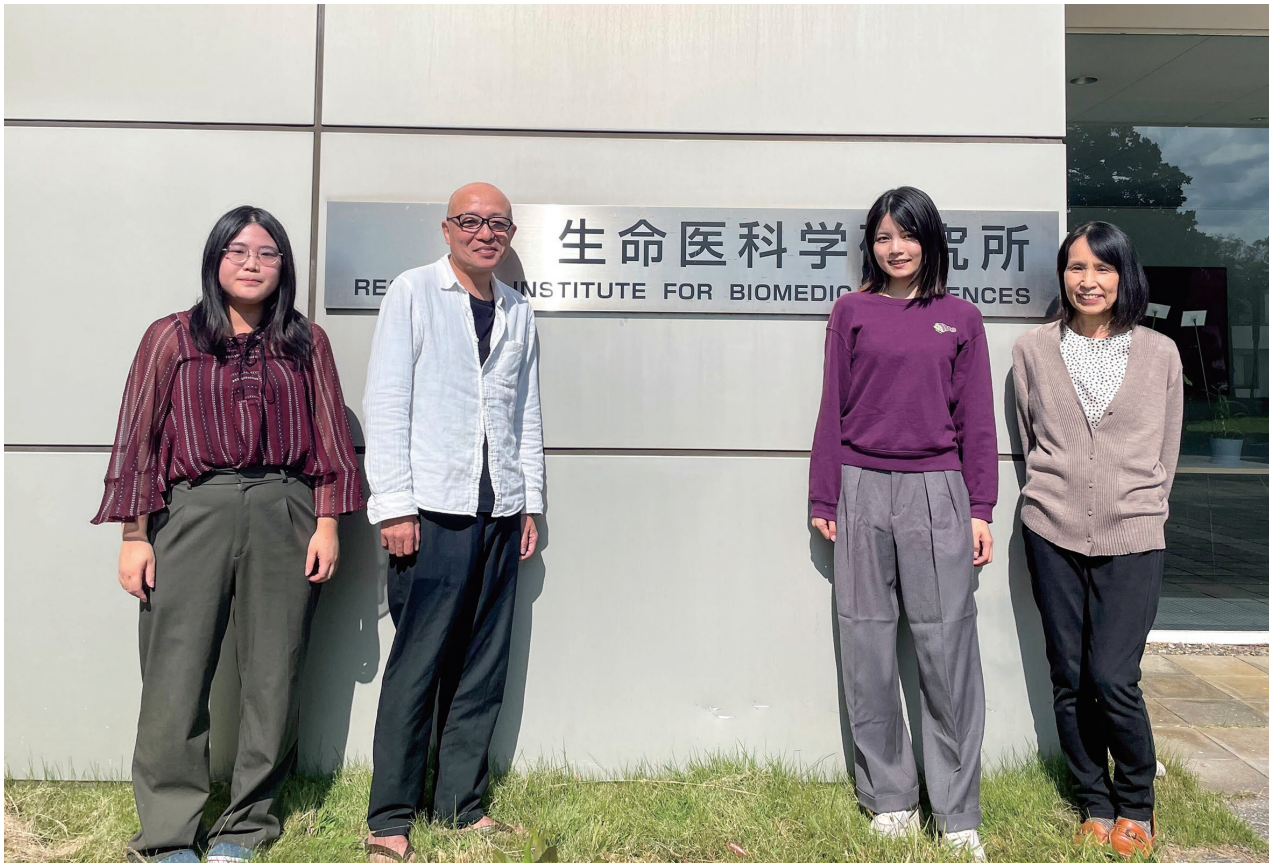
Oda, A., Furukawa, S., Kitabatake, M., Ouji-Sageshima, N., Sonobe, S., Horiuchi, K., Nakajima, Y., Ogiwara, K., Goitsuka, R., Shima, M., Ito, T., Nogami, K.: The spleen is the major site for the development and expansion of inhibitor producing-cells in hemophilia A mice upon FVIII infusion developing high-titer inhibitor. ***Thromb. Res.***, doi: 10.1016/j.thromres.2023.03.003. (2023)

Kugii, Y., Kuroki, Y., Hara, Y., Goitsuka, R. and Nakano, N.: Lack of HVEM signals in thymocytes impairs conventional CD8 T cell selection and promotes memory-like CD8 T cell development. ***J. Immunol***., 210, 1482-1493. (2023)

Katsumoto, T., Ogawara, Y., Yamagata, K., Aikawa, Y., Goitsuka, R., Nakamura, T. and Kitabayashi, I.: MOZ is critical for the development of MOZ/MLL-fusion-induced leukemia through regulation of Hoxa9/Meis1

expression. ***Blood Adv***. 6, 5527-5537. (2022)

Sato, Y., Tsuyusak, M., Takahashi-Iwanaga, H., Fujisawa, R., Masamune, A., Hamada, S., Matsumoto, R., Tanaka, Y., Kakuta, Y., Yamaguchi-Kabata, Y., Furuse, T., Wakana, S., Shimura, T., Kobayashi, R., Shinoda, Y., Goitsuka, R., Maezawa, S., Sadakata, T, Sano, Y, and Furuichi, Y.:Loss of CAPS2/Cadps2 Leads to Exocrine Pancreatic Cell Injury and Intracellular Accumulation of Secretory Granules in Mice. ***Frontier Mol. Bio***. doi: 10.3389/fmolb.2022.1040237. (2022)



Division of Cell Fate Regulation

Masayuki Sakurai, Ph.D.

Research Interest: Deciphering Molecular Mechanisms of Nucleobase Modification:

Gene expression, involving DNA transcription into RNA and protein synthesis, is regulated by nucleobase modifications (A, G, C, T/U). Our research focuses on these modifications, especially adenosine (A) to inosine (I) conversion via deamination, called A-to-I RNA editing event **(Fig.1)**. The edited A, namely I, comes to have a different property from A. The most significant change is I base pairs with G, which changes the genetic code almost the same as the A-to-G transition. ADAR (Adenosine Deaminase Acting on dsRNAs) primarily conducts this editing in metazoans, impacting RNA metabolism such as splicing and defense against foreign RNA. Our recent discoveries reveal ADAR's role extends to RNA:DNA hybrids, suggesting the presence of hidden endogenous A-to-I DNA editing mechanisms in mammalian DNA **(Fig.1)**. To date, our research includes insights into RNA:DNA hybrid dissociation at the telomeric regions in

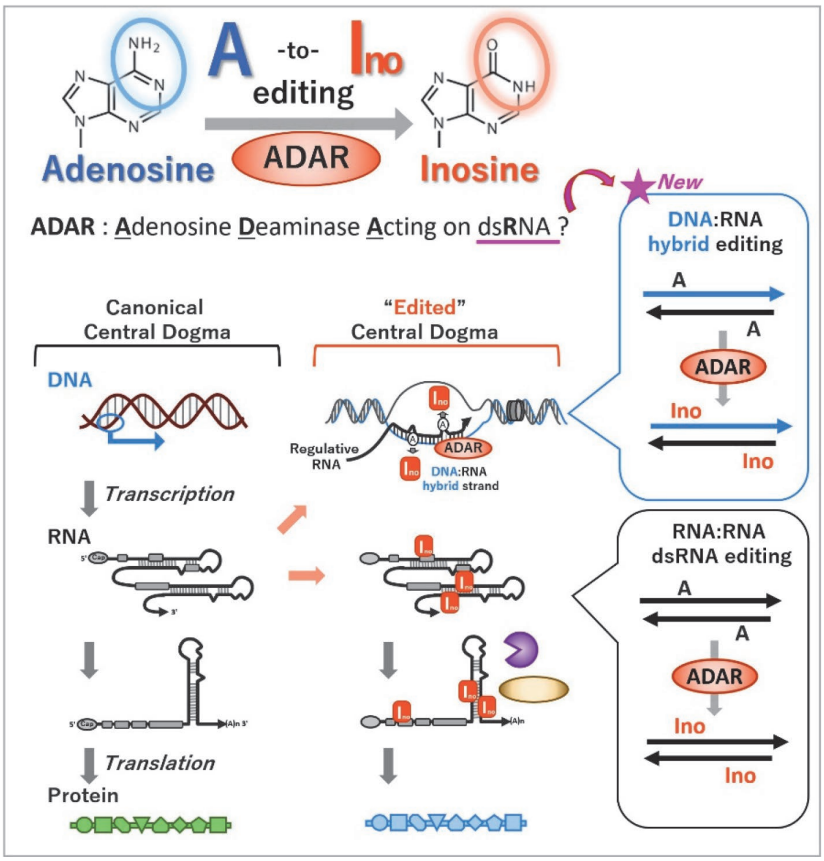


Fig.1. EpiNucleome and ADAR in the Central Dogma: ADAR, known for converting adenosine to inosine in double-stranded RNA, traditionally focuses on A-to-I editing in dsRNA. This editing alters genetic information, influencing protein function and RNA stability. Our findings reveal ADAR's new activity in editing DNA within RNA:DNA hybrids, highlighting its role in modifying DNA structure and sequences.

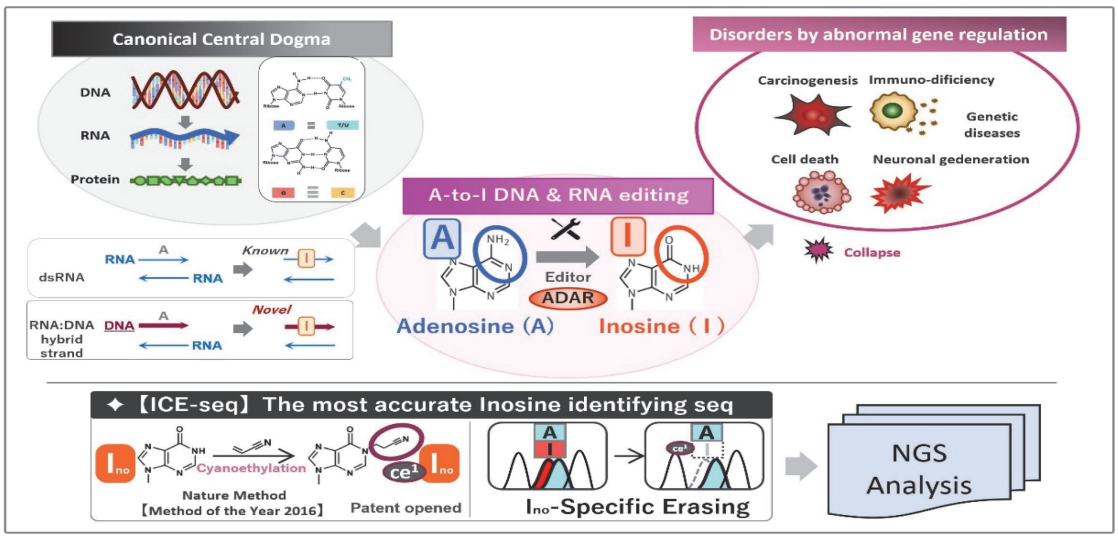


Fig.2. A-to-I RNA & DNA Editing Importance and Detection: A-to-I RNA&DNA editing by ADAR extends beyond traditional roles, impacting genetic information essential for gene expression and cell phenotype. The collapse of this system can cause various diseases. We have identified over 30,000 RNA editing sites using "ICE-seq," demonstrating the breadth of ADAR's editing.

cancer cells, ADAR1's stress response role against dsRNA digestion and apoptosis system, and breast cancer metastasis regulation via GABAA receptor mRNA editing. We've also identified ADAR1's function in a microRNA processing complex with Dicer during embryogenesis. Collaborating with Dr. Zhang Rugang (Nat. Cell Biol. 2022), we've shown that ADAR repression leads to cell senescence. Those findings redefine genetic architecture, emphasizing RNA's role and endogenously edited I in genomic DNA. Our goals are developing inosine labeling technologies in DNA, understanding DNA:RNA strand formation's impact on gene expression, and exploring practical applications in medical science and genetic engineering **(Fig.2)**.

Specific Aim 1: Impact of ADAR1 Downregulation on Cellular Senescence

In our exploration of cellular senescence, we have linked the depletion of p16INK4a-expressing cells, a critical senescence regulative factor, to the reduction in ADAR1, an enzyme vital for RNA editing. This discovery is crucial for understanding the regulation of human cell senescence and associated pathologies such as neurodegeneration **(Fig.3)**. ADAR1 is reduced post-transcriptionally via autophagic degradation, increasing p16INK4a levels and subsequent cellular senescence. This effect, evident in both in vitro and in vivo models, indicates that ADAR1's reduction is a primary trigger for senescence, independent of its RNA-editing function. Mechanistically, ADAR1's downregulation affects SIRT1 expression, controlled by the RNA-binding protein HuR, which influences SIRT1 mRNA stability and, consequently, p16INK4a mRNA translation. Thus, the coordinated downregulation of ADAR1 and SIRT1 is critical for upregulating p16INK4a. In mouse models, reductions in Adar1 expression in aging tissues, such as the brain, ovary, and intestine, correlate with decreased Sirt1 expression. These findings underscore an RNA-editing-independent role for ADAR1 in senescence, primarily through p16INK4a modulation.

This research, enhancing our understanding of senescence mechanisms, was published in Nat. Cell Biol. (2022) and represents a collaborative effort with Xue Hao, Yusuke Shiromoto, Martina Towers, Qiang Zhang, Shuai Wu, Aaron Havas,

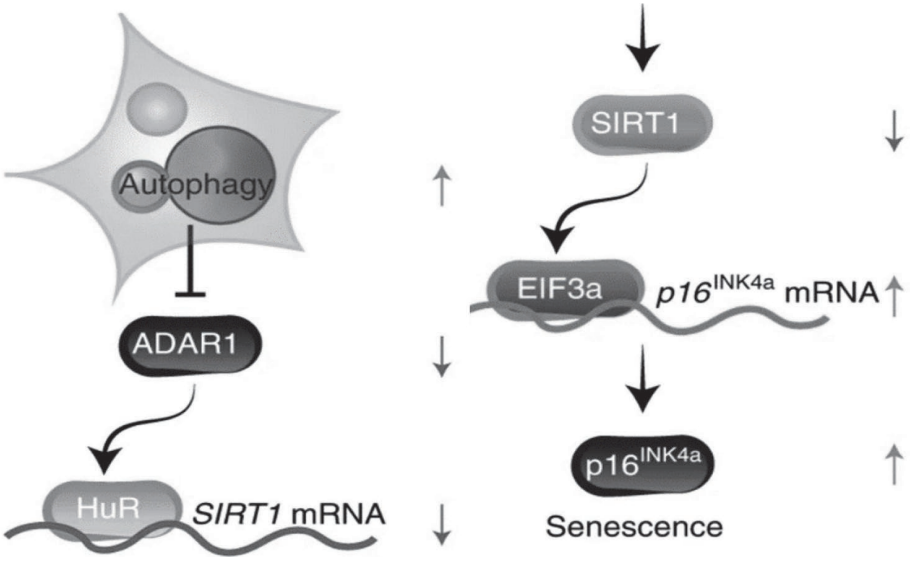


Fig.3. ADAR1's Role in Cellular Aging: This model depicts how ADAR1 downregulation by autophagy induces cellular senescence via p16INK4a upregulation, influenced by SIRT1. It outlines the complex interaction between ADAR1, SIRT1, and p16INK4a in cellular aging.

Lu Wang, Shelly Berger, Peter D. Adams, Bin Tian, Kuzuko Nishikura, Andrew V Kossenkov, and Pingyu Liu from The Wistar Institute, PA, the U.S.

Specific Aim 2: Advancements in Inosine Detection Technologies

Our research has made significant strides in detecting *inosine (Ino)* in DNA, addressing historical challenges in identifying active A-to-I DNA editing sites. Traditional difficulties included attributing A-to-G mutations to spontaneous chemical changes caused by except for A-to-I editing, Ino's replacement by G during PCR amplification, and the challenges of detecting inosine in DNA with only two molecules for each chromosome region only using standard sequencing methods. To overcome these obstacles, we developed "ICE-seq" (Inosine Chemical enrichment sequencing), a groundbreaking inosine identification method. Awarded "Method Of The Year 2016" by Nature Methods, ICE-seq uses inosine-specific cyanoethyl group addition reactions for differentiation in PCR, achieving 97% accuracy and next-generation sequencing compatibility. This method marks a significant leap in epigenetics and molecular biology. Further building on ICE-seq, we have innovated fluorescent and tag-functional labeling techniques for inosine, applicable to RNA and DNA. These techniques have led to new protocols for inosine-specific labeling and affinity purification, enabling the enrichment of nucleic acids with inosine. We apply these advanced methods to identify inosine sites within genomic DNA from cultured human cells and mouse tissues. As of October 2023, the patent application for this technology is complete, ushering in a new phase of inosine research. This project, a collaboration with Dr. Takeshi Wada and Dr. Kazuki Sato from the Faculty of Pharmaceutical Sciences, combines expertise from diverse fields, furthering our understanding and capabilities in genetic research.

This project is a collaborative venture with Dr. Takeshi Wada and Dr. Kazuki Sato from the Faculty of Pharmaceutical Sciences, reflecting a synergy of expertise across multiple disciplines.

Specific Aim 3: Exploring RNA Dynamics in A-to-I Editing and Nova EpiNucleome

Our research focuses on the dynamics of double-stranded RNA and DNA-RNA hybrid strands in cells, examining their influence on gene expression via A-to-I editing. This editing process, mediated mainly by ADAR, is crucial in the intricate 'Nova epinucleome' network, which involves interactions with nucleobase modifying enzymes and cofactor proteins. We are particularly interested in ADAR's role in A-to-I DNA editing and its interactions within DNA-RNA strands, as these are key to understanding various biological processes.

A central element of our study is the R-loop, a complex formed when RNA pairs with its template DNA, potentially acting as a site for A-to-I DNA editing. While typically part of cellular processes, these R-loops can also compromise genomic stability. Our research indicates that suppressing ADAR expression in cells triggers an increase in DNA damage and repair markers, such as γH2AX, RPA32, and DNA-PKCs. There is a notable correlation between R-loops and ADAR expression in HeLa cells and various cancer types. Further, we have delved into the molecular mechanisms behind cell division arrest during mitosis linked to ADAR repression. Our findings suggest that ADAR interacts with genes involved in mitosis regulation and the Spindle Checkpoint during the M-phase, influenced by protein phosphate modifications. Additionally, our studies have identified a specific transcription factor family that responds to R-loop elevation when ADAR expression is suppressed, potentially altering promoter structures and gene expression. This sheds light on the intricate regulation of transcription concerning R-loop formation.

Collaborating with Dr. Michiaki Hamada from Waseda University and Dr. Nobuyoshi Akimitsu from the University of Tokyo, this project aims to elucidate the complexities of RNA-based gene regulation and its impact on cellular functions.

Specific Aim 4: Advancing A-to-I Editing Techniques with Guide Oligonucleotides

In advancing A-to-I DNA editing research, we utilize ADAR's capabilities to introduce inosine artificially at targeted DNA sites using synthetic guide RNA. This method simplifies genome DNA base editing, offering significant potential for genetic engineering and medical science. A key achievement is our novel reporter system for real-time editing efficiency measurement in cells, utilizing targeted editing sequence followed by GFP, and integrated human cell genome. This system signals successful editing by expressing GFP fluorescent when editing occurs, and it is applicable for both RNA and DNA. This innovation marks a significant advancement in monitoring and validating editing processes. Our work now focuses on refining guide-oligonucleotides for high-ratio editing, exploring various chemical structures and sequence designs. This technology's potential spans from in-depth genetic research to practical disease treatment applications.

This project, a collaboration with Dr. Yosuke Katsuta from Kumamoto University and Dr. Daichi Ota from the National Cancer Center, aims to explore new frontiers in genetic editing, contributing significantly to molecular biology and genetics.

Specific Aim 5: Investigating ADAR's Role in Innate Immune Response to SARS-CoV-2

We investigate how cytoplasmic ADARs aid innate immunity by targeting double-stranded RNAs from viruses like SARS-CoV-2. Our study explores ADARs' interaction with SARS-CoV-2

RNAs post-cell entry, addressing gaps in understanding ADAR-mediated A-to-I RNA editing's role in viral mutations. Our approach includes extensive sequence analysis of viral RNA from human airway epithelial-derived cells and organoids post-infection. We aim to pinpoint A-to-I RNA editing sites, employing edited inosine site detection technology. Despite RNA quantity limitations, we are progressing in validating these sites as likely ADAR editing targets. Early results indicate a broad distribution of editing sites across the SARS-CoV-2 genome, especially in the ORF1ab region crucial for viral growth. Notably, the type I interferon response, promoting RNA editing, appears delayed in coronaviruses, hinting at a potential SARS-CoV-2 evasion strategy against the innate immune response.

This research, conducted with Dr. Ken Maeda and Dr. Yudai Kuroda from the National Institute of Infectious Diseases, aims to enhance our understanding of ADAR's function in viral immunity and the complex dynamics of host-pathogen interaction during SARS-CoV-2 infection.

Review articles and books

1. Hao, X., Shiromoto, Y., **Sakurai, M.**, Havas, A., Wang, L., Berger, S., Adams, P.D., Nishikura K., Kossenkov, A.V., Liu,P., Zhang, R. " ADAR1 downregulation by autophagy drives senescence independently of RNA editing by enhancing p16INK4a levels." ***Nat. Cell Biol.*** 1202-1210. [DOI: 10.1038/s41556-022-00959-z.] (2022).
2. **Sakurai, M.**, "Regulation of Genome Stability by RNA and DNA Editing," Tokyo University of Science, Science Education Magazine, Monthly " Kagaku Forum," in Japanese (2022).

Outreach activities

1. **Sakurai, M.**, "New aspects of A-to-I editing by adenosine deamination: molecular mechanisms and cellular dynamic control of RNA & DNA editing," The 45th Annual Meeting of the Molecular Biology Society of Japan Workshop [1PW-04-1] (2022).
2. **Sakurai, M.**, "Development of technology for detection and evaluation of mutation rates and sites by cellular endogenous base editing," Business meeting for companies BioJapan2022 in Japanese (2022).
3. **Sakurai, M.**, "Discovery of a guide RNA-dependent A-to-I DNA editing mechanism intrinsic to mammalian cells and elucidation of its function," JSPS HP "Grant-in-Aid for Scientific Research on Priority Areas" (https://www.jsps.go.jp/j-grantsinaid/37_topics/ index.html) in Japanese (2022).
4. **Sakurai, M.**, "A New Phase of Nucleobase A-to-I Editing by Adenosine Deamination," Joint Symposium of the Division of Data Science and Medical Research and the Division of Nucleic Acid Drug Discovery Research, the National Institute for General Research in Japanese (2022).