Corporate Sponsored Research Program

Project Division of Molecular and Developmental Biology
再生基礎医科学寄付研究部門(ロート製薬, 慈照会, VICX, トミー)

Project Professor Sumiko Watanabe, Ph.D.
Project Senior Assistant Professor Hideto Koso, MD, Ph.D.

特任教授 博士(医学) 渡 辺 すみ子
特任講師 博士(医学) 高 祖 秀 登

Our long-term goal is to understand the molecular mechanisms which coordinately regulate growth and differentiation of stem cells and differentiated cells with emphasis on intracellular signal transduction. Furthermore, recently we are focusing on failure of maintenance of differentiated cells, that ultimately lead to degeneration and cancer. For this purpose we are using models ranging from iPS and various culture cells, zebrafish, mouse, to clinical samples. Based on our research background on the area of cytokine signals, we now focus on the analysis of development and regeneration of neural retina and mechanisms of development of glioblastoma.

The neural retina is a part of the central nervous system (CNS), and regeneration of the retina from retinal stem cells or other sources by transplantation is a critical issue from both clinical and neurobiological points of view. Although reports of successful regeneration of the CNS from neural stem cells (NSC) have appeared in the literature, such has not been the case for the vertebrate neural retina. Furthermore, the nature of retinal stem cells has not been clarified, making it difficult to attempt regeneration of the retina. Based on the techniques and knowledge that have been accumulated through work on of haematopoietic systems in our laboratory, we attempt to identify mammalian retinal stem cells and following developmental processes by revealing the expression pattern of cell surface proteins. We found that various CD antigens mark spatiotemporally distinct populations of retinal cells, and genes specifically expressed in such populations has been revealed by microarray analyses. Various signaling molecules and transcriptional factors are under investigation for their roles on retinal development. We recently focuse on roles of Histone methylation for retinal development and maintenance. For developmental biological analyses, we use zebrafish in addition to mouse as model animals. We also work on molecular analysis of glioma causative genes using mouse model. Projects, which gave major findings during 2017 are as follows.

Cancer gene discovery using the Sleeping Beauty transposon-based insertional mutagenesis

Shingo Ito[1], Hideto Koso, Sumiko Watanabe: [1]Department of Coloproctological Surgery, Juntendo University Graduate School of Medicine

The *Sleeping Beauty* (*SB*) transposon mutagenesis is an unbiased and high-throughput method to profile the landscape of driver genes in a mouse model system. To better understand genes and signaling pathways that are able to transform neural stem cells into brain cancer-initiating cells, we have performed a transposon mutagenesis screen in mice. We identified an RNA-binding protein, DHX15, as a candidate tumor suppressor gene in glioma. *DHX 15* expression was consistently decreased in human glioma cell lines compared with normal neural stem cells. Overexpression of LARP4B in glioma cell lines suppressed proliferation and foci forma-

tion in vitro. Moreover, DHX15 suppressed tumor formtion in a genograft mouse model. ATPase activity was not required for the growth-inhibitory function of DHX15; however, the Ia, Ib, IV, and V motifs, which act as RNA-binding domains in DHX 15, were essential. qPCR analysis revealed that DHX15 suppressed expression of NF-κB downstream target genes as well as the genes involved in splicing. These data provide strong evidence that DHX15 serves as a tumor suppressor gene in glioma.

Analysis of Müller glia specific genes and their histone modification using Hes1-promoter driven EGFP expressing mouse

Toshiro Iwagawa, Masao Nagasaki[2], Yutaka Suzuki[3], Sumiko Watanabe: [2]Division of Biomedical Information Analysis, Department of Integrative Genomics, Tohoku Medical Megabank Organization, Tohoku University, [3]Department of Medical Genome Sciences, Graduate School of Frontier Sciences

Background: Retinal neurons and Müller glia are generated from a common population of multipotent retinal progenitor cells by a process that is regulated by various molecular mechanisms, including transcriptional and post-transcriptional regulation. To clarify the contribution of histone K4 and K27 methylation in this process, we identified Müller glia-specific molecular signatures during retinal development.

Using transgenic mice carrying the *Hes1* promoter (pHes1) and the enhanced green fluorescent protein (EGFP) gene, we purified EGFP-positive Müller glia and other EGFP-negative retinal cells from developing retinas and subjected them to RNA sequence analysis. EGFP-positive cell-specific genes (pHes1-EPGs) were identified by comparing the gene expression patterns of the pHes1-EGFP-positive and -negative fractions. The pHes1-EPGs were similar to genes expressed in retinal progenitors, suggesting that most of the pHes1-positive cells were not induced prior to commitment to the Müller glia lineage, but rather were downregulated in other cell lineages. We examined the modification profiles of H3K27me3 and H3K4me3 at pHes1-EPG-loci by referring to chromatin immunoprecipitation-sequence data of the Cd73-positive and -negative retinal fractions, which represented rods and other cells, respectively. Clustering of pHes1-EPGs by the H3K4me3 and H3K27me3 values followed by ontology analysis using an Ingenuity Pathway Analysis revealed a high incidence of transcription factors in clusters with high H3K27me 3 levels. *Hes1* was included in the cluster, and its expression level decreased dramatically in postnatal retinas. The H3K27me3 level at the *Hes1*-locus was upregulated strongly during retinal development, and the *Hes1* expression level was upregulated in an Ezh2-knockout retina, suggesting that *Hes1* expression was attenuated, at least partly, by modifying H3K27me3. In contrast, no correlation was detected between neurogenic gene expression levels and H3K27me3 levels, except for *Neurod1*. The H3K 27me3 level at the *Neurod1* locus was suppressed after treating the retina with DAPT, which inhibits differentiation of Müller glia, suggesting that commitment to the Müller glia lineage modulates H3K 27me3 status. Müller glia-specific genes overlapped with genes expressed in progenitors, and loci of transcription factors were highly modified with H3 K27me3. These results suggest that downregulation of Müller glia-related genes in other lineage rather than upregulation of them in Müller glia contributed Müller-specific molecular features, and a role for modified H3K27me3 in suppressing Müller glia-related genes in other retinal cell lineages to avoid unfavorable expression.

Pivotal roles of Fezf2 in differentiation of cone OFF bipolar cells and functional maturation of cone ON bipolar cells in retina

Haruna Suzuki-Kerr, Toshiro Iwagawa, Yutaka Suzuki[3], Sumiko Watanabe

During development of the retina, common retinal progenitor cells give rise to six classes of neurons that subsequently further diversify into more than 55 subtypes of neuronal subtypes. Here, we have investigated the expression and function of Fezf2, Fez zinc finger family of protein, in the developing mouse retina. Expression of Fezf2 was strongly observed in the embryonic retinal progenitors at E14 and declined quickly in subsequent development of retina. Then, in postnatal stage at around post natal day 8, Fezf2 was transiently expressed then declined again. Loss-of-function analysis using retinas from mice in which Fezf2 coding region was substituted with β-D-galactosidase showed that Fezf2 is expressed in a subset of cone OFF bipolar cells and required for their differentiation. Using electroretinogram, we found that Fezf2 knockout retina exhibited significantly reduced photopic b-wave, suggesting functional abnormality of cone ON bipolar cells. Furthermore, reduced expression of synaptic protein Trpm1 and structural alteration of ON bipolar cell invagination, both of which affected cone photoreceptor terminal synaptic activity, was identified by transmission electron microscopy and immunohistochemistry, respectively. Taken together, our results show that Fezf2 is indispensable in differentiation of bipolar precursors into cone OFF bipolar cells and in functional maturation of cone ON bipolar cells during development of mouse retina. These results contribute to

our understanding of how diversity of neuronal subtypes and hence specificity of neuronal connections are established in the retina by intrinsic cues.

Analysis of the role of docosahexaenoic acid in retinal degeneration

Hideto Koso, Asano Tsuhako, Hideo Shindo[4], Takao Shimizu[4], Sumiko Watanabe: [4]Department of Lipid Signaling, National Center for Global Health and Medicine

We analyzed the role of docosahexaenoic acid (DHA) in the retina. DHA has essential roles in photoreceptor cells in the retina and is therefore crucial to healthy vision. Although the influence of dietary DHA on visual acuity is well known and the retina has an abundance of DHA-containing phospholipids (PL-DHA), the mechanisms associated with DHA's effects on visual function are unknown. By using comprehensive phospholipid analyses and imaging mass spectroscopy, we found that LPAAT3 is expressed in the inner segment of photoreceptor cells and that PL-DHA disappears from the outer segment in the LPAAT3-knock-out mice. Following loss of PL-DHA, LPAAT3-knock-out mice exhibited abnormalities in the retinal layers, such as incomplete elongation of the outer segment and decreased thickness of the outer nuclear layers and impaired visual function, as well as disordered disc morphology in photoreceptor cells. Our results indicate that PL-DHA contributes to visual function by maintaining the disc shape in photoreceptor cells and that this is a function of DHA in the retina. This study thus provides the reason why DHA is required for visual acuity and may help inform approaches for overcoming retinal disorders associated with DHA deficiency or dysfunction.

Publications

Ito S, Koso H, Sakamoto K, Watanabe S. (2017) RNA helicase DHX15 acts as a tumour suppressor in glioma. *Br. J. Cancer* 117, 1349-1359

Shindou H, Koso H, Sasaki J, Nakanishi H, Sagara H, Nakagawa KM, Takahashi Y, Hishikawa D, Iizuka-Hishikawa Y, Tokumasu F, Noguchi H, Watanabe S, Sasaki T, Shimizu T. (2017) Docosahexaenoic acid preserves visual function by maintaining correct disc morphology in retinal photoreceptor cells. *J. Biol. Chem.* 292, 12054-12064

Ueno, K., Iwagawa, T., Ochiai, G., Koso, H., Nakauchi, H., Nagasaki, M., Suzuki, Y., Watanabe, S. (2017) Analysis of Muller glia specific genes and their histone modifications using Hes 1-promoter driven EGFP expressing mouse. Sci Rep, 7, 3578

Suzuki-Kerr, H., Baba, Y., Tsuhako, A., Koso, H., Dekker, J. D., Tucker, H. O., Kuribayashi, H., Watanabe, S. (2017) Forkhead box protein P1 is dispensable for retina but essential for lens development. Invest. Ophthalmol. Vis. Sci. 158, 1916-1929

Social Cooperation Research Program

Project Division of RNA Medical Science
RNA医科学社会連携研究部門

Project Associate Professor	Masaki Takahashi, Ph.D.	
Project Senior Assistant Professor	Masahiko Imashimizu, Ph.D.	
Project Assistant Professor	Jeewoong Park, Ph.D.	
特任准教授	博士(理学)	高 橋 理 貴
特任講師	博士(農学)	今清水 正 彦
特任助教	博士(理学)	朴 智 雄

RNA no longer stands behind DNA or protein but stands in front of DNA and protein. Recent achievements and discovery in biological science clearly emphasize the importance of RNA in life: the discovery of RNA interference, molecular mimicry between protein and RNA, ribosome structure at atomic resolution, and RNA quality control triggered by aberrant mRNAs. Moreover, the completed human genome project revealed, to our great surprise, the existence of a large amount of protein-noncoding RNAs (ncRNAs). These ncRNAs can be classified into two types: one, like antisense and microRNA, those function with sequence complementarity to the target mRNA or DNA, while the other, like aptamer, those function independent of sequence complementarity. In our laboratory, we aim to: 1) create artificial aptamers to target proteins of therapeutic interest: and 2) uncover the molecular mechanism underlying the versatile interaction between nucleic acid and protein of biological significance.

1. RNA Aptamer Discovery

Masaki Takahashi

Therapeutic molecules can be classified as low-, middle- and high-molecular weight drugs depending on their molecular masses. Antibodies represent high-molecular weight drugs and their clinical applications have been developing rapidly. Aptamers, on the other hand, are middle-molecular weight molecules that are short, single-stranded nucleic acid sequences that are selected in vitro from large oligonucleotide libraries based on their high affinity to a target molecule. Hence, aptamers can be thought of as a nucleic acid analog to antibodies. However, several viewpoints hold that the potential of aptamers arises from interesting characteristics that are distinct from, or in some cases, superior to those of antibodies. Recently, therapeutic middle molecules gain considerable attention as protein-protein interaction (PPI) inhibitors.

a. Aptamers targeting cell surface proteins.

High affinity binders targeting specific cell surface proteins are vital for development of basic and applied biosciences. However, despite sustained efforts to generate such binders by chemicals and antibodies, there are still many cell surface proteins that lack high affinity binders. Nucleic acid aptamers have potential as binding molecules for cell surface proteins, because they form distinct structures that have high affinity and specificity for a wide range of targets. Aptamers are isolated from large combinatorial libraries using a unique iterative selection-amplification process known as systematic evolution of ligands by exponential enrichment (SELEX). Among advantages of this method, purified and complex heterogeneous targets, such as bacteria, viruses, and whole-living cells, can be used for selection of aptamers. Moreover, SELEX allows generation of cell-surface-specific aptamers without prior knowledge of expression profiles in target

cells. Therefore, the technology has been widely used as a valid and feasible method to generate specific binders against cell surface proteins with intact structure.

b. NMR monitoring of the SELEX process to confirm enrichment of structured RNA.

Ryo Amano[1], Kazuteru Aoki[2], Shin Miyakawa[2], Yoshikazu Nakamura, Tomoko Kozu[3], Gota Kawai[1], Taiichi Sakamoto[1]: [1]Department of Life and Environmental Sciences, Faculty of Engineering, Chiba Institute of Technology, Chiba, Japan, [2]RIBOMIC Inc., Tokyo, Japan, [3]Research Institute for Clinical Oncology, Saitama Cancer Center, Saitama, Japan,

RNA aptamers are selected from an RNA pool typically comprising up to 10^{15} different sequences generated by SELEX procedure. Over several rounds of SELEX, the diversity of the RNA pool decreases and the aptamers are enriched. Hence, monitoring of the enrichment of these RNA pools is critical for the successful selection of aptamers, and several methods for monitoring them have been developed. In this study, we measured one-dimensional imino proton NMR spectra of RNA pools during SELEX. The spectrum of the initial RNA pool indicates that the RNAs adopt tertiary structures. The structural diversity of the RNA pools was shown to depend highly on the design of the primer-binding sequence. Furthermore, we demonstrate that enrichment of RNA aptamers can be monitored using NMR. The RNA pools can be recovered from the NMR tube after measurement of NMR spectra. We also can monitor target binding in the NMR tubes. Thus, we propose using NMR to monitor the enrichment of structured aptamers during the SELEX process.

c. Alkaline-tolerant RNA aptamers useful to purify acid-sensitive antibodies in neutral conditions.

Emire Inomata[1], Erika Tashiro[2], Shin Miyakawa[1], Yoshikazu Nakamura, Kazumasa Akita[1]: [1]RIBOMIC Inc., Tokyo, Japan, [2]EVEC, Inc., Hokkaido, Japan.

Aptamer could be used for various purposes, not only therapeutics but also diagnostics, and applicable to affinity chromatography as a carrier molecule to purify proteins of interest. Here we demonstrate the usage and advantages of RNA aptamer to Fc region of human IgG (i.e., IgG aptamer) for purification of human antibodies. IgG aptamer requires divalent cations for binding to IgG and bound IgG dissociates easily upon treatment with chelating reagent, such as EDTA, under neutral conditions. This elution step is very mild and advantageous for maintaining active conformations of therapeutic antibodies compared to the widely used affinity purification with Protein A/G, which requires acidic elution that often damages the active conformation of antibodies. In fact, of several monoclonal antibodies tested, three antibodies were prone to aggregate on acidic elution from the Protein A/G resin, while remained fully active upon neutral elution from the IgG aptamer resin. The IgG aptamer was fully manipulated to alkaline resistant by ribose 2′-modifications, and thereby reusable numerous times with 1 N NaOH washing. The capacity of the aptamer resin to bind IgG was equivalent to that of the Protein A/G resin. Therefore, the IgG aptamer will provide us with a unique tool to uncover and purify human monoclonal antibodies, which hold therapeutic potential but lose the activity upon acidic elution from Protein A/G-based affinity resin.

2. Molecular Mechanism Underlying the Versatile Interaction between Nucleic Acid and Protein

a. Nonstop-mRNA decay machinery is involved in the clearance of mRNA 5′-fragments produced by RNAi and NMD in *Drosophila melanogaster* cells.

Yoshifumi Hashimoto, Masaki Takahashi, Eri Sakota, Yoshikazu Nakamura

When translating mRNAs are cleaved in protein-coding regions, 5′ fragments of mRNAs are detached from stop codons (i.e., nonstop mRNAs) and protected from 3′-5′ exonucleases by ribosomes stalled at the 3′ termini. It has been shown in yeast that the nonstop mRNA decay (NSD) machinery triggers nonstop mRNA degradation by removing stalled ribosomes in the artificial reporter mRNAs. However, it is not known well whether NSD is involved in the degradation of endogenous nonstop mRNAs in higher eukaryotes. In this work, we addressed the question of whether 5′-nonstop-mRNA fragments generated by siRNA cleavage or nonsense-mediated-mRNA decay (NMD) are degraded by the NSD pathway in *Drosophila melanogaster* cells by knocking down three NSD components, Pelota (a yeast Dom34 homolog), Hbs1 and ABCE1 (a ribosome-recycling factor). We found that double, but not single, knockdown of any two of these three factors efficiently stabilized nonstop reporter mRNAs and triple knockdown of Pelota, Hbs1 and ABCE1 further stabilized nonstop mRNAs in highly ribosome-associated state. These findings demonstrated that Pelota, Hbs1 and ABCE1 are crucial for NSD in *Drosophila* cells as in yeast for rescuing stalled ribosomes and degrading nonstop mRNAs. To our knowledge, this is the first comprehensive

report to show the involvement of the NSD machinery in the clearance of mRNA 5′-fragments produced by RNAi and NMD in eukaryotes.

b. Transcription pausing: biological significance of thermal fluctuations biased by repetitive genomic sequences.

Masahiko Imashimizu, David B. Lukatsky[1]: [1]Department of Chemistry, Ben-Gurion University of the Negev, Be'er Sheva, Israel.

Transcription of DNA by RNA polymerase (RNAP) takes place in a cell environment dominated by thermal fluctuations. How are transcription reactions including initiation, elongation, and termination on genomic DNA so well-controlled during such fluctuations? A recent statistical mechanical approach using high-throughput sequencing data reveals that repetitive DNA sequence elements embedded into a genomic sequence provide the key mechanism to functionally bias the fluctuations of transcription elongation complexes. In particular, during elongation pausing, such repetitive sequence elements can increase the magnitude of one-dimensional diffusion of the RNAP enzyme on the DNA upstream of the pausing site, generating a large variation in the dwell times of RNAP pausing under the control of these genomic signals.

Publications

1 Hashimoto, Y., Takahashi, M., Sakota, E., Nakamura, Y.: Nonstop-mRNA decay machinery is involved in the clearance of mRNA 5′-fragments produced by RNAi and NMD in Drosophila melanogaster cells. Biochem Biophys Res Commun. Feb 26; 484(1): 1-7. (2017)

2 Amano, R., Aoki, K., Miyakawa, S., Nakamura, Y., Kozu, T., Kawai, G., Sakamoto, T.: NMR monitoring of the SELEX process to confirm enrichment of structured RNA. Sci Rep., Mar 21; 7 (1): 283. (2017)

3 Nakamura, Y.: Aptamers as therapeutic middle molecules. Biochimie. Accepted online Oct 16. (2017)

4 Sakamoto, T., Ennifar, E., Nakamura, Y.: Thermodynamic study of aptamers binding to their target proteins. Biochimie. Accepted online Oct 18. (2017)

5 Inomata, E., Tashiro, E., Miyakawa, S., Nakamura, Y., Akita, K.: Alkaline-tolerant RNA aptamers useful to purify acid-sensitive antibodies in neutral conditions. Biochimie. Accepted online Nov 10. (2017)

6 Imashimizu, M., Lukatsky, DB.: Transcription pausing: biological significance of thermal fluctuations biased by repetitive genomic sequences. Transcription. Dec 1: 1-8. (2017)

7 Nomura, Y., Yamazaki, K., Amano, R., Takada, K., Nagata, T., Kobayashi, N., Tanaka, Y., Fukunaga, J., Katahira, M., Kozu, T., Nakamura, Y., Haishima, Y., Torigoe, H., Sakamoto, T.: Conjugation of two RNA aptamers improves binding affinity to AML1 Runt domain. J Biochem. Dec 1; 162(6): 431-436. (2017)

8 Takahashi, M.: Aptamers targeting cell surface proteins. Biochimie. Accepted online Dec 2. (2017)

9 Takada, K., Amano, R., Nomura, Y., Tanaka, Y., Sugiyama, S., Nagata, T., Katahira, M., Nakamura, Y., Kozu, T., Sakamoto, T.: Characterisation of an aptamer against Runt domain of AML 1 (RUNX1) by NMR and mutational analyses. FEBS Open Bio, Accepted online Dec 15 (2017).

10 Goto, R., Miyakawa, S., Inomata, E., Shioyama, S., Nishida, Y., Yamaura, J., Nakamura, Y.: De novo sequencing of highly modified therapeutic oligonucleotides by hydrophobic tag sequencing coupled with LC-MS. J Mass Spectrom., 52 (2): 78-93 (2017).

Social Cooperation Research Program

Project Division of Systems Immunology Research
システム免疫学社会連携研究部門(医学生物学研究所)

Project Associate Professor Takeshi Satoh, Ph.D.
Project Assistant Professor Yasumasa Kimura, Ph.D.

特任准教授 博士(理学) 佐 藤 毅 史
特任助教 博士(理学) 木 村 恭 将

The immune system in mammals consists of many types of cells. They interact with each other and construct a complex network to maintain homeostasis and protect from pathogens. Our goal is to investigate the function of each immune cell from various points of view and analyze the multicellular events using bioinformatics. Furthermore, we will apply the knowledge from our research for the discovery of novel drugs or methods for the treatment of immune diseases.

1. Construction of fast bioinformatics pipelines for meta-genome analysis

Yasumasa Kimura, Shuji Suzuki[1], Masanori Kakuta[1], Rui Yamaguchi[2], Seiya Imoto[3], Yasushi Akiyama[1], Hiroshi Kiyono[4], Satoru Miyano[2], Satoshi Uematsu[5], Takeshi Satoh: [1]Department of Computer Science, Graduate School of Information Science and Engineering, Tokyo Institute of Technology. [2]Human Genome Center, The Institute of Medical Science, The University of Tokyo. [3]Division of Health Medical Data Science, Health Intelligence Center, The Institute of Medical Science, The University of Tokyo. [4]Division of Mucosal Immunology, Department of Microbiology and Immunology, The Institute of Medical Science, The University of Tokyo. [5]Division of Innate Immune regulation, International Research and Development Center for Mucosal Vaccines, The Institute of Medical Science, The University of Tokyo.

There are huge numbers of intestinal commensal bacteria (more than 100 trillions in human) mutually interacting host organism and regulating host immune system. In recent studies, bacterial 16S rRNA sequencing method is used for analysis of bacterial flora. However, the analysis with whole genome sequencing method provides more detailed and precise data to investigate populations of microorganisms in the gut. To develop a workflow for meta-genome analysis with whole genome sequencing method, we initiated collaboration with Human Genome Center in our institute and Tokyo Institute of Technology for high performance computing to analyze meta-genome sequence produced by next generation sequencers. Using already acquired meta-genome sequencing data from human feces DNA, we constructed new meta-genome analysis pipeline with ultra rapid software, GHOST-MP, and super computer. When general homology search method is used for meta-genome analysis without super computer, the analysis time is more than two weeks. Combining GHOST-MP with super computer, we archived "10 minutes analysis." This indicates that the pipeline enables us to multiple sample analysis in the short time and also various-type analysis. With this pipeline, we will collect various meta-genome sample data from genetically modified mice associated with diseases and human. Analysis of these huge and various kinds of data will lead to the discovery of etiology and new therapies.

2. Development of new method for "virome" analysis

Yasumasa Kimura, Kosuke Fujimoto[1], Shanai Yin, Yuki Usui, Naoki Takemura[1], Yasuo Ouchi[1], Shuji Suzuki[2], Masanori Kakuta[3], Rui Yamaguchi[3], Seiya Imoto[4], Yasushi Akiyama[2], Hiroshi Kiyono[5], Satoru Miyano[3], Satoshi Uematsu[1], Takeshi Satoh: [1]Division of Innate Immune regulation, International Research and Development Center for Mucosal Vaccines, The Institute of Medical Science, The University of Tokyo. [2]Department of Computer Science, Graduate School of Information Science and Engineering, Tokyo Institute of Technology. [3]Human Genome Center, The Institute of Medical Science, The University of Tokyo. [4]Division of Health Medical Data Science, Health Intelligence Center, The Institute of Medical Science, The University of Tokyo. [5]Division of Mucosal Immunology, Department of Microbiology and Immunology, The Institute of Medical Science, The University of Tokyo.

Numerous numbers of microorganisms reside in the mammal gut. Many studies showed that commensal bacteria are involved in the host health. In fact, various viruses, including bacteriophage, also exist in this tissue. Only a few studies about mucosal virus meta-genome (virome) have been done. Therefore, roles of the viruses in the gut for the health remain unclear. We focused on the virus population in the gut and tried to establish virome analysis method. To recover the viruses in the mucosal tissue, we need to apply different protocols from bacteria separation. We invented a new method to separate viruses from feces samples effectively using detergents and lytic enzymes. This protocol increases the recovery of virus comparing with that of other researchers. With this powerful technique, we are going ahead with virome study for mice and human.

3. Construction of bioinformatics pipelines for virome analysis

Yasumasa Kimura, Seiya Imoto[1], Shuji Suzuki[2], Masanori Kakuta[3], Rui Yamaguchi[3], Yasushi Akiyama[2], Hiroshi Kiyono[4], Satoru Miyano[3], Satoshi Uematsu[5], Takeshi Satoh: [1]Division of Health Medical Data Science, Health Intelligence Center, The Institute of Medical Science, The University of Tokyo. [2]Department of Computer Science, Graduate School of Information Science and Engineering, Tokyo Institute of Technology. [3]Human Genome Center, The Institute of Medical Science, The University of Tokyo. [4]Division of Mucosal Immunology, Department of Microbiology and Immunology, The Institute of Medical Science, The University of Tokyo. [5]Division of Innate Immune regulation, International Research and Development Center for Mucosal Vaccines, The Institute of Medical Science, The University of Tokyo.

The vast majority of viral metegenomic reads do not align to currently known viral sequences; these are termed "viral dark matter" and cause a major obstacle in comprehensively defining viromes. We constructed a computational pipeline that reconstructs viral genomes by assembling sequence reads (contig generation), predicts and annotates open reading frames (ORFs) on the contigs, and estimates their taxa. The pipeline further enabled us to analyze unknown viruses, which are the source of "viral dark matter." We use our virus separation method with this pipeline and accelerate virome study.

4. Analysis of phage-bacteria associations

Yasumasa Kimura, Seiya Imoto[1], Georg Tremmel[2], Kosuke Fujimoto[3], Yuki Usui, Rui Yamaguchi[2], Hiroshi Kiyono[4], Satoru Miyano[2], Satoshi Uematsu[3], Takeshi Satoh: [1]Division of Health Medical Data Science, Health Intelligence Center, The Institute of Medical Science, The University of Tokyo. [2]Human Genome Center, The Institute of Medical Science, The University of Tokyo. [3]Division of Innate Immune regulation, International Research and Development Center for Mucosal Vaccines, The Institute of Medical Science, The University of Tokyo. [4]Division of Mucosal Immunology, Department of Microbiology and Immunology, The Institute of Medical Science, The University of Tokyo.

The majority of intestinal phages are unknown. One way of defining those phages is to know their host bacteria. We performed bacteriome metagenome analyses of the same fecal samples that were used to analyze viromes for examining phage-bacteria associations. The lysogenized prophage sequences on bacterial contigs were comprehensively detected by searching homologous sequences of the viral contigs. CRISPR (clustered regularly interspaced short palindromic repeats) loci hold phage genomic fragments (CRISPR spacers) as a record of past infections by both temperate and virulent phages. By detecting CRISPR spacers on bacterial contigs and confirming their origins on viral contigs, we identified CRISPR spacer-based host-parasite associations. The identified phage-bacteria associations provide fundamental information for characterizing unknown phages.

5. Optimization of 16S rRNA metagenome analysis protocol

Yasumasa Kimura, Hiroko Ohmiya, Kosuke Fujimoto[1], Yuki Usui, Rui Yamaguchi[2], Seiya Imoto[3], Hiroshi Kiyono[4], Satoru Miyano[2], Satoshi Uematsu[1], Takeshi Satoh: [1]Division of Innate Immune regulation, International Research and Develop-

ment Center for Mucosal Vaccines, The Institute of Medical Science, The University of Tokyo. [2]Human Genome Center, The Institute of Medical Science, The University of Tokyo. [3]Division of Health Medical Data Science, Health Intelligence Center, The Institute of Medical Science, The University of Tokyo. [4]Division of Mucosal Immunology, Department of Microbiology and Immunology, The Institute of Medical Science, The University of Tokyo.

In our body, a huge number of microbes are colonized. They have beneficial and occasionally detrimental effects for our health. Therefore, many researchers focused on analysis of microbial community with 16S rRNA sequence method. Although this method has been used generally in the world, each researcher uses slightly different protocol. Here, we focused on target regions of 16S rRNA gene sequencing and compared coverage and phylum spectrum. From our results, the region including V4 was the best for detecting wide diversity of microbiota.

Publications

Eri T, Kawahata K, Kanzaki T, Imamura M, Michishita K, Akahira L, Bannai E, Yoshikawa N, Kimura Y, Satoh T, Uematsu S, Tanaka H, Yamamoto K. Intestinal microbiota link lymphopenia to murine autoimmunity via PD-1+ CXCR5−/dim B-helper T cell induction. Sci Rep. 2017 Apr 26; 7: 46037.

Takase Y, Usui K, Shimizu K, Kimura Y, Ichihara T, Ohkawa T, Atsumi J, Enokida Y, Nakazawa S, Obayashi K, Ohtaki Y, Nagashima T, Mitani Y, Takeyoshi I. Highly sensitive detection of a HER2 12-base pair duplicated insertion mutation in lung cancer using the Eprobe-PCR method. PLoS one. 2017; 12(2): e0171225.

Social Cooperation Research Program

Project Division of Advanced Regenerative Medicine
先端的再生医療社会連携研究部門

Professor	Arinobu Tojo, M.D., D.M.Sc.	教授(兼務) 医学博士	東 條 有 伸
Project Associate Professor	Satoshi Yamazaki, Ph.,D	特任准教授 博士(生命科学)	山 﨑 聡

Our major goal is to develop the regenerative medicine and to restore the physical impairments with various types of stem cells, which include mesenchymal stem/stromal cells (MSC) and pluripotent stem cells (PSC), such as embryonic stem cells (ESC) and induced pluripotent stem cells (iPSC). Currently our efforts are focused on 1) characterization of MSCs derived from various sources (adipose tissue, umbilical cord, and iPSC) for their tissue regenerative ability, 2) application of patient-derived iPSCs to delineate the pathogenesis of intractable disorders and to develop their breakthrough therapies.

1. Elucidation of regulatory mechanisms underlying self-renewal and differentiation of hematopoietic stem cells

Izawa K, Tojo A, Satoshi Yamazaki

Our studies focus mainly on investigation of stem cell biology using the hematopoietic stem cell (HSC) as a research model. Recent identification of a variety of stem cell sources including embryonic and somatic (tissue-specific) stem cells has brought about substantial progress in the field of stem cell research. The HSC represents the first stem cell for which identity and existence were determined. Studies on HSCs have provided us with some basic concepts applying to different types of stem cells, yet many of these concepts remain unverified. It therefore is very important to continue basic studies to answer many questions left unsolved and thus to permit contributions to the field of biological research and clinical medicine. HSCs are capable of continuous supply of all lineages of blood cells to each individual for his or her entire life. Both self-renewal and multilineage differentiation potentials enable this task. One major advantage in HSC research lies in that established assay systems allow clonal analysis of each individual stem cell. Using a defined assay system, we can test capabilities of self-renewal and multilineage differentiation at single cell levels using either in vitro or in vivo assays. We believe that HSC research will eventually make great contributions to the development of safe and efficacious regenerative medicine and gene therapy.

2. Basic research for developing cell-based therapy using human mesenchymal stem/stromal cells

Nonaka H, Ota S, Yumoto M, Hasegawa H, Nagamura T, Tojo A, Satoshi Yamazaki

Mesenchymal stromal/stem cells (MSCs) have great potential for use in regenerative medicine and cell-based therapies. There are growing expectations that such advanced therapeutic procedures will provide solutions for unmet medical needs. Currently, more than 300 clinical trials using MSCs are ongoing worldwide for the treatment of various diseases. However, to bring safe and efficient MSC-based therapies into practical use, fundamental and therapeutic properties of MSCs remain to be understood. In this project, we examined basic, immunosuppressive and anti-fibrotic properties of MSCs

derived from different tissues and showed that MSCs exhibited distinct characteristics depending on their origin. We also compared MSCs expanded in culture medium supplemented with FBS to MSCs expanded in in-house formulated serum-free medium. Interestingly, MSCs exhibited distinct therapeutic potential depending on the expansion condition in several in vitro assays. Moreover, the similar effects of the culture condition on the characteristics of MSCs obtained by were observed within MSCs derived from different tissues. To understand molecular mechanisms underlying the distinct properties of MSCs, we analyzed gene expression profiles and identified a set of genes that were differentially expressed in MSCs either from different tissues or expanded in different medium. Outcomes from our research lead to identifying a new biomarker and a better understanding of MSCs' therapeutic potential.

3. Generation of disease-specific human iPS cells

Izawa K, Tojo A, Satoshi Yamazaki

Here, using intractable disease-specific human iPS, we are intended to elucidate the pathogenetic mechanism and develop a new agent for the treatment of disease. Myelodysplastic symdrome (MDS) is a group of disorders that healthy blood cells are poorly formed in bone marrow (BM). However, the disease developing mechanism is not clear. First, we used a MDS patient sample, which has a point mutation in *splicing factor 3B, subunit1 (SF3B1) gene,* to generate MDS-specific iPS. 12 iPS clones were generated from $CD34^+$ cells that were isolated from the patient BMCs. So far, unfortunately, 8 of 12 clones had no variation in *SF3B1*. The second generation sequencing analysis of $CD34^+$ cells revealed a mutant allele burden (MAB) of 37.6%. *SF3B1* mutation is the herterozygous one, therefore, MAB of 50% implies that 100% of the cells carry the mutation. These results suggest that it is more difficult to reprogram the *SF3B1* variant $CD34^+$ cells than normal that. We will analyze the *SF3B1* mutation of other 4 clones and then generate new iPSC clones.

Publications

1. Kon A, Yamazaki S, Nannya Y, Kataoka K, Ota Y, Nakagawa MM, Yoshida K, Shiozawa Y, Morita M, Yoshizato T, Sanada M, Nakayama M, Koseki H, Nakauchi H, Ogawa S. 2017. Physiological *Srsf2* P95H expression causes impaired hematopoietic stem cell functions and aberrant RNA splicing in mice. *Blood*. pii: blood-2017-01-762393. doi: 10.1182/blood-2017-01-762393. [Epub ahead of print] PMID: 29146882
2. Tsukada M, Ota Y, Wilkinson AC, Becker HJ, Osato M, Nakauchi H, Yamazaki S. In Vivo Generation of Engraftable Murine Hematopoietic Stem Cells by Gfi1b, c-Fos, and Gata2 Overexpression within Teratoma. *Stem Cell Reports*. 2017 Oct 10; 9(4): 1024-1033. doi: 10.1016/j.stemcr.2017.08.010. Epub 2017 Sep 21. PMID: 28943250
3. Masai H, Kanoh Y, Moriyama K, Yamazaki S, Yoshizawa N, Matsumoto S. Telomere-binding factors in the regulation of DNA replication. *Genes Genet Syst*. 2017 Jun 30. doi: 10.1266/ggs.17-00008. [Epub ahead of print] PMID: 28674277
4. Tomura T, Nagashima S, Yamazaki S, Iizuka T, Fudou R, Ojika M. An Unusual Diterpene-Enhygromic Acid and Deoxyenhygrolides from a Marine Myxobacterium, Enhygromyxa sp. *Mar Drugs*. 2017 Apr 6; 15(4). pii: E109. doi: 10.3390/md15040109. PMID: 28383484
5. Ieyasu A, Ishida R, Kimura T, Morita M, Wilkinson AC, Sudo K, Nishimura T, Ohehara J, Tajima Y, Lai CY, Otsu M, Nakamura Y, Ema H, Nakauchi H, Yamazaki S. An All-Recombinant Protein-Based Culture System Specifically Identifies Hematopoietic Stem Cell Maintenance Factors. *Stem Cell Reports.* 2017 Mar 14; 8(3): 500-508. doi: 10.1016/j.stemcr.2017.01.015. Epub 2017 Feb 23. PMID: 28238792
6. Tajima Y, Ito K, Umino A, Wilkinson AC, Nakauchi H, Yamazaki S. Continuous cell supply from Krt7-expressing hematopoietic stem cells during native hematopoiesis revealed by targeted in vivo gene transfer method. *Sci Rep.* 2017 Jan 18; 7: 40684. doi: 10.1038/srep40684. PMID: 28098173

Social Cooperation Research Program

Project Division of International Advanced Medical Research

国際先端医療社会連携研究部門

Project Associate Professor　Koichiro Yuji, M.D., Ph.D.　特任准教授　博士(医学)　湯　地　晃一郎

The mission of the Project Division is to apply changes in advanced medical research at the Institute of Medical Science at the University of Tokyo (IMSUT). Our activities include field research in which innovative medicine will be implemented; cross-disciplinary education of physicians, researchers, and professionals; collaboration in innovative projects in the Coastal Area Life Innovation Comprehensive Special Zone for International Competitiveness Development; and establishing projections of the future healthcare system of Japan, which will be the first fully fledged aged society.

Implementing advanced medical research at IMSUT

Yuji, K.

The Project Division was established in November 2014. Our mission is to contribute to the progress of advanced medical research at IMSUT; to perform field research in which innovative medicine will be implemented; and to further the cross-disciplinary education of physicians, researchers, and professionals. Our future plans include collaboration in innovative projects in the Coastal Area Life Innovation Comprehensive Special Zone for International Competitiveness Development.

Projections on the future healthcare system in Japan, the first fully fledged aged society

Yuji, K.

Japan is rapidly becoming a fully fledged aged society, and the increasing dependence of the elderly population is a significant concern. We have simulated both the supply and demand features of Japan's future healthcare system.

Publications

1. Oshima Y, Tanimoto T, Yuji K, Tojo A. EGFR-TKI-Associated Interstitial Pneumonitis in Nivolumab-Treated Patients With Non-Small Cell Lung Cancer. JAMA Oncology, in press.
2. Tanimoto T, Oshima Y, Yuji K, Ozaki A, Kami M. Organophosphate poisoning and terrorism. Malaysian Journal of Medical Sciences, 24(4): 111-112, 2017.
3. Oshima Y, Tanimoto T, Yuji K, Tojo A. Association between Graft-versus-Host Disease and Nivolumab in the FDA Adverse Event Reporting System. Bone marrow transplant, 52(10): 1463-1464, 2017.
4. Shimada N, Ohno N. Tanosaki R, Fuji S, Suzuki Y, Yuji K, Uchimaru K, Tojo A. Therapy-related acute myeloid leukemia after the long-term ad-

ministration of low-dose etoposide for chronic-type adult T-cell leukemia/lymphoma: A case report and literature review. Intern Med, 56(14): 1879-1884, 2017.
5. Oshima Y, Tanimoto T, Yuji K, Tojo A. Association between aortic dissection and systemic exposure of vascular endothelial growth factor pathway inhibitors in the Japanese Adverse Drug Event Report database. Circulation, 135 (8): 815-817, 2017.
6. Nagai A, Hirata M, Kamatani Y, Muto K, Matsuda K, Kiyohara Y, Ninomiya T, Tamakoshi A, Yamagata Z, Mushiroda T, Murakami Y, Yuji K, Furukawa Y, Zembutsu H, Tanaka T, Ohnishi Y, Nakamura Y; BioBank Japan Cooperative Hospital Group., Kubo M. Overview of the BioBank Japan Project: Study Design and Profile. J Epidemiol., 27(3S) S2-S8, 2017.
7. Hirata M, Kamatani Y, Nagai A, Kiyohara Y, Ninomiya T, Tamakoshi A, Yamagata Z, Kubo M, Muto K, Mushiroda T, Murakami Y, Yuji K, Furukawa Y, Zembutsu H, Tanaka T, Ohnishi Y, Nakamura Y; BioBank Japan Cooperative Hospital Group., Matsuda K. Cross-sectional analysis of BioBank Japan Clinical Data: A Large Cohort of 200,000 Patients with 47 Common Diseases. J Epidemiol., 27(3S) S9-S21, 2017.
8. Hirata M, Nagai A, Kamatani Y, Ninomiya T, Tamakoshi A, Yamagata Z, Kubo M, Muto K, Kiyohara Y, Mushiroda T, Murakami Y, Yuji K, Furukawa Y, Zembutsu H, Tanaka T, Ohnishi Y, Nakamura Y; BioBank Japan Cooperative Hospital Group., Matsuda K. Overview of BioBank Japan Follow-up Data in 32 Diseases. J Epidemiol., 27(3S) S22-S28, 2017.
9. 湯地晃一郎．人工知能が切り拓く未来医療の展望．医学のあゆみ，263(8)：663–667，2017.
10. 湯地晃一郎．ゲノム編集が与える社会的インパクト．ファルマシア，54(2)：136–138，2018.

Social Cooperation Research Program

Project Division of ALA Advanced Medical Research

ALA先端医療学社会連携研究部門(SBIファーマ株式会社)

Project Professor	Kenzaburo Tani, M.D., Ph.D.	特任教授	医学博士	谷　憲三朗
Project Senior Assistant Professor	Hiroshi Kohara, Ph.D.	特任講師	博士(医学)	小原洋志
Project Assistant Professor	Shohei Miyamoto, Ph.D.	特任助教	博士(医学)	宮本将平

The overall mission of our lab is to contribute to develop new science, technology, and medical treatment based on or related with the comprehensive utilization of 5-aminolevulinic acid (5-ALA). To achieve this goal, we especially focus on the field of basic/clinical research on gene therapy and cell therapy for malignant tumors, and basic research on regenerative medicine for the treatment of intractable diseases.

A. Gene therapy, immune cell therapy and diagnosis of malignant tumors

The most commonly used therapies for malignant tumors include surgery, radiation therapy, chemotherapy, and some combination of these therapies. However, they have been not sufficiently effective for some types of tumors and the recurrent ones. In our lab, several approaches of immune therapy, which is expected to be an effective therapy for cancers refractory to conventional treatment, are under investigation.

a. A phase I clinical trial of immunotherapy combined with cyclophosphamide for patients with advanced solid tumors.

Yasuki Hijikata, Toshihiko Okazaki, Kazunari Yamada, Mutsunori Murahashi, Hisanobu Ogata Kenzaburo Tani:

We conducted a phase I clinical trial of RNF43 peptide-specific immune cell therapy combined with low dose cyclophosphamide (CPM) for patients with advanced solid tumors. The eligible patients were resistant to standard therapy, HLA-A*2402 or A*0201 positive and exhibiting high RNF43 expression in their tumor cells. Total adequate 10 patients were enrolled in this trial. Primarily, no severe adverse events greater than Grade 3 were observed. One patient exhibited PR 4 weeks after the completion of this trial. Six out of 10 patients had SD 7 weeks after initiation of treatment, among them, 2 patients experienced a decrease in the tumor markers with stabilized tumor sizes. On the other hand, 4 other patients showed PD. The frequency of Tregs in SD significantly decreased after the administration of CPM. In ICS assay, the ratio of IFN-gamma producing RNF43-specific CD8+T cells increased with time in SD, but conversely in PD. Consequently, the combination of immunotherapy and CPM may induce tumor specific immune cells accompanied by the decreased frequency of Tregs. Our phase I clinical trial exhibited safe tolerability and could bring clinical benefit against advanced solid tumors.

b. The development of novel recombinant oncolytic Coxsackievirus B3 therapy

Miyako Sagara, Shohei Miyamoto, Yuto Takishima, Hiroyuki Shimizu, Yoichi Nakanishi, Kenzaburo Tani:

Oncolytic virotherapy using enteroviruses emerges as a promising anticancer strategy. As therapeutic advantages, enteroviruses immediately induce robust oncolytic activity and do not have oncogenes that may lead to tumorigenesis. We recently showed coxsackievirus B3 wild type (CVB3-WT) infection elicited remarkably oncolytic activity against human non-small cell lung cancer cells (NSCLC). However, CVB3-WT infection caused adverse events of weight loss, pancreatitis, and myocarditis in mice. To overcome these pathogenicity, we engineered CVB3-WT genome for the development of microRNA (miRNA)-regulated oncolytic virus. We focused on two miRNAs (miR-1 and miR-217) expressed mainly normal muscle or pancreas. We successfully genetically constructed a novel recombinant CVB3-miR-1&217T (CVB3-miRT) by inserting 4 tandem target sequences complementary to two miR-1 and two miR-217 into the 3' UTR of CVB3-WT genome.

Recently, we investigated whether an infection with CVB3-miRT displays oncolytic activities against NSCLC. We found that CVB3-miRT infection induced potent oncolytic activity comparable to CVB3-WT in human NSCLC in vitro and in vivo. Here, we attempted to explore the oncolysis to triple-negative breast cancer (TNBC) because TNBC are highly aggressive and intractable tumors with dismal prognosis. We performed in vitro crystal violet staining to examine the effect of CVB3-miRT on TNBC. These results showed that CVB3-miRT had potent oncolytic activity against TNBC cell lines in a MOI-dependent manner. Furthermore, consecutive administrations of CVB3-miRT into subcutaneous xenografts of human TNBC pre-established in athymic nude mice significantly suppressed the tumor growth with a prolonged survival rate. The intratumoral CVB3-miRT administrations into human TNBC xenograft tumor mice model displayed dramatically decreased side effects of CVB3-WT-induced pathogenicity.

Collectively, we showed that CVB3-miRT infection indicated marked oncolytic activity against human NSCLC and TNBC cells in vitro and in vivo as well as CVB3-WT. This approach could be a promising new therapeutic modality to improve survival in patients suffering from NSCLC and TNBC in advanced stage.

c. Oncolytic Coxsackievirus therapy as an immunostimulator

Shohei Miyamoto, Miyako Sagara, Lisa Hirose-Yotsuya, Hiroshi Kohara, Hiroyuki Shimizu, Kenzaburo Tani:

Oncolytic virotherapy emerges as a novel anticancer therapeutic modality because of its distinctive cytotoxic mechanism of conventional therapies such as chemotherapy and radiotherapy. Oncolytic viruses are self-replicating, tumor-selective viruses, with an ability to directly induce cancer cell death, and have emerged as a promising treatment platform for cancer therapy.

Enteroviruses have recently been used as an oncolytic virus for cancer virotherapy. We carried out the screening of 38 enteroviral strains and found that coxsackievirus B3 (CVB3) possessed specific oncolytic activity against cell lines of human lung cancer, malignant pleural mesothelioma and breast cancer. In addition, We previously demonstrated that CVB3 had potent oncolytic activity with immunostimulatory properties, abundant cell surface calreticulin expression and secreted ATP as well as translocated extranuclear high-mobility group box 1 (HMGB1) in CVB3-infected lung cancer cell lines, which are required for immunogenic cell death. Moreover, intratumoral CVB3 administration markedly recruited natural killer (NK) cells and granulocytes, both of which contributed to the antitumor effects as demonstrated by depletion assays, macrophages, and mature dendritic cells (DCs) into tumor tissues.

However, CVB3 showed several organ toxicities. To overcome this situation, we constructed a novel recombinant CVB3-miRT by genetically incorporating two distinct normal tissue-specific miRNA target sequences into the CVB3 genome. The administrations into human lung cancer xenograft tumor in athymic nude mice with CVB3-miRT, but not parental CVB3, dramatically decreased serum level of amylase and mitigated both pancreatitis and myocarditis with a significant tumor regression.

For acquisition of non-clinical proof of concept, we developed the production method of CVB3-miRT reagent using 293 cells grown in a serum-free bag culture system. The harvested CVB3-miRT was concentrated and purified by tangential flow filtration and sucrose gradient zonal ultracentrifugation for non-clinical safety testing using mice and monkeys.

Our results provide important information for the development of novel anti-tumor enterovirus virotherapy.

d. A novel, polymer-coated oncolytic measles virus overcomes immune suppression and induces robust antitumor activity

Kaname Nosaki, Katsuyuki Hamada, Yuto Takishima, Miyako Sagara, Yumiko Matsumura, Sho-

hei Miyamoto, Yasuki Hijikata, Toshihiko Okazaki, Yoichi Nakanishi, Kenzaburo Tani:

Although various therapies are available to treat cancers, including surgery, chemotherapy, and radiotherapy, cancer has been the leading cause of death in Japan for the last 30 years, and new therapeutic modalities are urgently needed. As a new modality, there has recently been great interest in oncolytic virotherapy, with measles virus being a candidate virus expected to show strong antitumor effects. The efficacy of virotherapy, however, was strongly limited by the host immune response in previous clinical trials. To enhance and prolong the antitumor activity of virotherapy, we combined the use of two newly developed tools: the genetically engineered measles virus (MV-NPL) and the multilayer virus-coating method of layer-by-layer deposition of ionic polymers. We compared the oncolytic effects of this polymer-coated MV-NPL with the naked MV-NPL, both in vitro and in vivo. In the presence of anti-MV neutralizing antibodies, the polymer-coated virus showed more enhanced oncolytic activity than did the naked MV-NPL in vitro. We also examined antitumor activities in virus-treated mice. Complement-dependent cytotoxicity and antitumor activities were higher in mice treated with polymer-coated MV-NPL than in mice treated with the naked virus. This novel, polymer-coated MV-NPL is promising for clinical cancer therapy in the future.

e. Characterization of tumor-infiltrating CD8+ T lymphocytes in malignant lymphoma

Mutsunori Murahashi, Hiroyuki Kishi, Taichi Matsumoto, Shuji Hara, Atsushi Muraguchi, Kazuo Tamura, Kenzaburo Tani:

Characterization of tumor-specific CD8+ tumor-infiltrating T lymphocytes (TILs) is in progress for application to adoptive cell transfer. However, the immunological roles of these cells have not yet been clarified for malignant lymphoma. In this study, we studied the TCR sequences of TILs to identify their clonality. Three patients who were pathologically diagnosed as diffuse large B cell lymphoma were evaluated. CD8+ TILs were flow cytometrically analyzed for their CTL expression markers of 4-1BB and PD-1. In addition, TCRs of these T cells were identified using PCR method. The flow cytometric analyses of CD8+ TILs showed that the frequency of 4-1BB+ and PD-1+ cells were 17.2% ± 5.7% and 59.8% ± 1.3%, respectively in CD8+CD45RA− cells. Both of 4-1BB and PD-1 were significantly highly expressed in CD8+ CD45RA− cells compared to CD8+CD45RA+ cells. Our results suggest that CD8+ TILs characterized by high PD-1 expression existed in malignant lymphoma. Identification and functional analysis of their TCRs are now underway and such information would be helpful to develop new gene therapy modality for malignant lymphoma.

f. Pilot study to detect circulating tumor cells in human peripheral blood using 5-aminolevulinic acid

Hiroshi Kohara, Satoshi Takahashi, Masaru Shinozaki, Kaoru Uchimaru, Arinobu Tojo, Naohide Yamashita, Yasuki Hijikata, Shohei Miyamoto, Kenzaburo Tani:

Circulating tumor cells (CTCs) have been detected in peripheral blood of patients with a variety of cancers, and expected to be of its potential diagnostic and prognostic value. Although the United States Food and Drug Administration (FDA)-approved CellSearch™ system has been commonly used for counting CTCs in peripheral blood, this system has limitation in terms of its clinical sensitivity. Namely, this system detect only EpCAM-expressing CTCs.

5-aminolevulinic acid (5-ALA) has been shown to be a useful fluorescent sensitizing agent for photodynamic therapy, photodynamic detection, and other approaches in cancer therapeutics and diagnosis. Here we show the construction of a novel detection system of CTCs in human peripheral blood by taking advantage of 5-ALA. We performed flow cytometric detection of 5-ALA-derived Protoporphyrin IX (PpIX) fluorescence in EpCAM-positive and EpCAM-negative human cancer cell lines. Not only in EpCAM-positive cell lines including A549 (lung carcinoma), PC-3 (prostate cancer), Caco-2 (colon cancer) and AsPc-1 (pancreatic tumor), 5-ALA-derived PpIX fluorescence was detected also in EpCAM-negative cell lines including A498 (kidney carcinoma) and MMAc (melanoma) after 6 hour incubation in the presence of 5-ALA. We then evaluated the detection efficiency of rare cancer cells in human blood using serial dilution samples of pre-labeled cancer cells spiked into 1-2 mL of blood from healthy donor. The number of cancer cells detected by using 5-ALA-based labeling were in a good correlation with the number of the cells expected. We noticed that faint fluorescence was also detected in some leukocytes of healthy donor as predicted. Flow cytometric analysis of blood samples revealed CD45+CD14 high monocyte lineage cells showed relatively higher PpIX fluorescence than the other cell types in peripheral blood.

These data indicated that CTC detection system using 5-ALA-based cell labeling might be useful for early diagnosis of cancer patients. It is highly expected that 5-ALA-based labeling with the negative selection of CD45 and/or CD14 positive population would increase the sensitivity and specificity of

CTCs in patients' blood samples. We are now preparing to analyze peripheral blood samples from patients suffered from various malignancies with different clinical stages to evaluate the potential of 5-ALA-based CTC labeling.

g. Therapeutic vaccination based on side population cells transduced by the granulocyte-macrophage colony-stimulating factor gene elicits potent antitumor immunity

Chika Sakamoto, Hiroshi Kohara, Hiroyuki Inoue, Megumi Narusawa, Yoshie Ogawa, Lisa Hirose-Yotsuya, Shohei Miyamoto, Yumiko Matsumura, Kazunari Yamada, Atsushi Takahashi, and Kenzaburo Tani:

Among cancer immunotherapies, granulocyte-macrophage colony-stimulating factor (GM-CSF) gene-transduced tumor cell vaccines (GVAX) therapies appears promising and have been shown to be safe and effective in multiple clinical trials. However, the antitumor efficacies of GVAX therapy alone are in some cases limited. Here we showed that GVAX therapy targeting cancer stem cells (CSCs) substantially suppressed tumor development in syngeneic immunocompetent mice recapitulating normal immune systems. CSCs were isolated as side population (SP) cells from 4T1 murine breast carcinoma cell line, and transduced with GM-CSF gene delivered by non-transmissible Sendai virus (4T1-SP/GM). Impaired tumorigenicity of subcutaneously injected 4T1-SP/GM depended on CD8+ T cells in concert with CD4+ T cells and natural killer (NK) cells. Mice therapeutically vaccinated with irradiated 4T1-SP/GM cells had markedly suppressed tumor development of subcutaneously transplanted 4T1-SP cells compared with those treated with irradiated cells of non-transduced 4T1-SP cells or non SP (4T1-NSP/GM) cells. Tumor suppression was accompanied by the robust accumulation of mature dendritic cells at vaccination sites and Th1-skewed systemic cellular immunity. Our results suggested that CSCs cell-based GVAX immunotherapy might be clinically useful for inducing potent tumor-specific antitumor immunity.

B. Regenerative medicine and related technique development

Regenerative medicine is expected to be another essential therapeutic strategy for intractable diseases. We have been actively investigating some novel strategies to yield cell sources in regenerative medicine, and evaluated efficiency and safety of the cells for regenerative medicine.

a. Generation and functional analysis of congenital dyserythropoietic anemia (CDA) patient-specific induced pluripotent stem cells

Hiroshi Kohara, Hiromi Ogura, Takako Aoki, Chika Sakamoto, Yoshie Ogawa, Shohei Miyamoto, Hitoshi Kanno, Kenzaburo Tani:

The congenital dyserythropoietic anemias (CDAs) are inherited red blood cell disorders representing ineffective erythropoiesis and dyserythropoietic changes in the bone marrow. We recently diagnosed a female patient with undiagnosed congenital anemia as type IV CDA caused by a heterozygous missense mutation of the erythroid-specific transcription factor, *KLF1*; c.973G>A, p. E325K. Although the mutation has been reported in a male patient characterized as hydrops fetalis, severe neonatal jaundice and transfusion-dependent anemia (Arnaud L *et al*., Am J Hum Genet, 2010), the proband showed relatively mild phenotype showing moderate dyserythropoietic anemia. In order to investigate the pathological significance of mutant *KLF1* during erythroid cell development and differentiation, we generated induced pluripotent stem cells (iPSCs) from peripheral blood of the CDA patient (CDA-iPSCs), and utilized these cells to establish *in vitro* CDA model for better understanding of its molecular basis. CDA-iPSCs were generated from T lymphocytes in peripheral blood mononuclear cells. Hematopoietic precursors were induced from CDA-iPSCs by embryoid bodies formation. CD34+ precursor cells were isolated and further cultured in liquid culture with cytokine cocktail (erythropoietin (EPO), interleukin (IL)-3, and stem cell factor (SCF)) for additional 1-3 weeks. Flow cytometric analysis showed that CDA-iPSC-derived cells contained significantly lower percentage of CD235a+/CD71+ erythroid lineage cells than the cells derived from control iPSCs, and lack expression of the adhesion molecule CD44, which is known to be down regulated in peripheral blood erythroid cells of CDA patients (Arnaud L *et al*., Am J Hum Genet, 2010). In addition, colony-forming unit (CFU) assay indicated that CD34+ fraction derived from CDA-iPSCs contained a lower number of erythroid colony-forming cells and the most of the cells in these colonies are morphologically abnormal, in comparison with control iPSCs. We next evaluated mRNA expression levels of fetal (*HBG1* and *HBG2*), embryonic (*HBE*), and adult (*HBB*) globins, resulting that *HBG1* and *HBG2* were significantly increased in CDA-iPSCs-derived erythroid lineage cells, whereas *HBE* showed no significant change and *HBB* was decreased in CDA-iPSCs-derived erythroid lineage cells. However, *BCL11A*, one of the target genes of *KLF1* and also known as a suppressor of *HBG1* and *HBG2*, was not decreased in the presence *KLF1* gene mutation,

indicating that elevated *HBG1* and *HBG2* in CDA-iPSCs-derived erythroid cells was mediated by other mechanism like Leukemia/lymphoma Related Factor (LRF; Masuda T *et al*., Science, 2016). Here we suggest that our model provides insights on understanding the mechanisms of type IV CDA and the effect of *KLF1* gene mutation on clinical phenotype and it would be a useful tool for drug screening and identification of novel biomarker for the rare congenital anemia.

b. Role of P53 on T lymphopoiesis from human embryonic stem cells

Liao Jiyuan, Hiroshi Kohara, Shohei Miyamoto, Yoko Nagai, Tomotoshi Marumoto, Kenzaburo Tani:

Although pluripotent stem cells are well recognized as a potential source of cell therapy, it is still needed to improve efficiency to differentiate into target cell lineages. Tumor suppressor P53 regulates multiple signaling pathways triggered by diverse cellular stresses including DNA damages, oncogenic stimulations, and hypoxic stress, resulting in cell-cycle arrest, apoptosis, and senescence. P53 signaling is also important for double-stranded DNA breaks (DSBs) induced during physiologic events, i. e., rearrangement of antigen-specific receptors. It has been reported that P53-mediated DSB checkpoint contribute to normal murine T lymphopoiesis, especially at the double-negative (DN) stage which is defined as CD4-CD8- fraction in thymus and requires rearrangements of the T cell receptor (TCR) β locus and successful pre-TCR signaling (Guidos CJ *et al*., Genes Dev, 1996; Jiang D *et al*., J Exp Med 2006; HaksMC *et al*., Immunity, 1999). Here we defined the role of P53 on hematopoietic development, especially lymphopoiesis, from human embryonic stem cells (ESCs).

Firstly we modified P53 gene of human ESC H1 by utilizing genome editing tool of zinc finger nuclease (ZFN) targeting the 5th exon of the P53 gene, kindly provided by Sangamo BioSciences. Sequencing analysis of the P53 knockout (KO) ES cells showed the successful deletion at the 5th exon which induced the frame shift of the downstream sequence in both of its alleles. qRT-PCR showed no stable expression of full length P53 mRNA and western blot analysis of P53 phosphorylation status in P53 KO ESCs showed undetectable levels of phosphorylated or non-phosphorylated P53 proteins when cultured in the presence or absence of apoptotic signal triggered by mitomycin C (MMC). In consistent with this, P53 KO ESCs showed significant resistance to MMC-induced cell death. In addition, P53 KO ESCs lacked apoptotic stimulation-induced upregulation of P53 downstream target genes including P53 up-regulated modulator of apoptosis (PUMA). On the other hand induction of P53 target gene P21 was not observed both in H1 and P53 KO ESCs, as reported previously by other groups (Ginis I *et al*., Dev Biol, 2004; Barta T *et al*., Stem Cells, 2010; García CP *et al*., Stem Cell Res, 2014; World J *et al*., Stem Cells, 2014).

We then induced hematopoietic differentiation of P53 KO ESCs through embryoid body formation. Erythroid lineage cells developed from human ESCs were significantly suppressed in the absence of P53 signaling during embryoid body maturation. Pharmacological inhibition of P53 had the same effect as genetic disruption of P53 gene. CD34 + hematopoietic precursors were isolated from embryoid bodies originated from H1 and P53 KO ECSs, plated on OP9-DL1 stromal cells, and cultured in the presence of stem cell factor (SCF), FLT3 ligand, and interleukin (IL)-7. After 3-4 weeks of culture, CD45 + CD3 + T lineage cells were induced from both H1 and P53 KO ECSs-derived CD34 + cells. Among these cells, most of the cells were in CD4 + CD8 + double-positive (DP) stage, with increase in the yield of DP cells in the absence of P53 signaling (H1: 343 cells/1 x 10^6 input CD34 + cells; P53 KO: 2476 cells /1 x 10^6 input CD34 + cells; Figure). Whether pharmacological inhibition of P53 had the similar effect on T lymphopoiesis as genetic disruption of P53 gene needs to be investigated furthermore.

Our data indicate that P53 mediated signaling regulate in vitro early T lymphopoiesis from human pluripotent stem cells, especially at the transition from double negative into DP stage. These observations promoted us to perform high throughput transcriptome analysis including cDNA microarray analysis between early T lineage cells derived from H1 and P53 KO ESCs. Genes associated with the early T lymphopoiesis from human ESCs were identified and currently under further characterization.

c. Single-Cell-State Culture of Human Pluripotent Stem Cells Increases Transfection Efficiency.

Takenobu Nii, Hiroshi Kohara, Tomotoshi Marumoto, Tetsushi Sakuma, Takashi Yamamoto, Kenzaburo Tani:

Efficient gene transfer into human pluripotent stem cells (hPSCs) holds great promise for regenerative medicine and pharmaceutical development. In the past decade, various methods were developed for gene transfer into hPSCs; however, hPSCs form tightly packed colonies, making gene transfer difficult. In this study, we established a stable culture method of hPSCs at a single-cell state to reduce cell density and investigated gene transfection efficiency followed by gene editing efficiency.

hPSCs cultured in a single-cell state were transfected using nonliposomal transfection reagents with plasmid DNA or mRNA encoding enhanced green fluorescent protein. We found that most cells (DNA>90%; mRNA>99%) were transfected without the loss of undifferentiated PSC marker expression or pluripotency. Moreover, we demonstrated an efficient gene editing method using transcription activator-like effector nucleases (TALENs) targeting the adenomatous polyposis coli (APC) gene. Our new method may improve hPSC gene transfer techniques, thus facilitating their use for human regenerative medicine.

Publications

1. Shitaoka K, Hamana H, Kishi H, Hayakawa Y, Kobayashi E, Sukegawa K, Piao X, Lyu F, Nagata T, Sugiyama D, Nishikawa H, Tanemura A, Katayama I, Murahashi M, Takamatsu H, Tani K, Ozawa T, Muraguchi A. Identification of tumor-associated 1 antigen-reactive T cell receptor with tumoricidal activity by single-cell repertoire analysis of tumor-infiltrating lymphocytes. Cancer Immunol Res 2018 (in press)
2. Wang, B., Ogata, H., Takishima, Y., Miyamoto, S., Inoue, H., Kuroda, M., Yamada, K., Hijikata, Y., Murahashi, M., Shimizu, H., Okazaki, T., Nakanishi, Y., and Tani, K. A novel combination therapy for human oxaliplatin-resistant colorectal cancer using oxaliplatin and coxsackievirus A11. Anticancer Res. (in press)
3. Hijikata Y, Okazaki T, Tanaka Y, Murahashi M, Yamada Y, Yamada K, Takahashi A, Inoue H, Kishimoto J, Nakanishi Y, Oda Y, Nakamura Y, Tani K. A phase I clinical trial of RNF43 peptide-related immune cell therapy combined with low-dose cyclophosphamide in patients with advanced solid tumors. Plos One. 13(1): e0187878, 2018
4. Miyamoto, S., Sagara, M., Kohara, H., and Tani, K. Oncolytic coxsackievirus therapy as an immunostimulator. Rinsho Ketsueki. 58(8): 977-82, 2017.
5. Kulkeaw K, Inoue T, Iino T, Tani K, Akashi K, Speck N, Nakanishi Y, Sugiyama D. Twist1 regulates embryonic hematopoietic differentiation through binding to Myb and Gata2 promoter regions. Blood Adv. 1(20): 1672-1681, 2017
6. Sakamoto, C., Kohara, H., Inoue, H., Narusawa, M., Ogawa, Y., Hirose-Yotsuya, L., Miyamoto, S., Matsumura, Y., Yamada, K., Takahashi, A., and Tani, K. Therapeutic vaccination based on side population cells transduced by the granulocyte-macrophage colony-stimulating factor gene elicits potent antitumor immunity. Cancer Gene Ther. 24(4): 165-74, 2017.

Corporate Sponsored Research Program

Project Division of Fundamental Study on Cutting Edge of Genome Medicine

先端ゲノム医療の基盤研究寄付研究部門

Professor	Arinobu Tojo, M.D., D.M.Sc.		教授(兼務)	医学博士	東 條 有 伸
Project Associate Professor	Hiroshi Yasui, M.D., D.M.Sc.		特任准教授	博士(医学)	安 井 寛

Our major goal is to realize advanced genomic medicine. Major advancements in genome analysis have recently been reported from researchers around the globe, along with improvements in next-generation sequencing, leading to an era where genomic information can be collected and analyzed at low cost and in a short period of time. Thus, it is necessary to establish a framework for developing genome analysis while expanding our understanding of general society, academia, and medical associations, etc., in order to identify different diseases, such as hereditary diseases or nongenetic diseases.

In our department, comprehensive basic research on advanced genome medicine has been realized through our multidisciplinary collaborations among scientific experts: the Ethical, Legal, and Social Implications (ELSI) program: specimen preservation: electronic medical records access: and personal information protection.

1. Construction of infrastructure for research on advanced genome medicine

Hiroshi Yasui, Mikiko Suzuki, Megumi Isobe, Arinobu Tojo

In order to establish a framework for developing genome analysis while expanding our understanding of diseases, including hereditary and nongenetic diseases, we are using comprehensive approaches to advanced genome medicine. These approaches include addressing various issues, such as multidisciplinary collaborations among scientific experts: the Ethical, Legal, and Social Implications (ELSI) program: management of specimen preservation, clinical information, and personal information protection for genomic medicine as well as biobanking. Our mission also includes enhancement of social acceptance for genomic medicine.

2. Japan-US Comparative study for the promotion of the cancer genomic medicine in Japan

Hiroshi Yasui, Mikiko Suzuki, Arinobu Tojo

Regarding the spread of cancer genomic medicine Japan is behind not only Western countries but also China and Korea. We study to compare the current situation and the future prospects of cancer genomic medicine in Japan and the United States in order to contribute to design a policy to promote dissemination and uniformization of cancer genomic medicine for cancer patients in Japan.

3. Program for supporting biospecimen analysis for the diagnosis and treatment of hematological malignancies

Hiroshi Yasui, Arinobu Tojo, Kaoru Uchimaru[1], Toshiki Watanabe[2]: [1]Department of Computational

Biology and Medical Sciences, Graduate School of Frontier Sciences, The University of Tokyo, [2]Department of Advanced Medical Innovation, St. Marianna University School of Medicine

To support cancer scientists in promoting translational research and genome medicine, we have established a platform for supporting cohort studies and biospecimen analysis. Under this program, we are collecting and managing clinical materials, including tumor cells, serum, and peripheral blood mononuclear cells from patients at high risk of hematologic malignancies as well as patients with blood cancer. We provide support for obtaining and/or analyzing biomaterials, as requested by researchers, and contribute to their clinical studies and publications.

4. Investigator-initiated clinical trials under an Investigational New Drug application for the development of novel cancer therapeutics and biomarkers

Hiroshi Yasui, Fumitaka Nagamura[1], Giichiro Tsurita[2], Kohzoh Imai[3], Arinobu Tojo: [1]Center for Translational Research, IMSUT Hospital, University of Tokyo, [2]Department of Surgery, IMSUT Hospital, University of Tokyo, [3]Office of Support for Platforms for Advanced Technologies and Research Resources

Genome medicine and genome research, including pharmacogenomics and pharmacogenetics, are important for developing novel therapeutic agents for cancer and incurable diseases and identifying biomarkers. Our research aims to develop efficient approaches for conducting investigator-initiated clinical trials under Investigational New Drug (IND) applications to promote translational research and discover biomarkers for prediction of the safety and efficacy of novel therapeutics through omics analyses, including genomics. In 2017, we were conducting, supporting, or preparing three investigator-initiated clinical trials under INDs applications for the development of academic-oriented innovative anticancer drug especially novel cancer immunotherapy.

5. Support and management of translational research

Hiroshi Yasui

To promote translational research and genome medicine, Dr. Yasui, as a member of the Translational Research Advancement Center at the University of Tokyo, participates in the "Translational Research Network Program, Japanese Translational Research and Clinical Trials Core Centers", supported by the Japan Agency for Medical Research and Development. The aim of the program is to promote translational research and investigator-led clinical trials aiming for practical applications of basic studies in academia, managing the assessment of scientific seeds and intellectual property rights, and therefore promoting the development of advanced medical arts, including genome medicine.

Publications

1. Kikuchi J, Kuroda Y, Koyama D, Osada N, Izumi T, Yasui H, Kawase T, Ichinohe T, Furukawa Y. Myeloma cells are activated in bone marrow microenvironment by the CD180/MD-1 complex which senses lipopolysaccharide Cancer Res. 2018, in press
2. Ogawa M, Yokoyama K, Hirano M, Jimbo K, Ochi K, Kawamata T, Ohno N, Shimizu E, Yokoyama N, Yamaguchi R, Imoto S, Uchimaru K, Takahashi N, Miyano S, Imai Y, Tojo A. Different clonal dynamics of chronic myeloid leukaemia between bone marrow and the central nervous system. Br J Haematol. 2017 Dec 19. doi: 10.1111/bjh.15065. [Epub ahead of print]
3. Kobayashi M, Yokoyama K, Shimizu E, Yusa N, Ito M, Yamaguchi R, Imoto S, Miyano S, Tojo A. Phenotype-based Gene Analysis Allowed Successful Diagnosis of X-linked Neutropenia Associated with a Novel WASP Mutation in a Japanese adult patient. Ann Hematol. 2017 Sep 27 doi: 10.1007/s00277-017-3134-3. [Epub ahead of print]
4. Sasahara A, Tominaga K, Nishimura T, Yano M, Kiyokawa E, Noguchi M, Kanauchi H, Ogawa T, Minato H, Tada K, Seto Y, Tojo A, Gotoh N. An autocrine/paracrine circuit of growth differentiation (GDF) 15 has a role for maintenance of breast cancer stem-like cells. Oncotarget. 8(15): 24869-81, 2017
5. Tominaga K, Shimamura T, Kimura N, Murayama T, Matsubara D, Kanauchi H, Niida A, Shimizu S, Nishioka K, Tsuji E, Yano M, Sugano S, Shimono Y, Ishii H, Saya H, Mori M, Akashi K, Tada K, Ogawa T, Tojo A, Miyano S, Goto N. Addiction to the IGF2-ID1-IGF2 circuit for maintenance of the breast cancer stem-like cells. Oncogene 36(9): 1276-86, 2017
6. Takahashi H, Okayama N, Yamaguchi N, Miyahara Y, Morishima Y, Suehiro Y, Yamasaki T, Tamada K, Takahashi S, Tojo A, Asano S, Tanabe T. Associations of interactions between NLRP3 SNPs and HLA mismatch with acute and

extensive chronic graft-versus-host diseases. Sci Rep. Oct 12: 7(1): 13097, 2017
7. Hattori A, Tsunoda M, Konuma T, Kobayashi M, Nagy T, McSkimming D, Beattie N, Kannan N, Tojo A, Edison A, Ito T. Cancer progression by reprogrammed BCAA metabolism in myeloid leukemia. Nature 545(7655): 500-4, 2017
8. Tojo A, Kyo T, Yamamoto K, Nakamae K, Takahashi N, Kobayashi Y, Tauchi T, Okamoto S, Miyamura K, Hatake K, Iwasaki H, Matsumura I, Usui N, Naoe T, Tugnait M, Narasimhan NI, Lustgarten S, Farin H, Haluska F, Ohyashiki K. Ponatinib in Japanese Patients with Philadelphia Chromosome-Positive Leukemia, a Phase 1/2 Study. Int J Hematol. 106: 385-97, 2017

Social Cooperation Research Program

Project Division of Advanced Biopharmaceutical Science

先進的バイオ医薬品学社会連携研究部門

Professor	Hirotoshi Tanaka, Ph.D.		教　授	医学博士	田　中　廣　壽
Professor	Kouhei Tsumoto, Ph.D.		教　授	博士(工学)	津　本　浩　平
Project Associate Professor	Satoru Nagatoishi, Ph.D.		特任准教授	博士(生命科学)	長門石　　曉

Various antibodies have been approved for therapeutic use and currently examined in clinical development. Developments and improvements of technology for the discovery and optimization of high-potency antibodies, therefore, have greatly increased to find the specific and stable antibody with desired biological properties. Biophysical analyses of therapeutic antibody, particularly those of protein interaction and stability, are recognized as one of the critical procedures in the development of biopharmaceuticals, which would be assessed as an essential step to develop next generation antibodies. Development of analytical methods with quantitative and high-sensitive detection of antigen interaction, protein stability and biological function of antibody, therefore, has been intriguing for the pharmaceutic companies. In this division, we study biophysical analyses of various antibody to propose new strategy for development of the next generation antibody.

1. Thermodynamic analyses of amino acid residues at the interface of an antibody B2212A and its antigen roundabout homolog 1

A. Yui, H. Akiba, S. Kudo, M. Nakakido, S. Nagatoishi and K. Tsumoto

Artificial affinity maturation of antibodies is promising but often shows difficulties because the roles of each amino acid residue are not well known. To elucidate their roles in affinity against the antigen and thermal stability, interface residues in single-chain Fv of an antibody B2212A with its antigen roundabout homolog 1 were mutated and analyzed. Some amino acids played important roles in the affinity while others contributed to thermal stability.

2. Structure of the triose-phosphate/phosphate translocator reveals the basis of substrate specificity

Y. Lee, T. Nishizawa, M. Takemoto, K. Kumazaki, K. Yamashita, K. Hirata, A. Minoda, S. Nagatoishi, K. Tsumoto, R. Ishitani and O. Nureki

The triose-phosphate/phosphate translocator (TPT) catalyses the strict 1: 1 exchange of triose-phosphate, 3-phosphoglycerate and inorganic phosphate across the chloroplast envelope, and plays crucial roles in photosynthesis. Despite rigorous study for more than 40 years, the molecular mechanism of TPT is poorly understood because of the lack of structural information. Here we report crystal structures of TPT bound to two different substrates, 3-phosphoglycerate and inorganic phosphate, in occluded conformations. The structures re-

veal that TPT adopts a 10-transmembrane drug/metabolite transporter fold. Both substrates are bound within the same central pocket, where conserved lysine, arginine and tyrosine residues recognize the shared phosphate group. A structural comparison with the outward-open conformation of the bacterial drug/metabolite transporter suggests a rocker-switch motion of helix bundles, and molecular dynamics simulations support a model in which this rocker-switch motion is tightly coupled to the substrate binding, to ensure strict 1: 1 exchange. These results reveal the unique mechanism of sugar phosphate/phosphate exchange by TPT.

3. Biophysical characterization of the interaction between heme and proteins responsible for heme transfer in S. pyogenes

M. Hoshino, M. Nakakido, S. Nagatoishi, J.M.M. Caaveiro, C. Aikawa, I. Nakagawa and K. Tsumoto

Streptococcus pyogenes, an important pathogen that causes a wide range of diseases, possesses the sia gene cluster, which encodes proteins involved in the heme acquisition system. Although this system was previously described, the molecular mechanism of effective heme transfer remains to be elucidated. Here, we have characterized the interactions between heme and each domain of Streptococcal hemoprotein receptor (Shr) and Streptococcal heme-binding protein (Shp). Our kinetic and thermodynamic analyses suggested that effective heme transfer within this system is achieved not only by affinity-based transfer but also by the difference of the binding driving force. The biophysical characterization of the above-mentioned interaction will lead to an indication for the selection of the target for a chemical screening of inhibitors as novel antibacterial agents based on biophysical approaches.

4. Structural Basis for Binding and Transfer of Heme in Bacterial Heme-Acquisition Systems.

Y. Naoe, N. Nakamura, MM. Rahman, T. Tosha, S. Nagatoishi, K. Tsumoto, Y. Shiro, H. Sugimoto.

Periplasmic heme-binding proteins (PBPs) in Gram-negative bacteria are components of the heme acquisition system. These proteins shuttle heme across the periplasmic space from outer membrane receptors to ATP-binding cassette (ABC) heme importers located in the inner-membrane. In the present study, we characterized the structures of PBPs found in the pathogen Burkholderia cenocepacia (BhuT) and in the thermophile Roseiflexus sp. RS-1 (RhuT) in the heme-free and heme-bound forms. The conserved motif, in which a well-conserved Tyr interacts with the nearby Arg coordinates on heme iron, was observed in both PBPs. The heme was recognized by its surroundings in a variety of manners including hydrophobic interactions and hydrogen bonds, which was confirmed by isothermal titration calorimetry. Furthermore, this study of 3 forms of BhuT allowed the first structural comparison and showed that the heme-binding cleft of BhuT adopts an "open" state in the heme-free and 2-heme-bound forms, and a "closed" state in the one-heme-bound form with unique conformational changes. Such a conformational change might adjust the interaction of the heme(s) with the residues in PBP and facilitate the transfer of the heme into the translocation channel of the importer.

5. Use of SpyTag/SpyCatcher to construct bispecific antibodies that target two epitopes of a single antigen

K. Yumura, H. Akiba, S. Nagatoishi, O. Kusano-Arai, H. Iwanari, T. Hamakubo and K. Tsumoto

Bispecific antibody targeting of two different antigens is promising, but when fragment-based antibodies are used, homogeneous production is difficult. To overcome this difficulty, we developed a method using the SpyTag/SpyCatcher system in which a covalent bond is formed between the two polypeptides. Using this method, we constructed a bispecific antibody that simultaneously interacted with two different epitopes of roundabout homologue 1 (ROBO1), a membrane protein associated with cancer progression. A bispecific tetravalent antibody with an additional functional moiety was also constructed by using a dimeric biotin-binding protein. An interaction analysis of ROBO1-expressing cells and the recombinant antigen demonstrated the improved binding ability of the bispecific antibodies through spontaneous binding of the two antibody fragments to their respective epitopes. In addition, multivalency delayed dissociation, which is advantageous in therapy and diagnosis.

6. The carboxyl-terminal region of Dok-7 plays a key, but not essential, role in activation of muscle-specific receptor kinase MuSK and neuromuscular synapse formation

R. Ueta, T. Tezuka, Y. Izawa, S. Miyoshi, S. Nagatoishi, K. Tsumoto and Y. Yamanashi

As the synapse between a motor neuron and skeletal muscle, the neuromuscular junction (NMJ) is required for muscle contraction. The formation and maintenance of NMJs are controlled by the muscle-specific receptor kinase MuSK. Dok-7 is the essential cytoplasmic activator of MuSK, and indeed mice lacking Dok-7 form no NMJs. Moreover, DOK7 gene mutations underlie DOK7 myasthenia,

an NMJ synaptopathy. Previously, we failed to detect MuSK activation in myotubes by Dok-7 mutated in the N-terminal pleckstrin homology (PH) or phosphotyrosine binding (PTB) domain or that lacked the C-terminal region (Dok-7-ΔC). Here, we found by quantitative analysis that Dok-7-ΔC marginally, but significantly, activated MuSK in myotubes, unlike the PH- or PTB-mutant. Purified, recombinant Dok-7-ΔC, but not other mutants, also showed marginal ability to activate MuSK's cytoplasmic portion, carrying the kinase domain. Consistently, forced expression of Dok-7-ΔC rescued Dok-7-deficient mice from neonatal lethality caused by the lack of NMJs, indicating restored MuSK activation and NMJ formation. However, these mice showed only marginal activation of MuSK and died by 3 weeks of age apparently due to an abnormally small number and size of NMJs. Thus, Dok-7's C-terminal region plays a key, but not fully essential, role in MuSK activation and NMJ formation.

7. Disruption of cell adhesion by an antibody targeting the cell-adhesive intermediate (X-dimer) of human P-cadherin

S. Kudo, J.M.M. Caaveiro, S. Nagatoishi, T. Miyafusa, T. Matsuura, Y. Sudou and K. Tsumoto

Human P-cadherin is a cell adhesion protein of the family of classical cadherins, the overexpression of which is correlated with poor prognosis in various types of cancer. Antibodies inhibiting cell-cell adhesion mediated by P-cadherin show clear therapeutic effect, although the mechanistic basis explaining their effectiveness is still unclear. Based on structural, physicochemical, and functional analyses, we have elucidated the molecular mechanism of disruption of cell adhesion by antibodies targeting human P-cadherin. Herein we have studied three different antibodies, TSP5, TSP7, and TSP11, each recognizing a different epitope on the surface of the cell-adhesive domain (EC1). Although all these three antibodies recognized human P-cadherin with high affinity, only TSP7 disrupted cell adhesion. Notably, we demonstrated that TSP7 abolishes cell adhesion by disabling the so-called X-dimer (a kinetic adhesive intermediate), in addition to disrupting the strand-swap dimer (the final thermodynamic state). The inhibition of the X-dimer was crucial for the overall inhibitory effect, raising the therapeutic value of a kinetic intermediary not only for preventing, but also for reversing, cell adhesion mediated by a member of the classical cadherin family. These findings should help to design more innovative and effective therapeutic solutions targeting human P-cadherin.

8. Through-bond effects in the ternary complexes of thrombin sandwiched by two DNA aptamer

A. Pica, I. Russo Krauss, V. Parente, H. Tateishi-Karimata, S. Nagatoishi, K. Tsumoto, N. Sugimoto and F. Sica

Aptamers directed against human thrombin can selectively bind to two different exosites on the protein surface. The simultaneous use of two DNA aptamers, HD1 and HD22, directed to exosite I and exosite II respectively, is a very powerful approach to exploit their combined affinity. Indeed, strategies to link HD1 and HD22 together have been proposed in order to create a single bivalent molecule with an enhanced ability to control thrombin activity. In this work, the crystal structures of two ternary complexes, in which thrombin is sandwiched between two DNA aptamers, are presented and discussed. The structures shed light on the cross talk between the two exosites. The through-bond effects are particularly evident at exosite II, with net consequences on the HD22 structure. Moreover, thermodynamic data on the binding of the two aptamers are also reported and analyzed.

Publications

Yui, A., Akiba, H., Kudo, S., Nakakido, M., Nagatoishi, S. and Tsumoto, K. Thermodynamic analyses of amino acid residues at the interface of an antibody B2212A and its antigen roundabout homolog 1. J. Biochem. 162: 255-258, 2017.

Lee, Y., Nishizawa, T., Takemoto, M., Kumazaki, K., Yamashita, K., Hirata, K., Minoda, A., Nagatoishi, S., Tsumoto, K., Ishitani, R. and Nureki, O. Structure of the triose-phosphate/phosphate translocator reveals the basis of substrate specificity. Nature Plants. 3: 825-832, 2017.

Hoshino, M., Nakakido, M., Nagatoishi, S., Aikawa, C., Nakagawa, I. and Tsumoto, K. Biophysical characterization of the interaction between heme and proteins responsible for heme transfer in Streptococcus pyogenes. Biochem. Biophys. Res. Commun. 493: 1109-1114, 2017.

Naoe, Y., Nakamura, N., Rahman, M.M., Tosha, T., Nagatoishi, S., Tsumoto, K., Shiro, Y. and Sugimoto, H. Structural basis for binding and transfer of heme in bacterial heme-acquisition systems. Proteins. 85: 2217-2230, 2017.

Yumura, K., Akiba, H., Nagatoishi, S., Kusano-Arai, O., Iwanari, H., Hamakubo, T. and Tsumoto, K. Use of SpyTag/SpyCatcher to construct bispecific antibodies that target two epitopes of a single an-

tigen. J. Biochem. 162: 203-210, 2017.
Ueta, R., Tezuka, T., Izawa, Y., Miyoshi, S., Nagatoishi, S., Tsumoto, K. and Yamanashi, Y. The carboxyl-terminal region of Dok-7 plays a key, but not essential, role in activation of muscle-specific receptor kinase MuSK and neuromuscular synapse formation. J. Biochem. 161: 269-277, 2017.
Kudo, S., Caaveiro, J.M., Nagatoishi, S., Miyafusa, T., Matsuura, T., Sudou, Y. and Tsumoto, K. Disruption of cell adhesion by an antibody targeting the cell-adhesive intermediate (X-dimer) of human P-cadherin. Sci. Rep. 7: 39518, 2017.
Pica A., Russo Krauss, I., Parente, V., Tateishi-Karimata, H., Nagatoishi, S., Tsumoto, K., Sugimoto, N. and Sica, F. Through-bond effects in the ternary complexes of thrombin sandwiched by two DNA aptamers. Nucleic Acids Res. 45: 461-469, 2017.
長門石曉，津本浩平．低分子薬剤開発に活かされる熱量測定の最前線．熱測定．熱測定学会．44(4)：2017.
津本浩平，長門石曉，中木戸誠．バイオ医薬品の分析のコツ　品質評価のための基礎と応用（第２回）タンパク質科学の基礎と取扱い．PHARM TECH JAPA．じほう．33(11)：2017.
長門石曉，津本浩平（分担執筆）．医療応用のための物理化学．CSJカレントビュー　医療・診断・創薬の化学．化学同人．2017.
長門石曉，津本浩平．抗体の品質・物性評価に関する解説(２)：プロセス・製剤における評価．製剤機械技術学会誌．製剤機械技術学会．26(1)：2017
長門石曉，津本浩平（分担執筆）．８章　酵素．生体分子化学．講談．2017.
長門石曉，津本浩平．物理化学的解析技術を利用したリガンドスクリーニング．バイオサイエンスとインダストリー．バイオインダストリー協会．75(1)：2017
津本浩平，長門石曉．日本発次世代創薬のための化学：抗体医薬品開発の現状と展望．化学と工業．日本化学会．70(1)：2017.