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.
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Our long-term goal is to understand the molecular mechanisms which coordinately regulate differentiation and maintenance of neural retina. Recently we are focusing on failure of maintenance of differentiated cells, that ultimately lead to neural degeneration and cancer. In addition, how immune cells, such as microglia, affect pathogenesis of central nervous sytem is important issue. For this purpose we use models ranging from iPS, mouse, monkey, to clinical samples.
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, transcriptional factors, and epigenetic modification are under investigation for their roles on retinal development. We also work on molecular analysis of glioma causative genes using mouse model. Projects, which gave major findings during 2018 are as follows.

Generation of a novel mouse model of glioma by using transposon vectors

Keisuke Sumiyoshi, Hideto Koso, Sumiko Watanabe

Glioma is the most common form of malignant brain cancer in adults. To facilitate in vivo analysis of gliomagenesis, we developed a transposon vector that encodes the triple combination of PDGFA, and shRNAs against Nf1 and Trp53. Transduction of the vector into neural stem cells in the subventricular zone of the neonatal brain induced proliferation of oligodendrocyte precursor cells, and promoted formation of highly penetrant malignant gliomas within 2-4 months. By labeling cells expressing PDGFA or shNf1/shp53 with unique fluorescent proteins, the spatial distribution of cells with different genetic alterations were visualized within the same tumor. Interestingly, intratumoral heterogeneity in PDGFA expression levels spontaneously developed within the same tumor. The transposon vectors we developed provide a useful tool for the functional analysis of candidate genes in glioma.

Analysis of the role of RAS signaling in the retina

Hideto Koso, Asano Tsuhako, Sumiko Watanabe

The RAS gene family members are frequently mutated in human cancer. A subset of retinal tumors displays K-RAS mutations; however, the specific role of RAS activation on retinal tumor formation is unclear. To examine the role of RAS in retinal development, we overexpressed the mutant H-RAS (G12V) gene in retinal progenitor cells (RPCs). Mosaic activation of Ras in the RPCs of the postnatal retina resulted in differentiation of RPCs into cells with glial characteristics. Ras-activated RPCs subsequently underwent apoptosis. In sharp contrast, RAS activation in a large population of RPCs in the embryonic retina resulted in the formation of eye tumors. Together, our data indicate that the tumorigenicity of RAS activation in RPCs is context dependent, with tumor formation occurring when RAS activity is present in a large cluster of embryonic RPCs.

The role of Zhx2 transcription factor in bipolar cell differentiation during mouse retinal development

Yuichi Kawamura, Kyohei Yamanaka, Boonmin Poh, Hiroshi Kuribayashi, Hideto Koso, Sumiko Watanabe

We found that the *Zhx2* gene (whose product is known to act as a tumor suppressor in hepatocellular carcinoma) is expressed in embryonic retinal progenitors and in developing cone bipolar cells in the postnatal retina, as well as in Müller glia in the mature retina. To examine the functions of Zhx2 protein during retinal development, we performed loss- and gain-of-function analyses using a retinal explant culture system. We introduced a plasmid encoding *Zhx2* shRNA into isolated mouse retinas at E17.5, and the retinas were cultured as explants. After 3 days of culture, proliferation was slightly enhanced, leading to retinas thicker than in the control, but this phenomenon was observed only transiently. After 14 days of the culture, the thickness and gross morphology of retinas expressing sh-*Zhx2* were indistinguishable from those of the control. The numbers of rod cells, amacrine cells, and Müller glia were the same in both groups. However, although the total number of bipolar cells was the same, the experimental group saw an increased population of ON bipolar cells, and decreased numbers of a subset of OFF bipolar cells. We also examined the effects of overexpression of *Zhx2*. Although Zhx2 acts as a tumor suppressor, its overexpression in developing retinas did not lead to any discernible difference in retinal thickness, suggesting that proliferation activity was not affected. After 14 days of explant culture, the total number of bipolar cells decreased, and subset composition was altered. Taken together, these results suggest that Zhx2 plays roles in the regulation of bipolar cell subset fate determination during retinal development.

Roles of Nmnat1 in the survival of retinal progenitors through the regulation of pro-apoptotic gene expression via histone acetylation

Hiroshi Kuribayashi, Yukihiro Baba, Toshiro Iwagawa, Eisuke Arai1, Sumiko Watanabe

Leber congenital amaurosis (LCA) is a severe, genetically heterogeneous dystrophy of the retina, and mutations in the nicotinamide mononucleotide adenylyltransferase 1 (NMNAT1) gene is one of causal factors of LCA. NMNAT1 is a nuclear enzyme essential for nicotinamide adenine dinucleotide (NAD) biosynthesis pathways, but the mechanisms underlying the LCA pathology, and whether NMNAT1 plays a role in normal retinal development remain unclear. Thus, we examined the roles of Nmnat1 in retinal development via short hairpin (sh)-RNA-mediated downregulation. Retinal explants expressing sh-Nmnat1 showed large numbers of apoptotic retinal progenitor cells in the inner half of the neuroblastic layer. Decreased intracellular NAD content was observed, and the addition of NAD to the culture medium attenuated sh-Nmnat1-induced apoptosis. Of the nuclear Sirtuin (Sirt) family, the expression of sh-Sirt1 and sh-Sirt6 resulted in a phenotype similar to that of sh-Nmnat 1. Sirt proteins are the histone deacetylase, and the expression of sh-Nmnat1 increased the levels of acetylated histones H3 and H4 in retina. Expression of sh-Nmnat1 resulted in significantly increased expression of *Noxa* and *Fas*, two pro-apoptotic genes. Acetylation of the genomic 5' untranslated regions of *Noxa* and *Fas* loci was upregulated by sh-Nmnat1 expression. The co-expression of sh-Noxa or sh-Fas with sh-Nmnat1 reduced the number of apoptotic cells induced by sh-Nmnat1 expression alone, suggesting that the increased expression of these genes explains, at least in part, the phenotype associated with sh-Nmnat1 in the retina. Taken together, these findings demonstrate the importance of the NAD biosynthesis pathway in normal development of the retina.

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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 Assistant Professor Jeewoong Park, Ph.D.

特任准教授 博士(理学) 高 橋 理 貴
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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 create artificial aptamers to target proteins of therapeutic interest.

1. A chymase inhibitory RNA aptamer improves cardiac function and survival after myocardial infarction.

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We have reported that mast cell chymase, an angiotensin II-generating enzyme, is important in cardiovascular tissues. Recently, we developed a new chymase-specific inhibitory RNA aptamer, HA28, and we evaluated the effects of HA28 on cardiac function and the mortality rate after myocardial infarction. Echocardiographic parameters, such as the left ventricular ejection fraction, fractional shortening, and the ratio of early to late ventricular filling velocities, were significantly improved by treatment with HA28 after myocardial infarction. The mortality rate was significantly reduced in the HA28-treated group. Cardiac chymase activity and chymase gene expression were significantly higher in the vehicle-treated myocardial infarction group, and these were markedly suppressed in the HA28-treated myocardial infarction group. The present study provides the first evidence that a single-stranded RNA aptamer that is a chymase-specific inhibitor is very effective in the treatment of acute heart failure caused by myocardial infarction. Chymase may be a new therapeutic target in post-myocardial infarction pathophysiology.

2. The FGF2 aptamer inhibits the growth of FGF 2-FGFR pathway driven lung cancer cells.

Junko Hamamoto[1], Hiroyuki Yasuda[1], Yosuke Nonaka[2], Masatoshi Fujiwara[2], Yoshikazu Nakamura, Kenzo Soejima[1], Tomoko Betsuyaku[1]: [1]Division of Pulmonary Medicine, Department of Medicine, Keio University School of Medicine, Tokyo, Japan, [2]RIBOMIC Inc., Tokyo, Japan.

Cancers, including lung cancer, are a leading cause of death worldwide. To overcome this deadly disease, multiple modality inhibitors have been de-

veloped. These include cytotoxic agents, molecular targeted small molecules, such as tyrosine kinase inhibitors, and neutralizing antibodies. An aptamer is a short single-stranded nucleic acid molecule that is selected in vitro from a large random sequence library based on its high and specific affinity to a target molecule. Aptamers can be applied to therapeutics of various types of diseases, including cancer, due to their strong and specific neutralizing activities. However, the efficacy of aptamer-based therapy for cancer cells is not well characterized. In this study, we aimed to show that the FGF2 aptamer is effective for the treatment of FGF2 dependent lung cancer cells. We previously developed PC9 GR lung cancer cells, whose proliferation is dependent on EGFR and FGF2-FGFR pathways in a cell autonomous manner. Using PC9GR cells, we demonstrate that the addition of the FGF2 aptamer induces more significant inhibition of PC9GR cell proliferation than does the addition of EGFR inhibitor alone. Furthermore, the addition of the FGF2 aptamer more significantly inhibits the downstream signals and induces apoptosis to a higher extent than does the addition of EGFR inhibitor alone. Our results show that the FGF2 aptamer inhibits the growth of FGF2-FGFR pathway-dependent lung cancer cells. The findings provide preclinical evidence that aptamers can be useful for cancer treatment.

3. Single-round isolation of diverse RNA aptamers from a random sequence pool.

Masahiko Imashimizu, Masaki Takahashi, Ryo Amano, Yoshikazu Nakamura.

Aptamers are oligonucleotide ligands with specific binding affinity to target molecules. Generally, RNA aptamers are selected from an RNA pool with random sequences, using the technique termed SELEX, in which the target-binding RNA molecules are repeatedly isolated and exponentially amplified. Despite several advantages, SELEX often produces uncertain results during the iterative amplifications of the rare target-binding RNA molecules. Here, we develop a non-repeated, primer-less and target immobilization-free isolation method for generating RNA aptamers, which is robust to experimental noise. Uniquely, this method focuses on finding and removal of non-aptamer sequences from the RNA pool by RNase digestion leaving target-bound aptamer molecules, and thus is independent of aptamer types. The undigested RNA sequences remaining are so few in number that they must be mixed with a large excess of a known sequence for further manipulations and this sequence is then removed by restriction digestion followed by high-throughput sequencing analysis to identify aptamers. Using this method, we generated multiple RNA aptamers targeting α-thrombin and TGFβ1 proteins, independently. This method potentially generates thousands of sequences as aptamer candidates, which may enable us to predict a common average sequence or structural property of these aptamers that is different from input RNA.

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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 Assistant Professor　Yasumasa Kimura, Ph.D.　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. Development of new protocols 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 mammalian 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. We also additionally established a protocol for quantification of both single and double strand DNA viruses in a same sample. This method is critical for understanding viral ecology in the gut.

2. Construction of bioinformatics pipelines for virome analysis

Yasumasa Kimura, Seiya Imoto[1], Masanori Kakuta[2], Rui Yamaguchi[2], Yasushi Akiyama[3], Hiroshi Kiyono[4], Satoru Miyano[2], 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]Human Genome Center, The Institute of Medical Science, The University of Tokyo, [3]Department of Computer Science, Graduate School of Informa-

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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 taxonomies. The pipeline further enabled us to analyze unknown viruses, which are the source of "viral dark matter." We further improved the informatics pipeline for easy use to non-informatic researchers such as medical doctors.

3. Construction of the bacteriome and virome database

Yasumasa Kimura, Seiya Imoto[1], Kosuke Fujimoto[2], Koji Kashima[3], Yoshikazu Yuki[3], Kiyoshi Yamaguchi[4], Yoichi Furukawa[4], Yuki Usui, Rui Yamaguchi[5], Hiroshi Kiyono[3], Satoru Miyano[5], Satoshi Uematsu[2], Takeshi Satoh: [1]Division of Health Medical Data Science, Health Intelligence Center, The Institute of Medical Science, The University of Tokyo, [2]Division of Innate Immune regulation, International Research and Development Center for Mucosal Vaccines, The Institute of Medical Science, The University of Tokyo, [3]Division of Mucosal Immunology, Department of Microbiology and Immunology, The Institute of Medical Science, The University of Tokyo, [4]Division of Clinical Genome Research, The Institute of Medical Science, The University of Tokyo, [5]Human Genome Center, The Institute of Medical Science, The University of Tokyo.

We performed virome and bacteriome metagenome sequences of the same fecal samples collected from healthy Japanese volunteers. Using the established analysis pipeline, the virome and bacteriome communities were analyzed at the contig level. The viral and bacterial compositions and diversities were measured and compared across the samples. We categorized the human gut viromes by clustering approach and characterized them by dominant viral groups. This data set is important for analyzing gut microorganisms from patients in various diseases.

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. Construction of 16S rRNA gene analysis protocol and bioinformatics pipeline

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 Development 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 gene 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. The bioinoformatics pipeline that analyzes 16S sequences of our target region was also constructed and used for in-house data and collaborative researches.

Publications

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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

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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(neo ALA株式会社)

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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. Successful clinical sequencing of an elderly patient with refractory Sézary syndrome.

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Advances in clinical sequencing have enabled the discovery of actionable alterations that yield clinical benefits. Here, we report successful clinical results for lenalidomide in a case of refractory Sézary syndrome (SS) case based on clinical sequencing. A 75-year-old male was diagnosed with SS (T4N3M0B1b) in 2015. He had received various treatments in multiple hospitals, including prednisone, PUVA, extracorporeal photopheresis, bexarotene, romidepsin, total skin electron beam therapy, brentuximab vedotin, IFNα-2b, methotrexate, and pembrolizumab, but his skin lesions did not respond adequately to

these therapeutic modalities. The patient was admitted to our hospital on November 24, 2016. Generalized erythroderma, clinical stage IIIA, was observed. Mogamulizumab was administered, and partial response as Stage 1A was observed on December 27, 2016. However, three new erythema nodosum-like lesions appeared on his upper back and expanded to the trunk (Stage IIB) in January 2017. VP-16 was started on February 27, 2017. Although a part of the skin tumor shrank temporarily, it regrew in April 2017. From April to July 2017, skin electron beam therapy was applied, followed by vorinostat from July 31 for a month, but both had limited effects. After obtaining informed consent, whole exome sequencing (WES) and RNA sequencing was performed on the biopsied skin tumor for comparison with normal tissue, followed by analysis by our hospital curators using GSEA (Gene Set Enrichment Analysis) and the IBM Watson artificial intelligence (AI). Although WES did not show any actionable mutation, actionable targets were identified based on RNA sequencing and GSEA. Manual curation excluded the ibrutinib, idelalisib, palbociclib, and dasatinib options chosen by AI because evidence for SS was lacking. Mogamulizumab, pembrolizumab, and brentuximab vedotin were already used during his protracted clinical course. Denileukin diftitox was excluded because the latest immunohistochemistry of the lesions showed that they were CD25(−). The two remaining candidates were ipilimumab and lenalidomide. The patient was reluctant to be treated with ipilimumab as its mechanism of action is similar to that of pembrolizumab. We chose lenalidomide because of NF-κB pathway activation in this patient. Lenalidomide binds the Cullin 4 ring-E3 ubiquitin ligase-cereblon complex and degrades lymphoid transcription factors IKZF1 and IKZF3, leading to a decrease in NF-κB. Additionally, a phase II trial of lenalidomide monotherapy for refractory SS revealed effectiveness, and a phase III trial of maintenance therapy was reported. Lenalidomide was administered for 21 days of a 28-day cycle from September 11, 2017. After the first course of treatment, all skin tumors except for one on his sternal region disappeared. The sternal tumor grew gradually and was irradiated with 20 Gy in December 2017. The fifth course of lenalidomide was completed on February 3, 2017. All tumors, including sternal lesions, disappeared, and he finally achieved a complete response. As far as we aware, this is the first case report to succeed in using clinical sequencing for SS patients. Further clinical studies using clinical sequencing and accumulation of information are needed for AI to be used for routine clinical practice.

b. A phase I clinical trial of RNF43 peptide-related immune cell therapy combined with low-dose cyclophosphamide in patients with advanced solid tumors

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The objective of this study was to investigate the safety and the tolerability of combined cellular immunotherapy with low-dose cyclophosphamide (CPA) in patients with advanced solid tumors. This study targeted a novel tumor-associated antigen, ring finger protein 43 (RNF43). Eligible patients were resistant to standard therapy, HLA-A*24:02- or A*02:01-positive and exhibiting high RNF43 expression in their tumor cells. They were administered 300 mg/m^2 CPA followed by autologous lymphocytes, preliminarily cultured with autologous RNF43 peptide-pulsed dendritic cells (DCs), RNF43 peptide-pulsed DCs and systemic low dose interleukin-2. The primary endpoint was safety whereas the secondary endpoint was immunological and clinical response to treatment. Ten patients, in total, were enrolled in this trial. Primarily, no adverse events greater than Grade 3 were observed. Six out of 10 patients showed stable disease (SD) on day 49, while 4 other patients showed progressive disease. In addition, one patient with SD exhibited a partial response after the second trial. The frequency of regulatory T cells (Tregs) in patients with SD significantly decreased after CPA administration. The ratio of interferon-γ-producing, tumor-reactive CD8 + T cells increased with time in patients with SD. We successfully showed that the combination of immune cell therapy and CPA was safe, might induce tumor-specific immune responses and clinical efficacy, and was accompanied by a decreased ratio of Tregs in patients with RNF43-positive advanced solid tumors.

c. The development of novel recombinant oncolytic Coxsackievirus B3 therapy

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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 coxsackievirus (CVB3-miRT). Two organ-specific (enriched in muscle and pancreas) miRNAs target sequences were constructed into the 3′ untranslated region (3′ UTR) of the CVB3 genome (CVB3-miR1& 217T) resulting in the markedly reduced occurrence of CVB3-induced pancreatitis and myocarditis. However, non-clinical acute toxicity testing of recombinant CVB3 in mice and monkeys showed mild hematological and histopathological abnormal findings in the highest dose group. To improve its safety profile, microRNA target sequence complementary to miR-34a/c (enriched in normal organs) were inserted into the 3′ UTR (CVB3-3A/C), 5′ UTR (CVB3-5A/C) or both of these regions (CVB3-53A/C) of the CVB3 genome (CVB3-3A/C). All viruses elicited massive viral lysis of tumor cells in vitro and in vivo. But the novel recombinant viruses showed reduced replications and cytotoxicity in human normal cells expressing high level of cognate microRNAs. Human tumor-bearing nude mice treated with wild type virus showed remarkable weight loss, but recombinant viruses did not. 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 CVB 3-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.

d. Oncolytic Coxsackievirus therapy as an immunostimulator

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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 virother-

apy.

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

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Circulating tumor cells (CTCs) have been detected in peripheral blood (PB) of patients with a variety of cancers and expected to have 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 PB, this system has limitation in terms of its clinical use. Namely, this system detects only EpCAM-expressing CTCs which remain the characteristic of the epithelial tumor cells. Recent studies revealed that a proportion of CTCs does not have epithelial characteristics and such non-epithelial CTCs could be excluded in the EpCAM-based detection. Therefore, it is crucial to develop a novel method to capture and detect all tumor subpopulations of CTCs to understand accurate heterogeneity of CTCs. That will provide a useful clinical information for diagnosis and a better medicine treatment. 5-Aminolevulinic acid (5-ALA) has been shown to be a useful fluorescent sensitizing agent for photodynamic therapy and photodynamic detection in cancer therapeutics and diagnosis. This study is a pilot study to develop a novel CTCs detection method using 5-ALA. ProtoporphyrinIX (PpIX), a metabolite of 5-ALA, accumulates in tumor cells. It is expected this new method can detect both EpCAM-positive and -negative CTCs. After labeling the cells in blood of cancer patients with PpIX, we analyzed the CTCs by flowcytometry. Spike-in experiment showed the correlation between PpIX-labeled cells and expected cancer cells, indicating the accuracy of detecting cancer cells in patients' blood using PpIX .We analyzed the blood of some cancer patients and detected the PpIX-positive populations, which were not in healthy donors. The most of cells in the PpIX-positive populations were EpCAM-negative cells. To increase the sensitivity, we next concentrated the specimens by removing blood cells such as CD45 and CD14 positive cells. After the negative selection, we analyzed the CTCs by flowcytometry. The analysis of 10 mL of blood from melanoma and gastric cancer patients showed that there were PpIX-positive populations with or without EpCAM positive. Our results indicated the possibility that the detection of CTCs using PpIX labeling is a novel useful method for CTCs detection. We are analyzing and confirming the PpIX-positive cells are cancer cells released from primary tumors.

f. Pathway-Based Drug Repositioning for Cancers: Computational Prediction and Experimental Validation.

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Developing drugs with anticancer activity and low toxic side-effects at low costs is a challenging issue for cancer chemotherapy. In this work, we propose to use molecular pathways as the therapeutic targets and develop a novel computational approach for drug repositioning for cancer treatment. We analyzed chemically induced gene expression data of 1112 drugs on 66 human cell lines and searched for drugs that inactivate pathways involved in the growth of cancer cells (cell cycle) and activate pathways that contribute to the death of cancer cells (e.g., apoptosis and p53 signaling). Finally, we performed a large-scale prediction of potential anticancer effects for all the drugs and experimentally validated the prediction results via three in vitro cellular assays that evaluate cell viability, cytotoxicity, and apoptosis induction. Using this strategy, we successfully identified several potential anticancer drugs. The proposed pathway-based method has great potential to improve drug repositioning research for cancer treatment.

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 evaluate 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

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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 CD 235a(+)/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. Efficient gene transduction and reprogramming of hematopoietic cells including T-cells by using a non-integrating measles virus vector.

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By the ectopic expression of reprogramming genes OCT, KLF4, SOX2 and MYC (OKSM), somatic cells can be reprogrammed to induced pluripotent stem cells (iPSCs). Human iPSCs are considered a promising cell source to provide an import tool for the basic investigation and the advanced medicine including gene therapy and regenerative medicine. To establish iPSCs, integration-free Sendai virus (SV) vectors have been most widely used do far, but transduction and reprogramming of T cells without stimulation is still very challenging. On the other hand, a great success of chimeric antigen receptor T cell (CAR-T) therapies highlighted the importance of anti-cancer immunity for the cancer treatment. Particularly, many refractory patients with acute lymphoblastic leukemia and B-cell lymphoma were successfully treated with CD19-CAR-T therapies, however, some patients died before receiving the treatment due to long preparation time of CAR-Ts. Therefore, rapid production systems of CAR-Ts are desired, and for this purpose, efficient and safe gene transduction systems to T cells should be developed. In this study, we developed a new non-integrating measles virus (MV) vector-based delivery system with F deletion to eliminate cell membrane fusion-associated cytotoxicity. MV vectors transduced genes through MV receptors including CD46 and signaling lymphocyte activation molecule (CD150/SLAM). First, we examined trans-

duction efficiencies of MV vectors and SV vectors in hematopoietic cells by using GFP expression vectors (MV-Gs and SV-Gs). Compared to SV-Gs, our MV-Gs allowed more efficient gene transfer into most hematopoietic cell type including T (3-fold) and B cells (7-fold). Furthermore, at the same multiplicity of infection (MOI) of viral transduction, MV-Gs induced less apoptosis in T cell subset compared to SV-Gs due to the slower kinetics of viral RNA amplification in the transduced cells 24 h ,48 h and 72 h post transduction. Those results encouraged us to examined if MV vectors are more potent than SV vectors in iPSC generation from unstimulated T cells. To address this question, we developed MV vectors harboring four reprogramming genes (MV-OKSMGs) and compared with SV vectors harboring these genes (SV-OKSMGs). As expected, with the MV-OKSMGs, we could generate high-quality iPSCs with the similar morphology, pluripotency markers, karyotype and differentiation capacity as human embryonic stem cells. Upon the less cytotoxicity, iPSC generation efficiency of MV-OKSMGs was much higher than that of SV-OKSMGs for unstimulated T cells ($0.47 \pm 0.25\%$ vs $0.008 \pm 0.009\%$). Considering the safe history of MV vaccine, carrying capabilities of multiple genes, more flexible receptors and higher transduction efficiency for resting T cells, our exclusive MV vector would be a potential gene transfer system for iPSC generation and lymphocyte-based-immunotherapies such as CAR-T therapies.

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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
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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 non-genetic 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

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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

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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. We were conducting, supporting, summarizing 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, Arinobu Tojo

To promote translational research and genome medicine, we participate in the "Translational Research Network Program, Japanese Translational Research and Clinical Trials Core Centers" supported by the Japan Agency for Medical Research and Development, as members of the Translational Research Advancement Center at the University of Tokyo. 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.

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Social Cooperation Research Program

Project Division of Advanced Biopharmaceutical Science

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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 and computational analyses reveal the functional roles of the galloyl group of tea catechins in molecular recognition.

Takahashi T, Nagatoishi S, Kuroda D, and Tsumoto K.

Catechins, biologically active polyphenols in green tea, exhibit various biological activities, such as anticancer and antiviral activities, arising from interactions with functional proteins. However, the molecular details of these interactions remain unclear. In this study, we investigated the interactions between human serum albumin (HSA) and various catechins, including some with a galloyl group, by means of isothermal titration calorimetry (ITC), differential scanning calorimetry (DSC), and docking simulations. Our results indicate that the galloyl group was important for recognition by HSA and was responsible for enthalpic gains derived from a larger buried surface area and more van der Waals contacts. Thus, our thermodynamic and computational analyses suggest that the galloyl group plays important functional roles in the specific binding of catechins to proteins, implying that the biological activities of these compounds may be due in part to the physicochemical characteristics of the galloyl group.

2. Molecular basis for governing the morphology of type-I collagen fibrils by Osteomodulin.

Tashima T, Nagatoishi S, Caaveiro JMM, Nakakido M, Sagara H, Kusano-Arai O, Iwanari H, Mimuro H, Hamakubo T, Ohnuma SI, Tsumoto K.

Small leucine-rich repeat proteoglycan (SLRP) proteins have an important role in the organization of the extracellular matrix, especially in the formation of collagen fibrils. However, the mechanism governing the shape of collagen fibrils is poorly understood. Here, we report that the protein Osteomodulin (OMD) of the SLRP family is a monomeric protein in solution that interacts with type-I collagen. This interaction is dominated by weak electrostatic forces employing negatively charged residues of OMD, in particular Glu284 and Glu303, and controlled by entropic factors. The protein OMD establishes a fast-binding equilibrium with collagen, where OMD may engage not only with individual collagen molecules, but also with the growing fibrils. This weak electrostatic interaction is carefully balanced so it modulates the shape of the fibrils without compromising their viability.

3. Biophysical Analysis of the Protein-Small Molecule Interactions to Develop Small Molecule Drug Discovery.

Nagatoishi S, Caaveiro JMM, Tsumoto K.

In small molecule drug discovery, researchers must find specific binders that interact with a target protein and inhibit its function in connection with human diseases. It is of critical importance to know the binding mode of compounds interacting with a target protein to assure hit validation and optimization. Biophysical analysis is a powerful quantitative approach to evaluate the binding modes of such candidates. Since the level of sensitivity of biophysical analysis is suitable to quantitatively detect the binding of fragment compounds, and because of the remarkable success of compound libraries of small molecules, the development and adaptation of biophysical analysis for these applications is in great demand. Herein, we describe the technical developments of biophysical methods, especially thermodynamic and kinetic analysis, for the purpose of screenings which employ small molecules. In addition, we discuss the interaction mechanisms of small molecules to find hit compounds based on these biophysical analyses.

4. Discovery and Optimization of Inhibitors of the Parkinson's Disease Associated Protein DJ-1.

Tashiro S, Caaveiro JMM, Nakakido M, Tanabe A, Nagatoishi S, Tamura Y, Matsuda N, Liu D, Hoang QQ, Tsumoto K.

DJ-1 is a Parkinson's disease associated protein endowed with enzymatic, redox sensing, regulatory, chaperoning, and neuroprotective activities. Although DJ-1 has been vigorously studied for the past decade and a half, its exact role in the progression of the disease remains uncertain. In addition, little is known about the spatiotemporal regulation of DJ-1, or the biochemical basis explaining its numerous biological functions. Progress has been hampered by the lack of inhibitors with precisely known mechanisms of action. Herein, we have employed biophysical methodologies and X-ray crystallography to identify and to optimize a family of compounds inactivating the critical Cys106 residue of human DJ-1. We demonstrate these compounds are potent inhibitors of various activities of DJ-1 in vitro and in cell-based assays. This study reports a new family of DJ-1 inhibitors with a defined mechanism of action and contributes toward the understanding of the biological function of DJ-1.

5. Inhibition of homophilic dimerization and disruption of cell adhesion by P-cadherin-specific small molecules from SPR-based assays.

Senoo A, Nagatoishi S, Moberg A, Babol LN, Mitani T, Tashima T, Kudo S, Tsumoto K.

The inhibitor for the homophilic dimerization of P-cadherin was discovered by SPR-based screening using fragment compounds. Our SPR assays identified a specific P-cadherin binder, which was able to inhibit the cell adhesion of living CHO cells that expressed P-cadherin.

6. A combination of 19F NMR and surface plasmon resonance for site-specific hit selection and validation of fragment molecules that bind to the ATP-binding site of a kinase.

Nagatoishi S, Yamaguchi S, Katoh E, Kajita K, Yokotagawa T, Kanai S, Furuya T, Tsumoto K.

19F NMR has recently emerged as an efficient, sensitive tool for analyzing protein binding to small molecules, and surface plasmon resonance (SPR) is also a popular tool for this purpose. Herein a combination of 19F NMR and SPR was used to find novel binders to the ATP-binding pocket of MAP kinase extracellular regulated kinase 2 (ERK2) by fragment screening with an original fluorinated-fragment library. The 19F NMR screening yielded a high primary hit rate of binders to the ERK2 ATP-binding pocket compared with the rate for the SPR screening. Hit compounds were evaluated and categorized according to their ability to bind to different binding sites in the ATP-binding pocket. The binding manner was characterized by using isothermal titration calorimetry and docking simulation. Combining 19F NMR with other biophysical methods allows the identification of multiple types of hit compounds, thereby increasing opportunities for drug design using preferred fragments.

7. Assessing the Heterogeneity of the Fc-Glycan of a Therapeutic Antibody Using an engineered FcγReceptor IIIa-Immobilized Column.

Kiyoshi M, Caaveiro JMM, Tada M, Tamura H, Tanaka T, Terao Y, Morante K, Harazono A, Hashii N, Shibata H, Kuroda D, Nagatoishi S, Oe S, Ide T, Tsumoto K, Ishii-Watabe A.

The N-glycan moiety of IgG-Fc has a significant impact on multifaceted properties of antibodies such as in their effector function, structure, and stability. Numerous studies have been devoted to understanding its biological effect since the exact composition of the Fc N-glycan modulates the magnitude of effector functions such as the antibody-dependent cell mediated cytotoxicity (ADCC), and the complement-dependent cytotoxicity (CDC). To date, systematic analyses of the properties and influence of glycan variants have been of great interest. Understanding the principles on how N-glycosylation modulates those properties is important for the molecular design, manufacturing, process optimization, and quality control of therapeutic antibodies. In this study, we have separated a model therapeutic antibody into three fractions according to the composition of the N-glycan by using a novel FcγRIIIa chromatography column. Notably, Fc galactosylation was a major factor influencing the affinity of IgG-Fc to the FcγRIIIa immobilized on the column. Each antibody fraction was employed for structural, biological, and physicochemical analysis, illustrating the mechanism by which galactose modulates the affinity to FcγRIIIa. In addition, we discuss the benefits of the FcγRIIIa chromatography column to assess the heterogeneity of the N-glycan.

8. Development of drug discovery screening system by molecular interaction kinetics-mass spectrometry.

Obi N, Fukuda T, Nakayama N, Ervin J, Bando Y, Nishimura T, Nagatoishi S, Tsumoto K, Kawamura T.

Six small-molecule binders of CAII were analyzed quantitatively using nPOI and MIK-MS, and the results were compared to published surface plasmon resonance (SPR) results. The nPOI and SPR results show good agreement, confirming the reliability of the analysis. Time-dependent binding results may be obtained by our MS sensorgram approach. Drugs that meet medical needs in a short period are required; this nPOI-LC-MS system is considered an important tool for rapid drug discovery.

9. A secondary RET mutation in the activation loop conferring resistance to vandetanib.

Nakaoku T, Kohno T, Araki M, Niho S, Chauhan R, Knowles PP, Tsuchihara K, Matsumoto S, Shimada Y, Mimaki S, Ishii G, Ichikawa H, Nagatoishi S, Tsumoto K, Okuno Y, Yoh K, McDonald NQ, Goto K.

Resistance to vandetanib, a type I RET kinase inhibitor, developed in a patient with metastatic lung adenocarcinoma harboring a CCDC6-RET fusion that initially exhibited a response to treatment. The resistant tumor acquired a secondary mutation resulting in a serine-to-phenylalanine substitution at codon 904 in the activation loop of the RET kinase domain. The S904F mutation confers resistance to vandetanib by increasing the ATP affinity and autophosphorylation activity of RET kinase. A reduced interaction with the drug is also observed in vitro for the S904F mutant by thermal shift assay. A crystal structure of the S904F mutant reveals a small hydrophobic core around F904 likely to enhance basal kinase activity by stabilizing an active conformer. Our findings indicate that missense mutations in the activation loop of the kinase domain are able to increase kinase activity and confer drug resistance through allosteric effects.

10. Characterization of glycoengineered anti-HER2 monoclonal antibodies produced by using a silkworm-baculovirus expression system.

Egashira Y, Nagatoishi S, Kiyoshi M, Ishii-Watabe A, Tsumoto K.

Silkworm-baculovirus expression systems are efficient means for the production of recombinant proteins that provide high expression levels and post-translational modifications. Here, we characterized the stability, glycosylation pattern and antibody-dependent cell-mediated cytotoxicity activity of anti-HER2 monoclonal antibodies containing native or glycoengineered mammalian-like N-glycans that were produced by using a silkworm-baculovirus expression system. Compared with a monoclonal antibody produced by using a Chinese hamster ovary cell expression system, the glycoengineered monoclonal antibody had comparable thermal stability and a higher antibody-dependent cell-mediated cytotoxicity activity. These results suggest that silkworm-baculovirus expression systems are next-generation expression systems potentially useful for the cost-effective production of therapeutic antibodies.

11. Production and characterization of a novel site-specific-modifiable anti-OX40-receptor single-chain variable fragment for targeted drug delivery.

Tanabe A, Nakano K, Nakakido M, Nagatoishi S, Tanaka Y, Tsumoto K, Uchimaru K, Watanabe T.

OX40 receptor (tumor necrosis factor receptor superfamily, member 4; CD134) is a T-cell co-stimulatory molecule that plays an important role in T-cell activation and survival. OX40 receptor is activated by its ligand, OX40L; and modulation of the OX40-OX40L interaction is a promising target for the treatment of autoimmune diseases and cancers. Here, we generated a high-affinity anti-OX40 single-chain variable fragment carrying a C-terminal cysteine residue (scFvC). Physicochemical and functional analyses revealed that the scFvC bound to OX40-expressing cells and was internalized via OX 40-mediated endocytosis without inducing phosphorylation of IκBα (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha), an important complex in the classical NFκB (nuclear factor kappa-light-chain-enhancer of activated B cells) signaling pathway. In addition, mutation of the 36th cysteine residue in variable region of light chain enabled site-specific chemical modification to carboxy terminal cysteine and improved the thermal stability of the scFvC. These results suggest that this novel high-affinity anti-OX40 scFvC may be useful as a transporter for targeted delivery of small compounds, proteins, peptides, liposomes, and nanoparticles, into OX40-expressing cells for the treatment of autoimmune diseases and cancers.

12. PRDM14 directly interacts with heat shock proteins HSP90α and glucose-regulated protein 78.

Moriya C, Taniguchi H, Nagatoishi S, Igarashi H, Tsumoto K, Imai K.

PRDM14 is overexpressed in various cancers and can regulate cancer phenotype under certain conditions. Inhibiting PRDM14 expression in breast and pancreatic cancers has been reported to reduce cancer stem-like phenotypes, which are associated with aggressive tumor properties. Therefore, PRDM14 is considered a promising target for cancer therapy. To develop a pharmaceutical treatment, the mechanism and interacting partners of PRDM14 need to be clarified. Here, we identified the proteins interacting with PRDM14 in triple-negative breast cancer (TNBC) cells, which do not express the three most common types of receptor (estrogen receptors, progesterone receptors, and HER2). We obtained 13 candidates that were pulled down with PRDM14 in TNBC HCC1937 cells and identified them by mass spectrometry. Two candidates-glucose-regulated protein 78 (GRP78) and heat shock protein 90-α (HSP90α)-were confirmed in immunoprecipitation assay in two TNBC cell lines (HCC1937 and MDA-MB231). Surface plasmon resonance analysis using GST-PRDM14 showed that these two proteins directly interacted with PRDM14 and that the interactions required the C-terminal region of PRDM14, which includes zinc finger motifs. We also confirmed the interactions in living cells by NanoLuc luciferase-based bioluminescence resonance energy transfer (NanoBRET) assay. Moreover, HSP90 inhibitors (17DMAG and HSP990) significantly decreased breast cancer stem-like $CD24^-$ $CD44^+$ and side population (SP) cells in HCC1937 cells, but not in PRDM14 knockdown HCC1937 cells. The combination of the GRP78 inhibitor HA15 and PRDM14 knockdown significantly decreased cell proliferation and SP cell number in HCC1937 cells. These results suggest that HSP90α and GRP78 interact with PRDM14 and participate in cancer regulation.

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Social Cooperation Research Program

Project Division of Cancer Biomolecular Therapy
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Our division has been conducting basic research projects for development of innovative cancer therapy using immunologic and gene therapy approaches. The reagents, modalities, and concepts developed in this division have been clinically applied as translational research projects. We believe that bidirectional information exchange between the bench and the bedside would be one of the most important requirements for the successful development of novel and effective therapies.

I. Immuno-regulatory influence of abundant MFG-E8 expression by esophageal cancer treated with chemotherapy

Takashi Kanemura[1], Hiroshi Miyata[1,2], Tomoki Makino[1], Koji Tanaka[1], Keijiro Sugimura[1,2], Mika Hamada-Uematsu, Yu Mizote, Hiroaki Uchida, Yasuhiro Miyazaki[1], Tsuyoshi Takahashi[1], Yukinori Kurokawa[1], Makoto Yamasaki[1], Hisashi Wada[1], Kiyokazu Nakajima[1], Shuji Takiguchi[1], Masaki Mori[1], Yuichiro Doki[1], Hideaki Tahara[2]: [1]Department of Gastroenterological Surgery, Graduate School of Medicine, Osaka University, [2]Osaka International Cancer Institute

Milk fat globule- epidermal growth factor- factor 8 (MFG-E8) is secreted from macrophages and is known to induce immunological tolerance mediated by regulatory T-cells (Tregs). However, the roles of the MFG-E8 that is expressed by cancer cells have not yet been fully examined. MFG-E8 expression was examined using immunohistochemistry in surgical samples from 134 patients with esophageal squamous cell carcinoma. The relationships between MFG-E8 expression levels and clinicopathological factors, including tumor-infiltrating lymphocytes (TILs), were evaluated. High MFG-E8 expression was observed in 23.9% of the patients. The patients with tumors highly expressing MFG-E8 had a significantly higher percentage of neoadjuvant chemotherapy (NAC) history ($P<0.0001$), shorter relapse-free survival (RFS; $P=0.012$) and overall survival (OS; $P=0.0047$). On subgroup analysis according to NAC history, patients with high MFG-E8 expression had significantly shorter RFS ($P=0.027$) and OS ($P=0.0039$) only when they had been treated with NAC. Furthermore, tumors with high MFG-E8 expression had a significantly lower ratio of CD8+ T-cell/Treg in TILs ($P=0.042$) only in the patients treated with NAC, and those with a lower ratio had a shorter OS ($P=0.026$). High MFG-E8 expression was also found to be an independent prognostic factor in multivariate analysis. The abundant MFG-E8 expression in esophageal squamous cell carcinoma might have a negative influence on the long-term survival of patients after chemotherapy by affecting T-cell regulation in the tumor microenvironment.

II. Development of cancer immunotherapy using the blockade of MFG-E8

Yu Mizote, Mika Uematsu-Hamada, Miho Kudo, Hiroaki Uchida, Hideaki Tahara

The secreted protein, milk fat globule-EGF factor

8 (MFG-E8), stimulates disease progression through coordinated αvβ3 integrin signaling in tumor and host cells. MFG-E8 enhances tumor cell survival, invasion, and angiogenesis, and contributes to local immune suppression.

We have shown that systemic MFG-E8 blockade cooperates with cytotoxic chemotherapy, molecularly targeted therapy, and radiation therapy to induce destruction of various types of established mouse tumors. The combination treatments evoke extensive tumor cell apoptosis that is coupled to efficient dendritic cell cross-presentation of dying tumor cells. Our previous findings suggest that systemic MFG-E8 blockade might intensify the antitumor activities of existing therapeutic regimens through coordinated cell-autonomous and immune-mediated mechanisms also in human. In order to further investigate these findings in mouse tumor systems, we have developed multiple means to abrogate the functions of MFG-E8 antibodies specific to the mouse MFG-E8. Furthermore, we are now seeking the opportunities of developing this agent for clinical application.

III. NK cells control tumor-promoting function of neutrophils in mice

Yoshihiro Hayakawa[3], Keisuke Ogura[3], Marimo Sato-Matsushita, Seiji Yamamoto[3], Takashi Hori[3], Masakiyo Sasahara[3], Yoichiro Iwakura, Ikuo Saiki, Hideaki Tahara: [3]Institute Natural Medicine, University of Toyama

Although the importance of NK cells as a direct anti-tumor effector is well appreciated, the immuno-regulatory function of NK cells to control cancer-associated inflammation, which facilitate tumor progression, remains unknown. In this study, we demonstrate that the novel function of NK cells to control tumor-promoting inflammation through the functional modification of neutrophils. NK cells control the tumor-promoting function of neutrophils via an IFN-γ-dependent mechanism and the tumor progression in an NK cell-depleted host is totally diminished when the IL-17A-neutrophils axis is absent. In NK cell-depleted mice, neutrophils acquire the tumor-promoting phenotype as seen in the up-regulation of VEGF-A expression to promote tumor growth and angiogenesis. Importantly, a VEGFR inhibitor preferentially suppressed tumor growth in NK cell-depleted mice and such a selective anti-tumor effect in NK cell-depleted mice was a neutrophil-dependent. Furthermore, the systemic neutropenia by an antimetabolite treatment shows a significant anti-cancer effect only in mice with no NK cells. Thus, NK cells likely play an important role in controlling the tumor-promoting and angiogenic function of neutrophils.

IV. Lung-resident natural killer cells control pulmonary tumor growth in mice

Yutaka Yamamoto[3], Kiho Miyasato[3], Kei Takahashi[3], Naoki Yoshimura[3], Hideaki Tahara, Yoshihiro Hayakawa[3]: [3]Institute Natural Medicine, University of Toyama

Accumulating evidence indicates the importance of natural killer (NK) cells in controlling tumor growth and metastasis. NK cell subsets display diversities in their function and tissue distribution and Mac-1^{hi} $CD27^{lo}$ NK cells are the predominant population of lung-resident NK cells. Although the lung is a major organ where primary tumor develops and cancer cells metastasize, there is no clear evidence whether circulating NK cells and/or tissue-resident NK cells control tumor growth in the lung. In the present study, we examined an antitumor function of lung-resident NK cells to control pulmonary tumor growth. In an orthotopic lung tumor model, NK cells controlled pulmonary tumor growth, and mature circulating NK cell subsets were increased in tumor-bearing lungs through a C-X-C motif chemokine receptor 3 (CXCR3)-dependent mechanism. Although such increase in migratory NK cell subsets can be blocked by anti-CXCR3 treatment, there was no difference in pulmonary tumor growth in anti-CXCR3-treated mice compared with control mice. In addition to pulmonary tumor growth, lung-resident NK cells, but not migratory NK cells, play a dominant role in controlling metastatic growth of cancer cells in lung. These results strongly indicate an importance of lung-resident NK cells for controlling pulmonary tumor growth.

V. Development of fully retargeted herpes simplex virus (HSV) vectors for oncolytic virotherapy

Hiroaki Uchida, Hitomi Ikeda, Tomoko Shibata, Takuma Suzuki, Ayumi Endo, Hideaki Ono, Yasuhiko Sasaki, Naoki Kabasawa, Rintaro Hayase, Naoya Nagata, Yukinari Kato[4], Hideaki Tahara: [4]Department of Antibody Drug Development, Tohoku University Graduate School of Medicine

Herpes simplex virus (HSV) vectors are promising agents for oncolytic virotherapy. Uchida established a fully retargeted HSV platform that mediates virus entry exclusively via tumor-associated antigens in the lab of Prof. Joseph Glorioso at the University of Pittsburgh. Entry of HSV is initiated by the binding of glycoprotein D (gD) to one of its receptors, herpesvirus entry mediator (HVEM) or nectin-1. This interaction results in a conformational change in gD, triggering sequential activation of gH

and gB to execute fusion between the viral envelope and cell membranes. We inserted single-chain antibodies (scFv) against a number of different cell surface molecules such as epidermal growth factor receptor (EGFR), carcinoembryonic antigen (CEA), and epithelial cell adhesion molecule (EpCAM), into the retargeted HSV platform that encodes a gD ablated for binding to natural receptors and a gB containing entry-enhancing mutations we previously identified. As a result, we observed specific virus entry into cells expressing the cognate target antigen for each of the retargeted constructs. Our results indicate the adaptability of our system to different targeting ligands, leading to a new generation of broadly applicable and effective oncolytic HSV vectors. Furthermore, we introduced syncytial mutations into the gB and/or gK genes of gD-retargeted HSVs and found that gD retargeting does not abolish the hyperfusogenic activity of syncytial mutations and that these mutations do not eliminate the dependence of HSV entry and spread on a specific gD-receptor interaction. These observations suggest that syncytial mutations may be valuable for increasing the tumor-specific spreading of retargeted oncolytic HSV vectors. We are now testing whether syncytium formation in tumors would be associated with more potent antitumor effects *in vivo*. We are also investigating whether our retargeted oncolytic HSV vectors would exert robust antitumor effects when administered not only intratumorally but also intravenously.

VI. Establishment of highly functional monoclonal antibodies through novel screening methods for targeted cancer therapy

Hiroaki Uchida, Hitomi Ikeda, Tomoko Shibata, Nanami Hayashi, Miki Yamaguchi[5], Hideaki Tahara: [5]Department of Molecular Medicine, Research Institute for Frontier Medicine, Sapporo Medical University School of Medicine

Monoclonal antibodies (mAbs) have become an established therapeutic modality in clinical oncology. In order to identify cell-surface molecules that may be useful for targeting various types of cancers, our group established a unique screening approach that employs an adenoviral vector harboring fiber proteins engineered to bind antibodies, Adv-FZ33. This approach led to the successful identification of an array of potential target molecules for cancer treatment. Immunotoxins (antibody-drug conjugates; ADC) are a promising class of cancer therapeutics composed of a cytotoxic agent linked covalently to a cancer-targeted antibody. To systematically hunt for cell-surface molecules that may be efficiently targeted by immunotoxins, our group created another method for screening highly functional cancer-targeted mAbs and cognate antigens. The receptor-binding domain of the Diphtheria toxin (DT) was replaced with the antibody-binding domain (3C) derived from the Streptococcal protein G. The resultant mutated toxin protein (DT-3C) was used for selection of mAbs for specific cell killing activity as components of immunotoxins. Our novel screening system is advantageous in that the selected antibodies bind to intact cancer cells and get internalized efficiently, which has been critically required for therapeutic applications but elusive thus far. Furthermore, we have successfully taken advantage of some of these in-house monoclonal antibodies for development of novel fully retargeted HSV vectors. Additionally, we have created an HSV-based probe for screening of Abs that could mediate HSV entry by recognition of unknown receptors. We have found that one of the Abs selected by this screening method is capable of mediating HSV entry when incorporated into gD as an scFv. We expect that this novel Ab-screening system may lead to a new generation of RR-oHSV vectors.

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