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)

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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. 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.

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. 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 2014 are as follows.

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

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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, La-related protein 4b (LARP4B), as a candidate tumor suppressor gene in glioma. *LARP4B* expression was consistently decreased in human glioma stem cells and cell lines compared with normal neural stem cells. Moreover, heterozygous deletion of *LARP4B* was detected in nearly 80% of glioblastomas in the TCGA database. *LARP4B* loss was also associated with low expression and poor patient survival. Overexpression of LARP4B in glioma cell lines strongly inhibited proliferation by inducing mitotic arrest and apoptosis in four of six lines as well as

in two patient-derived glioma stem cell populations. The expression levels of *CDKN1A* and *BAX* were also upregulated upon LARP4B overexpression, and the growth-inhibitory effects were partially dependent on p53 (TP53) activity in cells expressing wild-type, but not mutant, p53. We further found that the La module, which is responsible for the RNA chaperone activity of LARP4B, was important for the growth-suppressive effect and was associated with *BAX* mRNA. Finally, LARP4B depletion in mouse primary astrocytes promoted cell proliferation and led to increased tumor size and invasiveness in an orthotopic model. These data provide strong evidence that LARP4B serves as a tumor suppressor gene in glioma.

Analysis of the role of microglial in retinal degeneration

Hideto Koso, Asano Tsuhako, Sumiko Watanabe

The retina is an integral part of the CNS, and has long served as a model for studying CNS development and pathologies. Retinitis pigmentosa is the most common cause of inherited blindness, which is characterized by the progressive loss of photoreceptor cells. There are currently no effective treatments to stop or cure this disease. Thus, a better understanding of its pathogenesis is needed. Retinal degeneration often causes activation of microglia and infiltration of monocyte-derived macrophages into the brain. To understand distinct characteristics between the two phagocyte subsets, we generated a genetic model of rod photoreceptor degeneration. Rod injury induced proliferation of microglia, as well as invasion of blood-derived macrophages into the retina. By comparing expression profiles between microglia and infiltrating macrophages, we identified *Sall1* as a gene specifically expressed in microglia. *Sall1gfp* knock-in mice allowed us to evaluate contribution of microglia to the phagocyte pool in the *rd1* mouse model of retinal degeneration. We showed that Sall1gfp^{+} microglia were expanded and maintained during inflammatory responses. We also found that *Sall1* deficiency did not affect microglial colonization in the brain but impaired their maturation, suggesting that *Sall1* is involved in the specification of naïve progenitors into mature microglia.

Role of transcription factor Tgif2 in photoreceptor differentiation in the mouse retina

Hiroshi Kuribayashi, Asano Tsuhako, Hideto Koso, Sumiko Watanabe

5'TG3'-interacting factors (TGIFs) function as transcriptional repressors. Defects in TGIFs cause severe abnormalities in the developing brain and face. We found that *Tgif2* was highly expressed in the mouse retina at early stages of development and examined its role in retinal development. Knockdown of *Tgif2* in retinal explants at E14 using shRNA (sh-*Tgif2*) resulted in a decreased number of rod photoreceptors, whereas the number of cone photoreceptors increased without perturbation of cell proliferation and apoptosis. Concomitantly, the expression levels of photoreceptor-related genes were decreased in sh-*Tgif2*-introduced retinal explants. To examine the *in vivo* effects of Tgif2 overexpression, we generated *Tgif2* conditional knock-in mice (*Tgif2*cKI). Although retinal cell differentiation, based on the relative proportions of retinal subtypes in the mature retina, was not affected, we observed abnormal localization of cone photoreceptor cell nuclei in the outer nuclear layer of the *Tgif2* cKI retina. However, electrical retinography suggest that cones in *Tgif2*cKI were functionally equivalent to those of wild mice. Our study revealed that Tgif2 participates in photoreceptor cell differentiation in the early stages of retinal development and regulates proper subretinal localization of the cone photoreceptors.

Regulation of retinal development via the epigenetic modification of histone H3

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We are interested in the roles of epigenetic mechanisms in retinal development. By ChIP-qPCR using whole retinal extracts at various developmental stages, we found that the levels of methylation of histones H3K27 and H3K4 and acetylation of histone H3 at specific loci in various genes, which play critical roles in retinal proliferation and differentiation, changed dramatically during retinal development. We next focused on the roles of H3K27 trimethylation in retinal development. Ezh1 and Ezh2 are methyltransferases that act on H3K27, while Jmjd3 and Utx are demethylases. We found that Ezh2 and Jmjd3 were mainly expressed during retinal development, and a loss-of-function of these genes revealed a role for H3K27me3 in the maturation of subsets of bipolar cells. Furthermore, Ezh2 and Jmjd3 regulate H3K27 trimethylation at specific loci within Bhlhb4 and Vsx1, which play critical roles in the differentiation of subsets of bipolar cells. Utx is expressed weakly in retina, and the down-regulation of Utx by sh-RNA in retinal explants suggested that Utx also participates in the maturation of bipolar cells. Ezh1 is expressed weakly in postnatal retina, and the phenotype of Ezh2-knockout retina suggested that Ezh1 plays a role in the methylation of H3K27 in the late phase of retinal differentiation. Taken together, we found

that these four genes, which exhibit temporally and spatially unique expression patterns during retinal development, play critical roles in the differentiation of retinal subsets through the regulation of histone H3K27 methylation at critical genetic loci.

Transition of differential histone H3 methylation in photoreceptors and other retinal cells during retinal differentiation

Kazuko Ueno, Toshiro Iwagawa, Hiroshi Kuribayashi, Yukihiro Baba, Yutaka Suzuki[3], Sumiko Watanabe: [3]Department of Medical Genome Sciences, Graduate School of Frontier Sciences

To analyze cell lineage-specific transitions in global transcriptional and epigenetic changes during retinogenesis, we purified retinal cells from normal mice during postnatal development into two fractions, namely, photoreceptors and other retinal cells, based on Cd73 expression, and performed RNA sequencing and ChIP sequencing of H3K27me 3 and H3K4me3. Genes expressed in the photoreceptor lineage were marked with H3K4me3 in the Cd73-positive cell fraction; however, the level of H3 K27me3 was very low in both Cd73-positive and -negative populations. H3K27me3 may be involved in spatio-temporal onset of a subset of bipolar-related genes. Subsets of genes expressed in amacrine and retinal ganglion cells, which are early-born retinal cell types, were suggested to be maintained in a silent state by H3K27me3 during late-stage retinogenesis. In the outer nuclear layer, upregulation of Rho and rod-related genes were observed in Ezh2-ablated retina, suggesting a role for H3K27me3 in the maintenance of proper expression levels. Taken together, our data on the transition of lineage-specific molecular signatures during development suggest that histone methylation is involved in retinal differentiation and maintenance through cell lineage-specific mechanisms.

Smyd5 plays pivotal roles in both primitive and definitive hematopoiesis during zebrafish embryogenesis.

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Methylation of histone tails plays a pivotal role in the regulation of a wide range of biological processes. SET and MYND domain-containing protein (SMYD) is a methyltransferase, five family members of which have been identified in humans. SMYD1, SMYD2, SMYD3, and SMYD4 have been found to play critical roles in carcinogenesis and/or the development of heart and skeletal muscle. However, the physiological functions of SMYD5 remain unknown. To investigate the function of Smyd5 in vivo, zebrafish were utilized as a model system. We first examined smyd5 expression patterns in developing zebrafish embryos. Smyd5 transcripts were abundantly expressed at early developmental stages and then gradually decreased. Smyd5 was expressed in all adult tissues examined. Loss-of-function analysis of Smyd5 was then performed in zebrafish embryos using smyd5 morpholino oligonucleotide (MO). Embryos injected with smyd5-MO showed normal gross morphological development, including of heart and skeletal muscle. However, increased expression of both primitive and definitive hematopoietic markers, including pu.1, mpx, l-plastin, and cmyb, were observed. These phenotypes of smyd5-MO zebrafish embryos were also observed when we introduced mutations in smyd5 gene with the CRISPR/Cas9 system. As the expression of myeloid markers was elevated in smyd5 loss-of-function zebrafish, we propose that Smyd5 plays critical roles in hematopoiesis.

Publications

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渡辺すみ子　マイクログリア細胞と炎症·免疫,炎症と免疫 vol 25(1), 73
眼科臨床エキスパート　網膜変性疾患診療のすべて 網膜変性疾患を理解するための基礎A網膜の構造と生理 医学書院 page 24-32　2016

Social Cooperation Research Program

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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/polypeptide 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. Therapeutic RNA Discovery

a. The efficient cell-SELEX strategy, Icell-SELEX, using isogenic cell lines for selection and counter-selection to generate RNA aptamers to cell surface proteins

Masaki Takahashi, Eri Sakota, Yoshikazu Nakamura

Aptamers are short single-stranded nucleic acid molecules that are selected in vitro from a large random sequence library based on their high and specific affinity to a target molecule by a process known as SELEX. Cell-SELEX that employs whole living cells overexpressing the defined cell surface proteins (for selection) and appropriate mock cells (for counter-selection) has been widely used as a valid and feasible method for generating aptamers against specific cell surface proteins. However, the endogenous expression of target proteins in mock cells or the heterogeneity of surface proteins between selection and counter-selection cells often impeded the isolation of proper aptamers against target proteins. To solve this problem, we developed "Isogenic cell-SELEX" (Icell SELEX in short) method, in which isogenic cell lines were manipulated for counter-selection by microRNA-mediated silencing and for selection by overexpression of target proteins. As a model experiment, we targeted integrin alpha V (ITGAV), which is a major transmembrane receptor expressed in almost all the cells, and established ITGAV-overexpressed and -downregulated HEK293 cells for selection and counter-selection, respectively. By taking advantage of a hundred-fold difference in the expression level of ITGAV between these two isogenic cell lines, we easily isolated several anti-ITGAV aptamers, whose binding to the cell-surface ITGAV was confirmed by flow cytometry with the dissociation constant of 300-400 nM range. We assume that Icell-SELEX could be applicable to a wide range of cell-surface proteins including various transmembrane proteins of biological and pharmacological significance.

b. Structural basis for specific inhibition of Autotaxin by a DNA aptamer

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Autotaxin (ATX) is a plasma lysophospholipase D that hydrolyzes lysophosphatidylcholine (LPC) to produce lysophosphatidic acid (LPA), a lipid mediator involved in various physiological and pathophysiological processes. Although ATX is an attractive therapeutic target, no ATX inhibition-mediated treatment strategies for human diseases have been established. Here, we report anti-ATX DNA aptamers that inhibit ATX with high specificity and efficacy. We selected anti-ATX DNA aptamers by the SELEX (systematic evolution of ligands by exponential enrichment) method. We solved the crystal structure of ATX in complex with the anti-ATX aptamer RB011, at 2.0 Å resolution. The structure revealed that RB011 adopts a characteristic L-shaped hairpin structure, supported by non-canonical base pairs as well as Ca^{2+} coordination. RB011 binds in the vicinity of the active site through base-specific interactions, thus preventing the access of the choline moiety of LPC substrates. Based on the structural information, we developed the modified anti-ATX DNA aptamer RB014, which exhibits *in vivo* efficacy in bleomycin-induced pulmonary fibrosis model mice. Our findings provide the structural basis for the specific inhibition of ATX by the anti-ATX aptamer, and highlight the therapeutic potential of anti-ATX aptamers for the treatment of human diseases, such as pulmonary fibrosis.

c. Dual therapeutic action of a neutralizing anti-FGF2 aptamer in bone diseases and bone cancer pain

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Fibroblast growth factor 2 (FGF2) plays a crucial role in bone remodeling and disease progression. However, the potential of FGF2 antagonists for treatment of patients with bone diseases has not yet been explored. Therefore, we generated a novel RNA aptamer, APT-F2, specific for human FGF2 and characterized its properties in vitro and in vivo. APT-F2 blocked binding of FGF2 to each of its four cellular receptors, inhibited FGF2-induced downstream signaling and cells proliferation, and restored osteoblast differentiation blocked by FGF2. APT-F2P, a PEGylated form of APT-F2, effectively blocked the bone disruption in mouse and rat models of arthritis and osteoporosis. Treatment with APT-F2P also exerted a strong analgesic effect, equivalent to morphine, in a mouse model of bone cancer pain. These findings demonstrated dual therapeutic action of APT-F2P in bone diseases and pain, providing a promising approach to the treatment of bone diseases.

d. *De novo* sequencing of highly modified therapeutic oligonucleotides by hydrophobic tag sequencing coupled with LC-MS

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Correct sequences are prerequisite for quality control of therapeutic oligonucleotides. However, there is no definitive method available for determining sequences of highly modified therapeutic RNAs, and thereby most of the oligonucleotides have been used clinically without direct sequence determination. In this study, we developed a novel sequencing method called `hydrophobic tag sequencing'. Highly modified oligonucleotides are sequenced by partially digesting oligonucleotides conjugated with a 5'-hydrophobic tag, followed by liquid chromatography-mass spectrometry analysis. 5'- hydrophobic tag-printed fragments (5'-tag degradates) can be separated in order of their molecular masses from tag-free oligonucleotides by reversed-phase liquid chromatography. As models for the sequencing, the anti-VEGF aptamer (Macugen) and the highly modified 38-mer RNA sequences were analyzed under blind conditions. Most nucleotides were identified from the molecular weight of hydrophobic 5'-tag degradates calculated from monoisotopic mass in simple full mass data. When monoisotopic mass could not be assigned, the nucleotide was estimated using the molecular weight of the most abundant mass. The sequences of Macugen and 38-mer RNA perfectly matched the theoretical sequences. The hydrophobic tag sequencing worked well to get simple full mass data, resulting in accurate and clear sequencing. The present study provides for the first time a *de novo* sequencing technology for highly modified RNAs, and contributes to quality control of therapeutic oligonucleotides.

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. Control of transcriptional pausing by biased thermal fluctuations on repetitive genomic sequences

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In the process of transcription elongation, RNA polymerase (RNAP) pauses at highly nonrandom positions across genomic DNA, broadly regulating transcription; however, molecular mechanisms responsible for the recognition of such pausing positions remain poorly understood. Here, using a combination of statistical mechanical modeling and high-throughput sequencing and biochemical data, we evaluate the effect of thermal fluctuations on the regulation of RNAP pausing. We demonstrate that diffusive backtracking of RNAP, which is biased by repetitive DNA sequence elements, causes transcriptional pausing. This effect stems from the increased microscopic heterogeneity of an elongation complex, and thus is entropy-dominated. This work shows a linkage between repetitive sequence elements encoded in the genome and regulation of RNAP pausing driven by thermal fluctuations.

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Social Cooperation Research Program

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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 event by using bioinformatics technique. Furthermore, we will apply the knowledge from our research for the discovery of novel drug or method 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 aquired 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 enable 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 KO mouse, disease model and human. Analysis of the huge and various kinds of samples data will lead to the discovery of the cause of diseases and the therapy.

2. Development of new "virome" analysis method

Yasumasa Kimura, Shanai Yin, 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[5], 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 invent 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, 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.

Many unidentified viruses have been presence in mucosal tissue and it is not so simple to analyze virome data. We applied and optimize the pipeline for commensal bacteria meta-genome analysis for virome analysis. This pipeline is effective for unidentified viruses as well. We use our virus separation method with this pipeline and accelerate virome study.

4. Optimization of 16S rRNA metagenome analysis protocol

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

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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(ロート製薬株式会社)

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Associate Professor	Tokiko Nagamura MD., D.M.Sc	准教授(兼任)	医学博士	長 村 登紀子

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

Nonaka H, Tamai R, Ueno Y, Horiuchi Y, Ota S, Yumoto M, Hasegawa H, Nagamura T, Tojo A, Yamazaki S

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 were observed in 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 us to the identification of a new biomarker and a better understanding of MSCs' therapeutic potential.

2. Generation of disease-specific human iPS cells

Izawa K, Mochizuki S, Yokoyama K, Yusa N, Yamazaki M, Kobayashi M, Otsu M, Tojo A, Yamazaki S

Here, using intractable disease-specific human iPS, we are intended to elucidate the pathogenetic mechanism and develop a new agent for the treat-

ment 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.

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 医学博士 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.

HPV compensation program in Japan

Yuji, K, and Nakada, H.

We investigated HPV vaccine injury compensation programs for both the national and local governments. Approximately 3.38 million girls were vaccinated, and 2,584 complained of health problems. The majority of these received the vaccine shot as a non-routine vaccination. In total, 98 people developed health problems and applied for assistance from 2011 to 2014, but no cases have been processed since October 2014. Several local governments are providing their own compensation program for cases of vaccine adverse reactions, but the number is extremely low. The confusion regarding the national program for HPV vaccine injury was caused by the discrepancy between the compensation programs for those vaccinated under the immunization law and for those who received voluntary vaccinations.

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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(SBIファーマ株式会社)

Project Professor	Kenzaburo Tani, M.D., Ph.D.	医学博士	谷　憲三朗
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Project Assistant Professor	Shohei Miyamoto, Ph.D.	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[1], Toshihiko Okazaki[2], Kazunari Yamada[1], Mutsunori Murahashi[1], Hisanobu Ogata[1] Kenzaburo Tani[1,3]: [1]Department of Advanced Cell and Molecular Therapy, Kyushu University Hospital, Fukuoka, Japan, [2]ARO Advanced Medical center, Kyushu University Hospital, Fukuoka, Japan, [3]Project Division of ALA Advanced Medical Research, IMSUT

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[1], Shohei Miyamoto[1], Yuto Takishima[1], Hiroyuki Shimizu[2], Yoichi Nakanishi[3], Kenzaburo Tani[1]: [1]Project Division of ALA Advanced Medical Research, IMSUT, [2]Department of Virology II, National Institute of Infectious Diseases, Tokyo, Japan, [3]Research Institute of Diseases of the Chest, Kyushu University, Fukuoka, Japan.

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[1], Miyako Sagara[1], Lisa Hirose-Yotsuya[1], Hiroshi Kohara[1], Hiroyuki Shimizu[2], Kenzaburo Tani[1]: [1]Project Division of ALA Advanced Medical Research, IMSUT, [2]Department of Virology II, National Institute of Infectious Diseases, Tokyo, Japan

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[1,2,3], Katsuyuki Hamada[4], Yuto Takishima[2], Miyako Sagara[2], Yumiko Matsumura[2], Shohei Miyamoto[2,5], Yasuki Hijikata[2], Toshihiko Okazaki[2], Yoichi Nakanishi[3], Kenzaburo Tani[2,5]: [1]Department of Thoracic Oncology, National Kyushu Cancer Center, Fukuoka, Japan, [2]Division of Molecular and Clinical Genetics, Medical Institute of Bioregulation, Kyushu University, Fukuoka, Japan, [3]Research Institute for Diseases of the Chest, Kyushu University, Fukuoka, Japan, [4]Department of Obstetrics and Gynecology, Ehime University, Toon, Japan, [5]Project Division of ALA Advanced Medical Research, IMSUT

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

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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[1], Satoshi Takahashi[2], Masaru Shinozaki[3], Kaoru Uchimaru[4], Arinobu Tojo[2], Naohide Yamashita[5], Yasuki Hijikata[5], Shohei Miyamoto[1], Kenzaburo Tani[1]: [1]Project Division of ALA Advanced Medical Research, IMSUT, [2]Department of Hematology/Oncology, IMSUT, [3]Department of Surgery, Research Hospital, IMSUT, [4]Laboratory of Tumor Cell Biology, Graduate School of Frontier Sciences, The University of Tokyo, Tokyo, Japan, [5]Department of Advanced Medical Science, Research Hospital, IMSUT

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 sensi-

tivity. 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+CD14high 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[1], Hiroshi Kohara[2], Hiroyuki Inoue[3], Megumi Narusawa[3], Yoshie Ogawa[2], Lisa Hirose-Yotsuya[2], Shohei Miyamoto[2], Yumiko Matsumura[3], Kazunari Yamada[3], Atsushi Takahashi[1], and Kenzaburo Tani[2]: [1]Division of Molecular and Clinical Genetics, Medical Institute of Bioregulation, Kyushu University, Fukuoka, Japan, [2]Project Division of ALA Advanced Medical Research, IMSUT, [3]Department of Advanced Molecular and Cell Therapy, Kyushu University Hospital, Fukuoka, Japan

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 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

Hiroshi Kohara[1], Hiromi Ogura[2], Takako Aoki[2], Chika Sakamoto[3], Yoshie Ogawa[1], Shohei Miyamoto[1], Hitoshi Kanno[2], Kenzaburo Tani[1,3,4]: [1]Project Division of ALA Advanced Medical Research, IMSUT, [2]Department of Transfusion Medicine and Cell Processing, School of Medicine, Tokyo Women's Medical University, Tokyo, Japan, [3]Division of Molecular and Clinical Genetics, Medical Institute of Bioregulation, Kyushu University, Fukuoka, Japan, [4]Department of Advanced Molecular and Cell Therapy, Kyushu University Hospital, Fukuoka, Japan

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

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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 106 input CD34 + cells; P53 KO: 2476 cells /1 x 106 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.

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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.

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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.
Project Associate Professor Hiroshi Yasui, M.D., D.M.Sc.

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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 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; specimen preservation; electronic medical records access; and personal information protection. Our mission is to increase social acceptance of genomic medicine.

2. 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 2016, we were conducting, supporting, or preparing three investigator-initiated clinical trials under INDs applications for the development of academic-oriented innovative anticancer therapeutics. Currently, we are conducting a phase II study on the efficacy of a novel peptide vaccine therapy for patients with advanced or recurrent pancreatic cancer. In collaboration with the Center for Translation Research, we are involved in site management for this project. Additionally, we are supporting a phase II study of another novel peptide vaccine against solid tumors and are preparing a phase I trial of novel therapeutic cancer vaccines for hematological malignancies.

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 and 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. 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.

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