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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特任教授 医学博士 渡 辺 すみ子
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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.

Functional analysis of candidate tumor suppressor genes for glioma

Hideto Koso, Shingo Ito, Keisuke Sumiyoshi, Sumiko Watanabe

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. Glioblastoma (GBM) is the most common form of malignant brain cancer in adults. Patients with GBM have a uniformly poor prognosis, with a mean survival of one year. Thus, advances on all fronts, both basic and applied, are needed in order to combat this deadly disease. To better understand genes and signaling pathways that are able to transform neural stem cells into glioma-initiating cells, we have performed a transposon mutagenesis screen in mice. Two RNA-binding proteins were identified as novel tumor suppressor genes in glioma, and we have performed functional analyses of these genes. The expression levels of both of the RNA-binding proteins were decreased in glioma compared to normal neural stem cells. The overexpression of these genes in glioma cell lines suppressed proliferation of these cells *in vitro*. On the other hand, shRNA-mediated knockdowon of these genes in primary mouse astrocytes promoted tumor

outgrowth in a xenograft mouse model. We are currently investigating the molecular mechanisms responsible for the growth inhibitory effects of the RNA-binding proteins in glioma.

Functional analysis of candidate oncogenes for medulloblastoma

Boonmin Poh, Hideto Koso, Sumiko Watanabe

Medulloblastoma is the most common malignant brain tumor of childhood and tends to metastasize throughout the central nervous system. Despite overall improvements in survival with multimodal treatment, a substantial proportion of patients are still incurable. To develop better therapies, insights into the molecular mechanisms leading to the development of medulloblastomas are essential. By conducting a *SB* transposon mutagenesis screen, we have identified candidate oncogenes for medulloblastoma, including *FoxR2*, *Alx4* and *Tgif2*. To analyze the *in vivo* function of these genes, we generated conditional knock-in mice that allowed us to overexpress these genes in a tissue-specific manner. We have mated these knock-in strains with the *Nestin-cre* transgenic mouse strain, which induces recombination in the entire brain. We are currently investigating the effect of overexpression of *FoxR2*, *Alx4* and *Tgif2* on the proliferation of granule neuron precursors in the cerebellum, the cell-of-origin for medulloblastoma.

Analysis of the role of microglial in retinal degeneration

Hideto Koso, Asano Tsuhako, Sumiko Watanabe

The retina is an integral part of the central nervous system (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. Accumulating evidence suggests pathogenic roles of microglia and infiltrating macrophages in mouse models of retinal degeneration; however differential roles of these two populations remain unclear. As microglia and infiltrating macrophages often coexist in lesional tissues, a direct comparison of gene expression patterns between these cells in the same lesion of the CNS is needed. By combining bone marrow transplantation (BMT) with the conditional model of rod photoreceptor degeneration, we were able to isolate microglia and infiltrating macrophages from the injured retina and perform gene expression profiling. By comparing gene expression patterns, we identitified a set of genes that are speicifically expressed in activated microglia in the injured retina. We are currently investigating the functions of these genes in microglial activation.

Differential regulation of genes in photoreceptors and other neurons by cell lineage specific mechanisms of histone H3 methylation at K4 and K27 during retinal differentiation

Toshiro Iwagawa, Hiroshi Kuribayashi, Yukihiro Baba, Yutaka Suzuki[1], Sumiko Watanabe: [1]Department of Medical Genome Sciences, Graduate School of Frontier Sciences, University of Tokyo

Various retinal cell subtypes arise from common progenitor population. Temporal dissection of molecular signature of different cell lineage must be a powerful tool to understand developmental mechanisms. Histone H3K27me3 is a negative marker of transcription, and Ezh2 is a major methyltransferase of H3K27 in retina; its ablation in the retina results in microphtalmea, and molecular analysis suggested that different molecular mechanisms are involved in the reduction of photoreceptors and other cells. To analyze cell lineage-specific transitions in global transcriptional and epigenetic changes during retinogenesis, we purified retinal cells from postnatal normal mice at three different developmental stages into two fractions, namely, photoreceptors and other retinal cells, based on Cd73 expression, and performed RNA sequencing and ChIP sequencing of H3K27me3 and H3K4me3. Genes expressed in the photoreceptor lineage were marked with H3K4me3 in the Cd73-positive cell fraction; however, the level of H3K27me3 was very low in both Cd73-positive and -negative populations. Spatio-temporal onset of a subset of bipolar-related genes was regulated by H3K27me3. Subsets of genes expressed in amacrine- and retinal ganglion cells, which are early born retinal cells, were 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 roles of H 3K27me3 for maintain proper level of expression. Taken together, our data on the transition of lineage-specific molecular signatures during development revealed that histone methylation regulates retinal differentiation and maintenance through cell lineage-specific mechanisms.

MicroRNA-7a regulates Müller glia differentiation by attenuating Notch3 expression

Yukihiro Baba, Sumiko Watanabe

miRNA-7a plays critical roles in various biological aspects of health and disease. We aimed to re-

veal the roles of miR-7a in mouse retinal development using loss- and gain-of-function analyses of miR-7a. Plasmids encoding miR-7a or miR-7a-decoy (anti-sense miR-7a) were introduced into the mouse retina at P0, and the retina was cultured as an explant. Then, retinal progenitor cell proliferation and the differentiation of the retinal subtypes were examined using immunostaining. miR-7a had no apparent effect on the proliferation of retinal progenitor cells. However, the expression of the Müller glia marker cyclin D3 was reduced by the overexpression of miR-7a and upregulated by the miR-7a decoy, suggesting that miR-7a negatively regulates Müller glia differentiation. Potential miR-7a targets were predicted by using a public available prediction program miRNA.org, which suggested that Notch3 is candidate miR-7a target. The 3'-UTR of *Notch3* contained a sequence complementary a to the seed sequence of miR-7a. A reporter assay in NIH3T3 cells using a plasmid containing multiple repeats of the potential target sequence in the Notch3 3'-UTR demonstrated that miR-7a suppressed expression Notch3 via the 3'-UTR. The expression of sh-Notch3 and the overexpression of NICD3 in the retina suggested that miR-7a regulates Müller glia differentiation by attenuating Notch3 expression. Taken together, our results suggested that miR-7a regulates the differentiation of Müller glia by suppressing Notch3 expression.

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Social Cooperation Research Program

Project Division of Social Communication System for Advanced Clinical Research

先端医療社会コミュニケーションシステム社会連携研究部門

Project Professor　Masahiro Kami, M.D., Ph.D.

特任教授　医学博士　　上　　昌　広

The aim of our division is to establish and popularize state-of-art medicine and to promote translational research. We investigate ideal medical governance and methodology to develop national consensus in healthcare, especially through the media.
We also perform individual case studies on vaccine development, drug approval, and medical support for disaster-stricken area by the Great East Japan Earthquake on March 11, 2011. In each case, we also study the system of management, information circulation, and network.

Medical support for disaster-stricken area

Masaharu Tsubokura, Shigeaki Kato[1], Shuhei Nomura[2], Tomohiro Morita, Amina Sugimoto[3], Stuart Gilmour[4], Masahiro Kami, Tomoyoshi Oikawa[5], Yukio Kanazawa[5], Ryugo S. Hayano[6], Makoto Miyazaki[7], Hideo Satou[8], Katsumi Sato[8], Shin Masaki[8], Yu Sakuma[8], Tomoyuki Furutani[9], Daisuke Yoneoka[4], Yoshitaka Nishikawa[1], Yuji Fukuda[1], Yasutoshi Saito[1], Tetsuya Tanimoto[10], Kikugoro Sakaihara[5], Tatsuo Hanai[5], Jinichi Mori[11], Arinobu Hori[12], Takeaki Ishii[1], Sae Ochi[1], Kenji Shibuya[4], Yukihide Iwamoto[13], Hidekiyo Tachiya[14], Akihiko Ozaki[5], Yuni Watanabe[6], Michio Murakami[15], Kyoko Ono[16], Tosihiro Oka[17], Taikan Oki[15], Yuki Shimada[5], Toshiyuki Kambe[5], Tsuyoshi Nemoto[5], Masahiko Nihei[8], Hiroaki Shimmura[18], Junichi Akiyama[18], Michio Tokiwa[18], Koichiro Abe[19], Shuji Sakai[19], Takahiro Tetsuda[20], Junpei Kato[20]: [1]Soma Central Hospital, Fukushima, [2]Imperial College London, UK, [3]London School of Hygiene & Medicine, UK, [4]Department of Global Health Policy, Graduate School of Medicine, The University of Tokyo, [5]Minamisoma Municipal General Hospital, Fukushima, [6]Department of Physics, Graduate School of Science, the University of Tokyo, [7]Department of Radiation Health Management, Fukushima Medical University, [8]Hirata Central Hospital, Fukushima, [9]Faculty of Policy Management, Keio University, [10]Navitas Clinic, Tokyo, [11]Human Genome Center, Institute of Medical Science, the University of Tokyo, [12]Hibarigaoka Hospital, Fukushima, [13]Department of Orthopaedic Surgery, Graduate School of Medical Sciences, Kyushu University, [14]City Office of Soma, Fukushima, [15]Institute of Industrial Science, the University of Tokyo, [16]Research Institute of Science for Safety and Sustainability, National Institute of Advanced Industrial Science and Technology, Tsukuba, [17]Faculty of Economics, Fukui Prefectural University, Fukui, [18]Jyoban Hospital, Tokiwa Foundation, Iwaki, [19]Tokyo Women's Medical University, Tokyo, [20]Fukuoka Houeikai Hospital, Fukuoka

After Great East Japan Earthquake and subsequent Fukushima Daiichi nuclear power plant disaster, our team continues to provide the medical care for residents in the disaster-stricken area, especially in Soma, Minamisoma, and Iwaki cities. Col-

laborating with medical staff who work in the areas and support physicians, we conducted researches regarding the levels of internal and external radiation exposure, and gave medical advice to the local people through radiation seminars. We also examined the impacts of the nuclear disaster on public health other than cancer development by radiation exposure. These include deterioration of chronic diseases, decreased strength and psycho-social impacts.[1-14] These results were widely published in newspapers and popular magazines.

Vaccine development in Japan

Masahiro Kami, Tetsuya Tanimoto[10], Eiji Kusumi[10], Claire Leppold[21]: [21]University of Edinburgh, UK

Vaccines are sometimes useful to prevent the disease development; however, they have potential adverse effects, and they are not always effective for every patient despite of extremely high cost. We studied problems about approval, development, and usage of vaccines in Japan.[15]

Clinically Oriented Research

Tsubokura Masaharu, Shigeaki Kato[1], Masahiro Kami, Yukio Kanazawa[5], Haruka Nakada, Yukiko Kishi, Koichiro Yuji, Tomoko Matsumura, Masaki Miyasaka[22], Yuukou Wada[23], Tetsuya Tanimoto[10], Akihiko Ozaki[5], Manabu Tsukada[5], Kazuo Watanabe[24], Hiromichi Ohira[5], Shigehira Saji[25], Hiroshi Kawaguchi[18]: [22]Sendaikousei Hospital, Sendai, [23]Miyagihokubu Cardiovascular Clinic, Miyagi, [24]Division of Diagnostic Pathology, Fukushima Pathology Laboratory, Fukushima, [25]Fukushima Medical University

We studied effective treatment for breast cancer, and neurofibroma.[16,17] And also we studied optimal treatment for heart diseases.[18,19] We examined the reactions of medical journalists against cancer problems.[20] These results were published in scientific journals

Economic Burden of Health Care on Patients

Yuko Kodama, Akihiko Matsui[26], Ryoko Morozumi[27], Masahiro Kami, Yurie Yoshino: [26]Faculty of Economics, the University of Tokyo, [27]Faculty of Economics, University of Toyama

We conducted a research about high medical expenses of long term patients with economists. This research will change the government's plan for the burden of high medical expenses of the patients. The economic burden on patients or the government with prevailing advanced medical care including anticancer drugs is an important issue, we continue further investigation.

New drug approval in Japan

Masahiro Kami, Tetsuya Tanimoto[10], Jinichi Mori[28], Yuji Miura[29]: [28]Human Genome Center, Institute of Medical Science, the University of Tokyo, [29]Division of Hematology, Teikyo University Chiba Medical Center, Chiba

New and emerging drugs are hopes for patients with incurable diseases; however, rigorous assessment of efficacy and adverse effects of these drugs are mandatory. We studied problems about process of drug approval in Japan.[21,22]

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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

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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 the sequence complementarity to the target mRNA or DNA, while the other, like aptamer, those function independent of the 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 mRNA surveillance and aberrant product clearance.

1. Therapeutic RNA Discovery

a. Development of an efficient Cell-SELEX using engineered isogenic cell lines to generate RNA aptamers against a cell surface protein of interest

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. Modified Cell-SELEX that employs whole living cells overexpressing the defined cell surface proteins (for selection) and corresponding 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 cell surface proteins in mock cells often impeded the isolation of aptamers against target proteins. To solve this problem, we manipulated 'negative' cells, whose endogenous expression of target proteins is silenced by shRNA-mediated gene knockdown, and used them for counter-selection at each round of selection to 'positive' (target-overexpressed) cells. 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 silenced HEK293 cells as positive and negative selection targets, respectively. By taking advantage of a hundred-fold difference in the expression level of ITGAV between these two isogenic cell lines, we successfully isolated several anti-ITGAV aptamers, whose binding to the cell-surface ITGAV was confirmed by flow cytometry with the dissociation constant of the nanomolar range. The refined Cell-SELEX targeting the specific proteins on cell surface may be broadly useful for efficient isolation of aptamers with high-affinity to various transmembrane proteins, including pharmacologically and biologically important targets.

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

Kazuki Kato[1], Hisako Ikeda[2], Shin Miyakawa[2], Satoshi Futakawa[2], Yosuke Nonaka[2], Masatoshi Fujiwara[2], Shinichi Okudaira[3], Kuniyuki Kano[3], Junken Aoki[3], Junko Morita[1], Ryuichiro Ishitani[1], Hiroshi Nishimasu[1], Osamu Nureki[1], Yoshikazu Nakamura: [1]Graduate School of Science, The University of Tokyo, [2]Ribomic Inc., [3]Graduate School of Pharmaceutical Sciences, Tohoku University.

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. Normalization of overexpressed α-Synuclein causing Parkinson's disease by a moderate gene silencing with RNA interference

Masaki Takahashi, Mari Suzuki[1], Masashi Fukuoka[1], Nobuhiro Fujikake[1], Shoko Watanabe[1], Miho Murata[1], Keiji Wada[1], Yoshitaka Nagai[1], Hirohiko Hohjoh[1]: [1]National Institute of Neuroscience, NCNP

The α-synuclein (SNCA) gene is a responsible gene for Parkinson's disease (PD); and not only nucleotide variations but also overexpression of SNCA appears to be involved in the pathogenesis of PD. A specific inhibition against mutant SNCA genes carrying nucleotide variations may be feasible by a specific silencing such as an allele-specific RNA interference (RNAi); however, there is no method for restoring the SNCA overexpression to a normal level. Here, we show that an atypical RNAi using small interfering RNAs (siRNAs) that confer a moderate level of gene silencing is capable of controlling overexpressed SNCA genes to return to a normal level; named "expression-control RNAi" (ExCont-RNAi). ExCont-RNAi exhibited little or no significant off-target effects in its treated PD patient's fibroblasts that carry SNCA triplication. To further assess the therapeutic effect of ExCont-RNAi, PD-model flies that carried the human SNCA gene underwent an ExCont-RNAi treatment. The treated PD-flies demonstrated a significant improvement in their motor function. Our current findings suggested that ExCont-RNAi might be capable of becoming a novel therapeutic procedure for PD with the SNCA overexpression, and that siRNAs conferring a moderate level of gene silencing to target genes, which have been abandoned as useless siRNAs so far, might be available for controlling abnormally expressed disease-causing genes without producing adverse effects.

2. mRNA surveillance and aberrant product clearance

a. The fate of naturally truncated nonstop mRNA in *Drosophila* cells

Yoshifumi Hashimoto, Masaki Takahashi, Eri Sakota, Yoshikazu Nakamura.

When translating mRNAs are cleaved in ORF regions, truncated mRNA intermediates are immediately degraded by exoribonuclease. However, how 5'-intermediates of mRNAs are degraded is not immediately obvious since 3' ends of these intermediates are protected from exonuclease by the stalled ribosome. The coding-region cleavage naturally occurs by siRNA digestion or nonsense-mediated mRNA decay (NMD), thus generating nonstop mRNAs. Therefore, we assumed that the mechanism of nonstop mRNA decay (NSD) is involved in the decay of naturally truncated mRNAs by siRNA cleavage or NMD. In yeast, it is known that Dom34 and Hbs1 play crucial roles in NSD by removing stalled ribosomes from nonstop mRNAs. In this study, we examined whether Pelota (a Dom34 homolog) and Hbs1 are involved in the decay of nonstop mRNAs generated by siRNA cleavage and NMD in *Drosophila* cells using siRNA-mediated gene knockdown. The result demonstrated, for the first time, that Pelota (a Dom34 homolog) and Hbs1 are crucial for NSD in *Drosophila* cells for rescuing stalled ribosomes and degrading nonstop mRNAs. Furthermore, it seemed that the degree of involvement of Pelota/Hbs1 in NSD is higher in *Drosophila* than in yeast, and that, unlike in yeast, 5'-3' degradation is less obvious in *Drosophila* cells. It is also demonstrated that Pelota/Hbs1 are involved in the

decay of the 5'-intermediates of siRNA digestion and NMD, including endogenous targets of NMD. These findings suggest that NSD plays pivotal role for clean up of naturally truncated mRNAs.

b. Calpain mediates processing of the translation termination factor eRF3 into the IAP-binding isoform p-eRF3

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The involvement of polypeptide chain-releasing factor eRF3 in translation termination and mRNA decay is well established. Moreover, the finding that the proteolytically processed isoform of eRF3 (p-eRF3) interacts with inhibitors of apoptosis proteins (IAPs) to activate caspase, implies that eRF3 is a cell death regulator. However, the protease(s) responsible for p-eRF3 production and how p-eRF3 regulates apoptosis remain unknown. Here, we show that calpain mediates p-eRF3 production in vitro and in living cells. p-eRF3 is produced in cells treated with ER stressors in a calpain-dependent manner. These findings suggest that p-eRF3 is a novel regulator of calpain-dependent cell death.

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Social Cooperation Research Program

Project Division of Bacterial Infection Biology
細菌感染生物学社会連携研究部門

Project Associate Professor Hiroshi Ashida, Ph.D.
Project Assistant Professor Shiho Suzuki, Ph.D.

特任准助教 医学博士 芦田 浩
特任助教 生命科学博士 鈴木志穂

Research in this division is directed toward understanding the complex interactions that occur between pathogenic bacteria and the gastrointestinal epithelium and the process of infectious diseases. Our special interest is focused upon the molecular pathogenicity of enteropathogenic bacteria, such as Shigella, Helicobacter pylori, enteropathogenic E. coli and enterohemorrhagic E. coli. We are also searching for effective methods to protect or regulate bacterial infection by using knowledge accumulated, and interested in developing animal model for studying the bacterial pathogens.

1. *Shigella* IpaH family effectors as a versatile model for studying pathogenic bacteria.

Ashida H[1] and Sasakawa C[1,2,3]: [1]Division of Bacterial Infection Biology, Institute of Medical Science, University of Tokyo, Japan. [2]Nippon Institute for Biological Science, Tokyo, Japan. [3]Medical Mycology Research Center, Chiba University, Chiba, Japan

Shigella spp. are highly adapted human pathogens that cause bacillary dysentery (shigellosis). Via the type III secretion system (T3SS), *Shigella* deliver a subset of virulence proteins (effectors) that are responsible for pathogenesis, with functions including pyroptosis, invasion of the epithelial cells, intracellular survival, and evasion of host immune responses. Intriguingly, T3SS effector activity and strategies are not unique to *Shigella,* but are shared by many other bacterial pathogens, including *Salmonella*, *Yersinia*, and enteropathogenic *Escherichia coli* (EPEC). Therefore, studying *Shigella* T3SS effectors will not only improve our understanding of bacterial infection systems, but also provide a molecular basis for developing live bacterial vaccines and antibacterial drugs. One of *Shigella* T3SS effectors, IpaH family proteins, which have E3 ubiquitin ligase activity and are widely conserved among other bacterial pathogens, are very relevant because they promote bacterial survival by triggering cell death and modulating the host immune responses.

2. *Shigella* manipulates host immune responses by delivering effector proteins with specific roles.

Ashida H[1], Mimuro H[2], and Sasakawa C[1,3,4]: [1]Division of Bacterial Infection Biology, Institute of Medical Science, University of Tokyo, Japan. [2]Division of Bacteriology, Department of Infectious Diseases Control, International Research Center for Infectious Diseases, The Institute of Medical Science, The University of Tokyo, Tokyo, Japan. [3]Nippon Institute for Biological Science, Tokyo, Japan. [4]Medical Mycology Research Center, Chiba University, Chiba, Japan

The intestinal epithelium deploys multiple defense systems against microbial infection to sense bacterial components and danger alarms, as well as to induce intracellular signal transduction cascades that trigger both the innate and adaptive immune system, which are pivotal for bacterial elimination. However, many enteric bacterial pathogens, includ-

ing *Shigella*, deliver a subset of virulence proteins (effectors) via the type III secretion system (T3SS) that enable bacterial evasion from host immune systems; consequently, these pathogens are able to efficiently colonize the intestinal epithelium. We have discovered examples of interactions between *Shigella* and host immune responses, with particular emphasis on strategies that bacteria use to manipulate inflammatory outputs of host cell responses such as cell death, membrane trafficking, and innate and adaptive immune responses.

Publications

Ashida H & Sasakawa C. *Shigella* IpaH family effectors as a versatile model for studying pathogenic bacteria. *Front Cell. Infect. Microbiol*. In press.

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

特任准教授 理学博士 佐 藤 毅 史
特任助教 理学博士 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 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, Naoki Takemura[1], Yasuo Ou-

chi[1], Shuji Suzuki[2], Masanori Kakuta[2], 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 researcher. 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.

Publications

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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)

Professor	Arinobu Tojo, M.D., D.M.Sc.		教授(兼任)	医学博士	東 條 有 伸
Project Associate Professor	Yasuhiro Ebihara, MD., D.M.Sc.		特任准教授	医学博士	海老原 康 博
Associate Professor	Otsu Makoto, MD., D.M.Sc.		准教授(兼任)	医学博士	大 津 真
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

Ueno Y, Ota S, Nonaka H, Ishii T, Yamada T, Nagamura T, Otsu M, Tojo A

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 exhibit distinct characteristics depending on their origin. 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 from different tissues. Outcomes from our research lead to identify biomarkers for MSCs, which is one of the keys to deliver successful MSC-based cell therapy.

2. Generation of disease-specific human iPS cells

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

Here, using intractable disease-specific human iPS, we are intended to elucidate the pathogenetic mechanism and develop a new agent for the treatment of disease. Myelodysplastic symdrome (MDS) is a group of disorders that healthy blood cells are poorly formed in bone marrow (BM). However, the disease developing mechanism is not clear. First, we used a MDS patient sample, which has a point mutation in *splicing factor 3B, subunit1 (SF3B1) gene*, to generate MDS-specific iPS. 12 iPS clones were generated from $CD34^+$ cells that were isolated from the patient BMCs. So far, unfortunately, 8 of 12 clones had no variation in *SF3B1*. The second generation sequencing analysis of $CD34^+$ cells revealed

a mutant allele burden (MAB) of 37.6%. *SF3B1* mutation is the herterozygous one, therefore, MAB of 50% implies that 100% of the cells carry the mutation. These results suggest that it is more difficult to reprogram the *SF3B1* variant $CD34^+$ cells than normal that. We will analyze the *SF3B1* mutation of other 4 clones and then generate new iPSC clones.

Publications

Konuma T, Kato S, Ooi J, Ebihara Y, Mochizuki S, Ishii H, Takei T, Oiwa-Monna M, Tojo A, Takahashi S. Second allogeneic transplantation using unrelated cord blood for relapsed hematological malignancies after allogeneic transplantation. *Leuk Lymphoma. 2016 Jan; 57(1): 103-9.*

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

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

A. Gene therapy and immune cell therapy against 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.

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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. Coxsackievirus A11 is a novel oncolytic virus that induces immunogenic cell death in human non-small cell lung cancer

Miyako Sagara[1], Shohei Miyamoto, Hiroyuki Inoue[1], Masaki Kuroda[1], Yuto Takishima[1], Kyosuke Kobayashi[2], Kazunari Yamada[1], Hiroyuki Shimizu[3], Masatoshi Tagawa[4], Yoichi Nakanishi[5], Hisanobu Ogata[1], Beibei Wang[1], Kenzaburo Tani[1,2,6].: [1]Division of Molecular and Clinical Genetics, Molecular and Clinical Genetics, Medical Institute of Bioregulation, Kyushu University, Fukuoka, Japan [2]Division of Translational Cancer Research Medical Institute of Bioregulation, Kyushu University, Fukuoka, Japan [3]Department of Virology II, National Institute of Infectious Diseases, Tokyo, Japan [4]Division of Pathology and Cell Therapy, Chiba Cancer Center Research Institute, Chiba, Japan [5]Research Institute of Diseases of the Chest, Kyushu University, Fukuoka, Japan [6]Project Division of ALA Advanced Medical Research, IMSUT

Non-small cell lung cancer (NSCLC) is the leading cause of cancer-related mortality worldwide. Oncolytic virotherapy using enteroviruses recently emerges as a promising anticancer strategy. Herein, we found that coxsackievirus A11 (CVA11) exhibited extensive oncolytic activity in all of 7 human NSCLC cell lines in a MOI-dependent manner, whereas no cytotoxicity in human normal keratinocyte. Flow cytometry analyses showed that CVA11-treated NSCLC cell lines generated a marked subpopulation of apoptotic (annexinV + /7-AAD −) cells and induced subsequent sub-G1 portion. An assay using a pan-caspase inhibitor showed that apoptosis significantly contribute to the CVA11-driven oncolysis in NSCLC cell lines. Furthermore we investigated whether CVA11 infection could induce immunogenic cell death by releasing DAMPs such as calreticulin exposure and HMGB1 translocation. Of note, CVA11 infection induced abundant surface exposure of CRT in a time dependent manner and robust release of HMGB1 from the nuclei of NSCLC cells into the cytosol. Collectively, we demonstrate that CVA11 displays remarkable oncolytic activity against human NSCLC with immunostimulatory properties.

c. 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.

B. Regenerative medicine and related technique development

Regenerative medicine is expected to be an 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. Development of novel measles virus vector for induction of pluripotent stem cells

Takafumi Hiramoto[1], Maino Tahara[2], Chika Sakamoto[1], Yuichiro Nakatsu[2], Toru Kubota[2], Hiroaki Ono[3], Hiroshi Kohara, Makoto Takeda[2], and Kenzaburo Tani[1,4].: [1]Division of Molecular and Clinical Genetics, Molecular and Clinical Genetics, Medical Institute of Bioregulation, Kyushu University, Fukuoka, Japan [2]Department of Viology 3, National Institute of Infectious Diseases. [3]Department of Pediatrics Graduate School of Medical Sciences, Kyushu University. [4]Project Division of ALA Advanced Medical Research, IMSUT

We reports newly developed virus vector, measles virus vector can transfer multiple genes into human hematopoietic cells effectively and induce ground state pluripotent stem cells from somatic cells without affecting the host genome. Measles virus (MV) which belongs to negative single strand RNA viruses has been known to have high affinity for human peripheral immune cells including monocytes, B cells and T cells. We recently have developed novel MV gene transfer vector which is non-transmissible, can transfer multiple genes simultaneously. The MV vector which carries5 genes (GFP, human OCT3/4, SOX2, KLF4, and L-MYC) (MV-dF-OSKL-EGFP) could express these genes in various human cells with differential expression levels depending on the arrangement of the gene in the vector. Especially, MV-dF-OSKL-EGFP was able to transduce genes into more than 80% of hematopoietic cells besides natural killer cells. Naive and stem cell memory T cells were also transduced by MV-dF-OSKL-EGFP. These results indicated that the newly developed MV vector had the significant character as the new gene transfer vector compared with conventional viral gene transfer vectors including Sendai virus vector, which be longs to the same RNA virus vector. We could successfully generated induced pluripotent stem cells (iPS cells) from human fibroblasts or peripheral blood T cells using MV-dF-OSKL-EGFP. These iPS cells expressed the pluripotent markers of NANOG and Tra-1-60and were demethylated. These iPS cells also differentiated into three germ line tissues in vitro and in vivo. Importantly, we also could establish the ground state like pluripotent cell (GSL-iPS cells) from hematopoietic cells by using MV-dF-OSKL-EGFP. In the presence of human leukemia inhibitory factor (LIF), GSK-3inhibitor (CHIR99021), and MEK inhibitor (PD0325901), GSL-iPS cells were able to be cultured from a dissociated single cell with rapid cell growth. GSL-iPS cells also expressed pluripotent markers of NANOG and Tra-1-60, and were able to differentiate into three germ line cells. In conclusion, our newly developed MV vector may induce revolutional advance in the field of gene and cell therapy using iPS cells.

b. Characterization of common marmoset dysgerminoma-like tumor induced by the lentiviral expression of reprogramming factors.

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Recent generation of induced pluripotent stem (iPSCs) has made a significant impact on the field of human regenerative medicine. Prior to the clinical application of iPSCs, testing of their safety and usefulness must be carried out using reliable animal models of various diseases. In order to generate iPSCs from common marmoset (CM; Callithrix jacchus), one of the most useful experimental animals, we have lentivirally transduced reprogramming factors, including POU5F1 (also known as OCT3/4), SOX2, KLF4, and c-MYC into CM fibroblasts. The cells formed round colonies expressing embryonic stem cell markers, however, they showed an abnormal karyotype denoted as 46, X, del(4q), +mar, and formed human dysgerminoma-like tumors in SCID mice, indicating that the transduction of reprogramming factors caused unexpected tumorigenesis of CM cells. Moreover, CM dysgerminoma-like tumors were highly sensitive to DNA-damaging agents, irradiation, and fibroblast growth factor receptor inhibitor, and their growth was dependent on c-MYC expression. These results indicate that DNA-damaging agents, irradiation, fibroblast growth factor receptor inhibitor, and c-MYC-targeted therapies might represent effective treatment strategies for unexpected tumors in patients receiving iPSC-based therapy.

c. Single-cell-state culture of human pluripotent stem cells increases transfection efficiency

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Human pluripotent stem cells (hPSCs), such as human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs), have the potential to self-renew indefinitely and differentiate into various cell types. hPSCs can differentiate into various stem or progenitor cell populations used for regenerative medicine and drug development. Newly developed genome editing technology has advanced the use of hPSCs for such purposes. However, to fully utilize hPSCs to achieve this goal, more efficient gene transfer methods under defined conditions are required. Development of efficient genome editing methods, such as zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated nuclease 9 (Cas9), for use in hPSCs holds great promise in the fields of basic and clinical research. Among these methods, TALENs are more efficient and safer for use in hPSCs to achieve specific gene editing, as ZFNs had a low gene editing efficiency and CRISPR/Cas9 was accompanied by more severe off-target effects than TALENs. Electroporation is a widely used transfection method for hPSC genome editing; however, this method results in reduced cell viability and gene editing efficiency.

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 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 non-liposomal transfection reagents with plasmid DNA driven by the human elongation factor 1-alpha 1 (EF1α) promoter or mRNA encoding enhanced green fluorescent protein (eGFP). The proportion of eGFP+ cells considerably increased in single-cell-state cultures (DNA: 95.80±2.51%, mRNA: 99.70±0.10%). Moreover, most of the cells were viable (control: 93.10±0.40%, DNA: 83.40±2.03%, mRNA: 86.71±0.19%). The mean fluorescence intensity (MFI) was approximately three-fold higher than that in cells transfected by electroporation (electroporation (EPN): 6631±992; transfection (TFN): 17933±1595). eGFP expression was detected by fluorescence microscopy until day seven post-transfection. Our results also demonstrate an inverse correlation between cell density and transfection efficiency. To test whether transfection using this method affected the "stemness" of hPSCs, we examined SSEA4 and NANOG expression in eGFP-transfected cells by flow cytometry analysis. The percentage of both SSEA4+ and NANOG+ cells was greater than 90%. Moreover, transplantation of eGFP-transfected cells into immunodeficient mice led to the formation of teratomas. These results strongly suggested that single-cell-state hPSC culture improved transfection efficiency without inducing differentiation or loss of pluripotency. Moreover, we used our efficient transfection method to edit the hPSC genome using TALENs. We constructed a Platinum TALEN driven by the EF1α promoter targeting the adenomatous polyposis coli (APC) gene and analyzed the efficiency of gene editing using the Cel-1 assay. Our efficient transfection method induced mutations more efficiently than electroporation (Transfection: 11.1±1.38%, Electroporation: 3.2±0.89). These results showed that TALENs increased gene editing efficiency in single-cell-state hPSC cultures. Overall, our efficient hPSC transfection method using single-cell-state culture provides an excellent experimental system to investigate the full potential of hPSCs. We expect that this method may contribute to the fields of hPSC-based regenerative medicine and drug discovery.

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