Center for Experimental Medicine and Systems Biology

Laboratory of Developmental Genetics
発生工学研究分野

Professor	Nobuaki Yoshida, M.D., D.M.Sc.	教　授	医学博士	吉　田　進　昭
Senior Assistant Professor	Hirotake Ichise, D.V.M., Ph.D.	講　師	獣医学博士	市　瀬　広　武
Assistant Professor	Taeko Ichise, Ph.D.	助　教	医学博士	市　瀬　多恵子
Assistant Professor	Manabu Ozawa, Ph.D.	助　教	農学博士	小　沢　　学

Gene targeting technologies not only for culturing cells but also for animals themselves are very powerful tools for understanding gene functions in vivo. In addition to the conventional gene targeting, tissue specific gene manipulation using Cre-loxP system, named conditional knockout, allows us to explore spatiotemporal gene function in many different tissue at many different developmental stages. We are also using CRISPR/Cas9 systems, which are very effective and rapid method for genome editing. Using these technologies, we are trying to investigate fundamentals which characterize 'stemness'in embryonic pluripotent stem cells or somatic multipotent stem cells. In the somatic stem cells, we are especially interested in germ cells and neural stem cells. Our research aims are to reveal molecular mechanisms which orchestrate spermatogenesis, especially focusing on roles of RNA processing proteins, e.g., PTBP1 or PTBP2, or histone modifiers, e. g., FBXL10, FBXL11 or RYBP, by using gene knockout technology, and to develop novel therapeutic strategies for infertile patients. We are also studying roles of Ras signaling in developmental, physiological, and pathological lymphangiogenesis, and roles of PLCγ2 signaling in initiating and maintaining the separation of the blood and lymphatic vasculature.

1. The histone demethylase fbxl11/kdm2a plays an essential role in embryonic development by repressing cell-cycle regulators

Eri Kawakami, Akinori Tokunaga[1], Manabu Ozawa, Reiko Sakamoto, Nobuaki Yoshida.: [1]The Tokunaga Laboratory, Faculty of Medicine, Oita University.

Methylation and de-methylation of histone lysine residues play pivotal roles in mammalian early development; these modifications influence chromatin architecture and regulate gene transcription. Fbxl11 (F-box and leucine-rich repeat 11)/Kdm2a is a histone demethylase that selectively removes mono- and di-methylation from histone H3K36. Previously, two other histone H3K36 demethylases (Jmjd5 or Fbxl10) were analyzed based on the phenotypes of the corresponding knockout (KO) mice; the results of those studies implicated H3K36 demethylases in cell proliferation, apoptosis, and senescence (Fukuda T et al., 2011; Ishimura A et al., 2012). To elucidate the physiological role of Fbxl11, we generated and examined Fbxl11 KO mice. Fbxl11 was expressed throughout the body during embryogenesis, and the Fbxl11 KO mice exhibited embryonic lethality at E10.5-12.5, accompanied with severe growth defects leading to reduced body size. Furthermore, knockout of Fbxl11 decreased cell proliferation and increased apoptosis. The lack of Fbxl11 resulted in downregulation of the Polycomb group protein (PcG) Ezh2, PcG mediated H2A

ubiquitination and upregulation of the cyclin-dependent kinase inhibitor $p21^{Cip1}$. Taken together, our findings suggest that Fbxl11 plays an essential role in embryonic development and homeostasis by regulating cell proliferation and survival.

2. Calpain-Mediated Degradation of Drebrin by Excitotoxicity In vitro and In vivo.

Takahiko Chimura, Thomas Launey[2], Nobuaki Yoshida: [2]RIKEN Brain Science Institute, Launey Research Unit.

The level of drebrin, an evolutionarily conserved f-actin-binding protein that regulates synaptic structure and function, is reduced in the brains of patients with chronic neurodegenerative diseases such as Alzheimer's disease (AD) and Down's syndrome (DS). It was suggested that excitotoxic neuronal death caused by overactivation of NMDA-type glutamate receptors (NMDARs) occurs in AD and DS; however, the relationship between excitotoxicity and drebrin loss is unknown. Here, we show that drebrin is a novel target of calpain-mediated proteolysis under excitotoxic conditions induced by the overactivation of NMDARs. In cultured rodent neurons, degradation of drebrin was confirmed by the detection of proteolytic fragments, as well as a reduction in the amount of full-length drebrin. Notably, the NMDA-induced degradation of drebrin in mature neurons occurred concomitantly with a loss of f-actin. Furthermore, pharmacological inhibition of f-actin loss facilitated the drebrin degradation, suggesting a functional linkage between f-actin and drebrin degradation. Biochemical analyses using purified drebrin and calpain revealed that calpain degraded drebrin directly *in vitro*. Furthermore, cerebral ischemia also induced the degradation of drebrin *in vivo*. These findings suggest that calpain-mediated degradation of drebrin is a fundamental pathology of neurodegenerative diseases mediated by excitotoxicity, regardless of whether they are acute or chronic. Drebrin regulates the synaptic clustering of NMDARs; therefore, degradation of drebrin under excitotoxic conditions may modulate NMDAR-mediated signal transductions, including pro-survival signaling. Overall, the results presented here provide novel insights into the molecular basis of cellular responses to excitotoxicity *in vitro* and *in vivo*.

3. Foxc2 in pharyngeal arch mesenchyme is important for aortic arch artery remodelling and ventricular septum formation

Mohammad Khaja Mafij Uddin[3], Wataru Kimura[4], Tomoyuki Ishikura[5], Haruhiko Koseki[5], Nobuaki Yoshida, Mohammod Johirul Islam[3], Mohammed Badrul Amin[3], Kasumi Nakamura[3], Yi-Xin Wu[3], Eiji Sato[3], Kazushi Aoto[3], Naoyuki Miura[3]: [3]Department of Biochemistry, Hamamatsu University School of Medicine, [4]Division of Cardiology, Department of Internal Medicine, UT Southwestern Medical Center, [5]Laboratory for Developmental Genetics, RIKEN Center for Integrative Medical Sciences (IMS-RCAI)

The forkhead box C2 (Foxc2) protein is a member of the forkhead/winged helix transcription factor family and plays an essential role in cardiovascular development. Previous studies showed that *Foxc2* null mouse embryos die during midgestation or just after birth with severe cardiovascular defects, including interruption, coarctation of the aortic arch and ventricular septal defects. These are also seen in human congenital heart disease. However, the tissue specific role of Foxc2 in aortic arch remodelling is not yet fully understood. Here we show that Foxc2 is expressed in a restricted pattern in several cell populations, including the mesenchyme and endothelium of pharyngeal arch arteries, which are important for cardiovascular development. In this study, we use a conditional knockout approach to examine the tissue specific role of Foxc2 in aortic arch remodelling. We demonstrate that mouse embryos lacking Foxc2 in *Nkx2.5*-expressing mesenchyme and endothelium of pharyngeal arch arteries display aortic arch interruption type B and ventricular septal defects. In contrast, conditional deletion of *Foxc2* in *Tie2*-expressing endothelial cells does not result in aortic arch or ventricular septal defects, but does result in embryonic lethality due to peripheral oedema. Our data therefore provide for a detailed understanding of the role of mesenchymal Foxc2 in aortic arch remodelling and in the development of ventricular septum.

4. Loss of *Tifab*, a del(5q) MDS gene, alters hematopoiesis through derepression of Toll-like receptor-TRAF6 signaling

Melinda E. Varney[6], Madeline Niederkorn[7] Hiroyasu Konno[8], Takayuki Matsumura[8], Jin Gohda[8], Nobuaki Yoshida, Taishin Akiyama[8], Susanne Christie[6], Jing Fang[6], David Miller[6], Andres Jerez[9], Aly Karsan[10], Jaroslaw P. Maciejewski[9], Ruhikanta A. Meetei[6], Jun-ichiro Inoue[8], Daniel T. Starczynowski[11]: [6]Division of Experimental Hematology and Cancer Biology, Cincinnati Children's Hospital Medical Center. [7]Division of Experimental Hematology and Cancer Biology, Cincinnati Children's Hospital Medical Center, Department of Biological Sciences, University of Cincinnati. [8]Division of Cellular and Molecular Biology, Department of Cancer Biology, The Institute of Medical Science, the University of Tokyo. [9]Department of Translational Hematology and Oncology Research, Taussig Cancer Institute, Cleveland Clinic. [10]Michael Smith

Genome Sciences Centre and Department of Pathology and Laboratory Medicine, BC Cancer Research Centre, Canada Michael Smith Genome Sciences Centre and Department of Pathology and Laboratory Medicine, BC Cancer Research Centre. [11]Division of Experimental Hematology and Cancer Biology, Cincinnati Children's Hospital Medical Center, Department of Cancer Biology, University of Cincinnati College of Medicine.

TRAF-interacting protein with forkhead-associated domain B (*TIFAB*) is a haploinsufficient gene in del(5q) myelodysplastic syndrome (MDS). Deletion of *Tifab* results in progressive bone marrow (BM) and blood defects, including skewed hematopoietic stem/progenitor cell (HSPC) proportions and altered myeloid differentiation. A subset of mice transplanted with *Tifab* knockout (KO) HSPCs develop a BM failure with neutrophil dysplasia and cytopenia. In competitive transplants, *Tifab* KO HSPCs are out-competed by wild-type (WT) cells, suggesting a cell-intrinsic defect. Gene expression analysis of *Tifab* KO HSPCs identified dysregulation of immune-related signatures, and hypersensitivity to TLR4 stimulation. TIFAB forms a complex with TRAF6, a mediator of immune signaling, and reduces TRAF6 protein stability by a lysosome-dependent mechanism. In contrast, *TIFAB* loss increases TRAF6 protein and the dynamic range of TLR4 signaling, contributing to ineffective hematopoiesis. Moreover, combined deletion of TIFAB and miR-146a, two genes associated with del(5q) MDS/AML, results in a cooperative increase in TRAF6 expression and hematopoietic dysfunction. Re-expression of TIFAB in del(5q) MDS/AML cells results in attenuated TLR4 signaling and reduced viability. These findings underscore the importance of efficient regulation of innate immune/TRAF6 signaling within HSPCs by TIFAB, and its cooperation with miR-146a as it relates to the pathogenesis of hematopoietic malignancies, such as del(5q) MDS/AML.

5. Hypergravity Provokes a Temporary Reduction in CD4+CD8+ Thymocyte Number and a Persistent Decrease in Medullary Thymic Epithelial Cell Frequency in Mice

Ryosuke Tateishi[8,12], Nobuko Akiyama[8,12], Maki Miyauchi[8,12], Riko Yoshinaga[8,12], Hiroki Sasanuma[12], Takashi Kudo[12,13,14], Miki Shimbo[12,13,14], Masahiro Shinohara[12,15,16], Koji Obata[12,17], Jun-ichiro Inoue[8], Masaki Shirakawa[12,18], Dai Shiba[12,18], Hiroshi Asahara[12,15], Nobuaki Yoshida, Satoru Takahashi[12,13,14], Hironobu Morita[12,17], Taishin Akiyama[8,12]: [12]Mouse epigenetics project, ISS/Kibo experiment, Japan Aerospace Exploration Agency, JAXA. [13]Laboratory Animal Resource Center, University of Tsukuba. [14]Department of Anatomy and Embryology, Faculty of Medicine, University of Tsukuba. [15]Department of Systems BioMedicine, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University. [16]JST, PRESTO. [17]Department of Physiology, Gifu University Graduate School of Medicine. [18]JEM Utilization Center, Human Spaceflight Technology Directorate, JAXA.

Gravity change affects many immunological systems. We investigated the effects of hypergravity (2G) on murine thymic cells. Exposure of mice to 2G for three days reduced the frequency of $CD4^+$ $CD8^+$ thymocytes (DP) and mature medullary thymic epithelial cells (mTECs), accompanied by an increment of keratin-5 and keratin-8 double-positive ($K5^+K8^+$) TECs that reportedly contain TEC progenitors. Whereas the reduction of DP was recovered by a 14-day exposure to 2G, the reduction of mature mTECs and the increment of $K5^+K8^+$ TEC persisted. Interestingly, a surgical lesion of the inner ear's vestibular apparatus inhibited these hypergravity effects. Quantitative PCR analysis revealed that the gene expression of Aire and RANK that are critical for mTEC function and development were up-regulated by the 3-day exposure and subsequently down-regulated by the 14-day exposure to 2G. Unexpectedly, this dynamic change in mTEC gene expression was independent of the vestibular apparatus. Overall, data suggest that 2G causes a temporary reduction of DP and a persistent reduction of mature mTECs in a vestibular system-dependent manner, and also dysregulates mTEC gene expression without involving the vestibular system. These data might provide insight on the impact of gravity change on thymic functions during spaceflight and living.

6. Generation of a FLP recombination-activated, tamoxifen-inducible Cre-driver mouse strain for spatio-temporal genetic modification

Hirotake Ichise, Akiko Hori, Seiji Shiozawa, Saki Kondo[19], Yumi Kanegae[19], Izumu Saito[19], Taeko Ichise, Nobuaki Yoshida: [19]Laboratory of Molecular Genetics, the Institute of Medical Science, the University of Tokyo.

Temporal and spatial genetic modification of mice using a site-specific recombination (SSR) system, such as the Cre/loxP-based conditional knockout technique, is well-established. Recently, *in vivo* SSR-based, multi-step, serial genetic modification in a particular cell lineage during cell differentiation, tissue morphogenesis, and tumorigenesis has been a useful technique in mouse genetics. However, the application of such a technique has been limited, owing to a small number of site-specific recombinase drivers and target mice other than Cre drivers and Cre-target mice. In this study, we generated

a FLP recombination-activated, tamoxifen-inducible Cre-driver mouse strain. The new line, named CM32, expresses the GFPneo-fusion gene in a wide variety of tissues before FLP recombination and expresses CreMer after FLP recombination. We observed tamoxifen-induced Cre recombination in various tissues of the CM32 mice that had FLP-recombined in the germline and in three independent Cre reporter mouse lines, although we also found tamoxifen-independent, leaky Cre activity in a locus-dependent manner. The CM32 transgenic mouse line will be a useful tool for two-step SSR-based genetic modification that consists of FLP recombination, followed by tamoxifen-dependent or spontaneous Cre recombination.

7. Histone demethylases Fbxl11 regulates balance between self-renew and differentiation of spermatogonia in mice

Ozawa M, Kawakami E, Tokunaga A[1], Sakamoto R, Yoshida N

Methylation and demethylation of histone residue are important modifications of epigenetics. Histone modifications fluctuate drastically during germ cell development or spermatogenesis. A number of gene knockout mice lacking gene(s) which catalyze these modifications show an infertile phenotype, suggesting that proper epigenetic modifications of histone residue are essential for germ cell development or spermatogenesis. Fbxl11 is a gene catalyzing demethylation of H3K4 or H3K36. Although these histone modifications has been reported to be important for normal germ cell development or sustainable sperm production, functional roles of Fbxl11 in male germ cell development or spermatogenesis are poorly understood until present. To determine the role of Fbxl11 in spermatogenesis, we have developed germ cell specific Fbxl11 knockout mouse (Fbxl11cKO) model by mating *Nanos3*-Cre mice in which Cre recombinase is expressed only in primordial germ cells from around embryonic day 7 (E7) with Fbxl11 floxed mice in order to escape embryonic lethal phenotype of conventional Fbxl11KO mice. Germ cell specific Fbxl11 mouse are totally infertile, and showed drastic abnormality in spermatogenesis, e.g., few sperm could be recovered from epididymis, or the average weight of testis is significantly reduced in the Fbxl11cKOs at 8 weeks old (33.9 ± 4.9mg in the Fbxl11cKO vs 76.5 ± 7.1mg in the control). Immunohistochemistry using anti-SCP3 antibody, a first meiotic spermatocyte marker, anti-cleaved Caspase3, an apotitic marker, and anti-PLZF antibody, an undifferentiated spermatogonia marker, revealed that about 80% of seminiferous tubules have abnormal spermatocyte layers (34 ± 3.2 % tubules are without spermatocytes and 45.7 ± 5.8 % tubules are with only a few spermatocytes), and apoptosis occurred more frequently in the Fbxl11 cKO testis than the control. Interestingly, of almost all the abnormal seminiferous tubules contained PLZF-positive undifferentiated spermatogonia in spite of the presence or absence of meiotic cells in the tubules, and the number of PLZF positive cells per tubule is rather greater in 3 weeks old Fbxl11cKO testis than the same-age control (average 13.8 ± 0.6 cells per tubule in the Fbxl11cKO vs 9.2 ± 1.4 in the control). Furthermore, flow-cytometrical analysis indicated that the number of EpCAM positive spermatogonia in Fbxl11cKO is comparable with the control at postnatal day 5 (P5), whereas development of c-KIT positive differentiating spermatogonia are strongly inhibited in the Fbxl11cKO at P12, suggesting that Fbxl11 might regulate phase-decision from self-renew to early differentiation in spermatogonia. In addition to the *in vivo* analysis, we have developed Fbxl11 KO germline stem cell (GSC) *in vitro* and compared transcriptome using Affymetrix GeneChip Mouse Transcriptome Array. By cutoff criteria of $P < 0.05$ and fold differences greater than or less than 2 or 0.5, respectively, 475 genes are downregulated whereas 170 genes are upregulated in the Fbxl11 KO GSC compared with the control. Gene ontology (GO) analysis revealed that GO terms related with germ cell development or spermatogenesis such as 'Male Gamete Generation', Spermatogenesis', or 'Meiosis' are significantly accumulated in gene group downregulated in the Fbxl11 KO GSC. Further and detail analysis to discover gene(s) which directly regulate balance between spermatogonial self-renew and differentiation under control of Fbxl11 are undergoing.

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Center for Experimental Medicine and Systems Biology

Laboratory of Systems Biology
システムズバイオロジー研究分野

Associate Professor　Susumu Nakae, Ph.D.

准教授　農学博士　　中　江　　進

Gene-modified mice are considered to be powerful tools for understanding of pathophysiological function of the targeted gene(s) in vivo. Our research focus is the understanding of pathogenesis of rejection and immune disorders such as allergy and autoimmunity using gene-modified mice.

An interleukin-33-mast cell-interleukin-2 axis suppresses papain-induced allergic inflammation by promoting regulatory T cell numbers.

Morita, H.[1,2,3], Arae, K.[1,4], Unno, H.[1,5], Miyauchi, K.[6], Toyama, S.[1,7], Nambu, A.[8,12], Oboki, K.[1], Ohno, T.[1,9], Motomura, K.[1], Matsuda, A.[10], Yamaguchi, S.[8], Narushima, S.[8], Kajiwara, N.[1], Iikura, M.[11], Suto, H.[12], McKenzie, A.N.[13], Takahashi, T.[2], Karasuyama, H.[7,14], Okumura, K.[12], Azuma, M.[9], Moro, K.[15,16], Akdis, C.A.[3], Galli, S.J.[17], Koyasu, S.[15], Kubo, M.[6,18], Sudo, K.[19], Saito, H.[1], Matsumoto, K.[1], Nakae, S.[1,8,16]: [1]Department of Allergy and Immunology, National Research Institute for Child Health and Development, [2]Department of Pediatrics, Keio University School of Medicine, [3]Swiss Institute of Allergy and Asthma Research, University of Zurich, Davos, Switzerland, [4]Department of Immunology, Faculty of Health Science, Kyorin University, [5]Department of Pediatrics, The Jikei University School of Medicine, [6]Laboratory for Signal Network, 15 Laboratory for Immune Cell System, Center for Integrative Medical Sciences, RIKEN Yokohama Institute, [7]Department of Immune Regulation, [9]Department of Molecular Immunology, Graduate School of Medical and Dental Science, Tokyo Medical and Dental University, [8]Laboratory of Systems Biology, Center for Experimental Medicine and Systems Biology, The Institute of Medical Science, The University of Tokyo, Tokyo, [10]Department of Ophthalmology, 12 Atopy Research Center, Juntendo University School of Medicine, [11]Department of Respiratory Medicine, National Center for Global Health and Medicine, [13]MRC Laboratory of Molecular Biology, Cambridge, UK, [14]Japan Science and Technology Agency, Core Research for Evolutional Science and Technology (CREST), and 16 Precursory Research for Embryonic Science and Technology (PRESTO), Japan Science and Technology Agency, [17]Departments of Pathology and of Microbiology and Immunology, Stanford University School of Medicine, Stanford, CA, USA, [18]Division of Molecular Pathology, Research Institute for Biological Sciences, Tokyo University of Sciences, [19]Animal Research Center, Tokyo Medical University.

House dust mite-derived proteases contribute to allergic disorders in part by disrupting epithelial barrier function. Interleukin-33 (IL-33), produced by lung cells after exposure to protease allergens, can induce innate-type airway eosinophilia by activating natural helper (NH) cells, a member of group 2 innate lymphoid cells (ILC2), to secrete Th2 type-cytokines. Because IL-33 also can induce mast cells (MCs) to secrete Th2 type-cytokines, MCs are thought to cooperate with NH cells in enhancing

protease or IL-33-mediated innate-type airway eosinophilia. However, we found that MC-deficient $Kit^{W\text{-}sh/W\text{-}sh}$ mice exhibited exacerbated protease-induced lung inflammation associated with reduced numbers of regulatory T (Treg) cells. Moreover, IL-2 produced by IL-33-stimulated MCs promoted expansion of numbers of Treg cells, thereby suppressing development of papain- or IL-33-induced airway eosinophilia. We have thus identified a unique anti-inflammatory pathway that can limit induction of innate-type allergic airway inflammation mediated by NH cells.

IL-25 and IL-33 Contribute to Development of Eosinophilic Airway Inflammation in Epicutaneously Antigen-Sensitized Mice.

Morita, H.[1,2], Arae, K.[2,4], Unno, H.[2,3], Toyama, S.[2], Motomura, K.[2], Matsuda, A.[2], Suto, H.[5], Okumura, K.[5], Sudo, K.[6], Takahashi, T.[1], Saito, H.[2], Matsumoto, K.[2], Nakae, S.[2,5,7,8]: [1]Department of Pediatrics, Keio University School of Medicine, [2]Department of Allergy and Clinical Immunology, National Research Institute for Child Health and Development, [3]Department of Pediatrics, Jikei University School of Medicine, [4]Department of Immunology, Faculty of Health Science, Kyorin University, Tokyo, [5]Atopy Research Center, Juntendo University School of Medicine, [6]Animal Research Center, Tokyo Medical University, [7]Laboratory of Systems Biology, Center for Experimental Medicine and Systems Biology, The Institute of Medical Science, The University of Tokyo, [8]Precursory Research for Embryonic Science and Technology (PRESTO), Japan Science and Technology Agency.

IL-25, IL-33 and TSLP are produced predominantly by epithelial cells and are known to induce Th2-type cytokines. Th2-type cytokines are involved not only in host defense against nematodes, but also in the development of Th2-type allergic diseases. TSLP was reported to be crucial for development of allergic airway inflammation in mice after inhalation of allergens to which they had been sensitized epicutaneously (EC) beforehand. However, the roles of IL-25 and IL-33 in the setting remain unclear. Normal OVA-specific Th2- and Th17-cell responses of lymph nodes and spleens were observed in IL-25-deficient ($IL\text{-}25^{-/-}$) and $IL\text{-}33^{-/-}$ mice after EC sensitization with OVA. Nevertheless, the number of eosinophils, but not neutrophils, in the BALFs, and the levels of Th2 cytokines, but not Th17 cytokines, in the lungs were significantly decreased in the $IL\text{-}25^{-/-}$ and $IL\text{-}33^{-/-}$ mice pre-sensitized EC with OVA, followed by inhalation of OVA, whereas their levels of AHR and OVA-specific serum IgE were normal. Both IL-25 and IL-33 are critical for induction of Th2-type cytokine-mediated allergic airway eosinophilia, but not Th17-type cytokine-mediated airway neutrophilia, at the local sites of lungs in the challenge phase of mice sensitized EC with OVA. They do not affect OVA-specific T-cell induction in the sensitization phase.

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Center for Experimental Medicine and Systems Biology

Laboratory of Innate Immunity
自然免疫研究分野

Professor Kensuke Miyake, M.D., Ph.D.

教 授 医学博士 三 宅 健 介

Pathogen sensors, such as Toll-like receptor (TLR), play sentinel roles in detecting pathogenic ligands during infection and induce both innate and acquired immune responses. Meanwhile, excessive TLR responses are strongly associated with fatal diseases such as septic shock and autoimmune diseases. For this reason, immune system must strictly control TLR responses to avoid disruption of homeostasis. However, molecular mechanisms involved in TLR regulation are not fully elucidated. We have previously shown that TLRs are regulated by various TLR associating molecules including MD-2, PRAT4A and Unc93B1. Our goal is to uncover molecular mechanism that is indispensable for appropriate TLR responses using genetically engineered mice.

1. Targeting the nucleic acids-sensing TLRs for therapeutic intervention in autoimmune diseases

Yuji Motoi[1], Yusuke Murakami, Takuma Shibata[1], Kensuke Miyake[1,2]: [1]Division of Infectious Genetics, Department of Microbiology and Immunology, [2]Laboratory of Innate Immunity, Center for Experimental Medicine and Systems Biology, The Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minatoku, TOKYO1208-8639, Japan.

TLR7 senses microbial-derived RNA in endolysosome, but can also erroneously respond to self-derived RNA. In fact, it has been reported that TLR7-dependent signaling promote autoimmune diseases. Thus, TLR7 can be therapeutic target. Although antibodies (Abs) are powerful tools for therapeutic intervention, TLR7 has been excluded from targets for Ab-mediated intervention because of its lack of cell surface expression. Despite this expectation, we found an anti-TLR7 Ab dose-dependently inhibits TLR7 responses in dendritic cells, macrophages and B cells. For this reason, we evaluated the therapeutic effect of anti-TLR7 Ab in *Unc93b1*$^{D34A/D34A}$ mice that cause thrombocytopenia, splenomegaly and chronic active hepatitis due to TLR7 hyper-responsiveness, and found that thrombocytopenia in *Unc93b1*$^{D34A/D34A}$ mice was significantly improved by the treatment with anti-TLR7 mAb. Furthermore, splenomegaly and hepatitis in mice treated with the anti-TLR7 mAb were also significantly remedy compared with control antibody.

On basis of these results, we established anti-human TLR7 Ab for blocking human TLR7 responses in vitro. Moreover, we generated human TLR7 transgenic (huTLR7 Tg) mice. We plan to use HuTLR7 Tg mice to evaluate the effects of anti-human TLR7 Ab *in vivo*.

In addition, TLR8 also recognize mouse TLR7 ligands in human and is involved in exacerbation of Rheumatoid Arthritis. Thus, in case of human disease, the anti-human TLR8 Ab that inhibits human TLR8 responses might work in clinical application. For this reason, we also constructed both anti-human TLR8 Abs and human TLR8 transgenic mice to verify our hypothesis.

2. Characterization of cleaved forms of TLR7 and TLR9

Chikako Yamamoto[2], Ryutaro Fukui[2], Nobuaki Yoshida[3] and Kensuke Miyake[1,2]: [1]Division of Infectious Genetics, Department of Microbiology and Immunology, [2]Laboratory of Innate Immunity & [3]Laboratory of Developmental Genetics, Center for Experimental Medicine and Systems Biology, The Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minatoku, TOKYO1208-8639, Japan.

Vertebrates have TLR3, 7, 8 and 9 as sensors of microbial nucleic acids, however it is suggested that TLR7/9 responses strongly associate with autoimmune diseases owing to inappropriate recognition of self nucleic acid. In endolysosome, TLR7 and TLR9 recognize a single-stranded RNA and an unmethylated CpG motif in microbial DNA, respectively. TLR7/9 ordinarily reside in Endoplasmic Reticulum, and ligand stimulation enhance the trafficking of TLR7/9 to endolysosome. Such strict regulation of TLR7/9 subcellular localization seems to have a role for blocking self nucleic acid recognition. Previous reports indicated novel posttranscriptional modification in TLR7/9 that ectodomains of TLR7/9 were cleaved in endolysosome. It seemed that ectodomain cleavage in TLR7/9 represents another strategy to restrict excessive TLR7/9 activation. Recently, we established new monoclonal anti-TLR7 and anti-TLR9 to detect endogenous or non-tagged TLRs and used these antibodies to clarify the mechanism of TLRs cleavage.

Our data showed that cleaved TLR7 N-terminal binds to C-terminal by its disulfide bond and the cysteines are important for response of TLR7 (Kanno et al., *Int. Immunol*. 2013). We focused on four cysteines of TLR7 (C98, C445, C475, and C722) and made serine mutants of these cysteines for analysis of cleavage pattern and the effect on response. As results, C98 and C475 were required for binding of TLR7 N-terminal to C-terminal, and proteolytic cleavage of TLR7. These cysteine mutants did not respond to TLR7 ligands, and also no response was observed in deletion mutants of TLR7 cleavage site or truncated form of TLR7 C-terminal alone. From these data, it is suggested that proteolytic cleavage of TLR7 and binding of N-terminal to C-terminal are essential for TLR7 response, and dependent on its cysteines.

In the case of TLR9, we found no effect of disulfide bond but the binding of N-terminal fragment to C-terminal fragment is important for TLR9 response. In addition, we also confirmed the importance of proteolytic cleavage of TLR9 in ligand recognition by analyzing crystal structure of TLR9.

3. Identification of regulatory molecules for TLR responses and constructing genetically engineered mice.

Takuma Shibata[1,2], Ryutaroh Fukui[1], Nobuaki Yoshida[2] and Kensuke Miyake[1,2]: [1]Division of Infectious Genetics, Department of Microbiology and Immunology, [2]Laboratory of Innate Immunity, Center for Experimental Medicine and Systems Biology, The Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minatoku, TOKYO1208-8639, Japan.

We are trying functional cloning using CRISPR/CAS9 based lentiviral Knock-Out Library to comprehensively identify regulatory molecules associating with TLR responses. Confirming the function of candidate genes *in vitro* using knock-out cell lines, we construct conventional/conditional knock-out mice or knock-in transgenic mice using ROSA26 locus to reveal the physiological function of novel TLR associating molecules *in vivo*.

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