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 Cd6 gene as a permissive locus for targeted transgenesis in the mouse

Hirotake Ichise, Taeko Ichise, Hiroki Sasanuma, and Nobuaki Yoshida

The introduction of a transgene into the genome through homologous recombination or sequence-specific enzymatic modification is a key technique for producing transgenic mice. The Rosa26 gene has been widely used to produce transgenic mice because the gene is transcriptionally active in various cell types and, at many developmental stages, is permissive for constitutive expression of integrated transgenes, and is dispensable for normal development. However, permissive loci other than Rosa26 are needed to generate mice that harbor multiple transgenes for complex studies. Here, we identified the Cd6 locus on mouse chromosome 19 as a transgene integration site in a transgenic mouse strain showing widespread reporter expression. Using this locus, we generated a knock-in mouse line that harbors a CAG promoter-driven reporter transgene, and found that the homozygous transgenic mice are viable and fertile, although transgene insertion disrupted Cd6 gene expression. The transgene on the Cd6 locus expressed reporter genes extensively throughout embryos, neonates, and adults. Combined with the Cre/loxP binary system, blood and lymphatic endothelial cell-specific reporter expression from the transgenic locus was achieved. These results suggest that Cd6 is valuable as an alterna-

tive site for targeted transgenesis.

2. FGF2-induced Ras-MAPK signalling maintains lymphatic endothelial cell identity by upregulating endothelial-cell-specific gene expression and suppressing TGFβ signalling through Smad2

Taeko Ichise, Nobuaki Yoshida, Hirotake Ichise

The lymphatic endothelial cell (LEC) fate decision program during development has been described. However, the mechanism underlying the maintenance of differentiated LEC identity remains largely unknown. Here, we show that fibroblast growth factor 2 (FGF2) plays a fundamental role in maintaining a differentiated LEC trait. In addition to demonstrating the appearance of LECs expressing α-smooth muscle actin in mouse lymphedematous skin in vivo, we found that mouse immortalised LECs lose their characteristics and undergo endothelial-to-mesenchymal transition (EndMT) when cultured in FGF2-depleted medium. FGF2 depletion acted synergistically with transforming growth factor (TGF) β to induce EndMT. We also found that H-Ras-overexpressing LECs were resistant to EndMT. Activation of H-Ras not only upregulated FGF2-induced activation of the Erk mitogen activated protein kinases (MAPK3 and MAPK1), but also suppressed TGFβ-induced activation of Smad2 by modulating Smad2 phosphorylation by MAPKs. These results suggest that FGF2 regulates LEC-specific gene expression and suppresses TGFβ signalling in LECs through Smad2 in a Ras-MAPK-dependent manner. Taken together, our findings provide a new insight into the FGF2-Ras-MAPK-dependent mechanism that maintains and modulates the LEC trait.

3. Development of FGF2-dependent pluripotent stem cells showing naive state characteristics from murine preimplantation inner cell mass

Manabu Ozawa, Eri Kawakami, Reiko Sakamoto, Takayuki Shibasaki, Akiteru Goto and Nobuaki Yoshida

Two distinct types of embryonic pluripotent stem cells can be established from either the inner cell mass (ICM) of preimplantation blastocyst (leukemia inhibitory factor (LIF)-dependent embryonic stem cell, ESC, called naive state) or the epiblast of post-implantation fetuses (fibroblast growth factor 2 (FGF2)-dependent epiblast stem cells, EpiSC, called primed state). Here, we report that naive pluripotent stem cell was established from the ICM, but maintained its self-renewal by treatment with FGF2 and mouse embryonic fibroblasts (MEFs) when they were exposed FGF2 during establishment. This cell line is competent to contribute to chimeric animals, including germ cells, at high efficiency. The ERK1/2, SMAD2/3, and JAK/STAT3 pathways are essential to maintain self-renewal. Inhibition of ERK1/2 or SMAD2/3 initiates transition to a naive state ESC-like state, whereas inhibition of JAK/STAT3 promotes a primed EpiSC-like character. Our present results could provide novel insights into understanding the growth factor environment and ICM plasticity, and mechanisms which orchestrate the pluripotency of embryonic stem cells and the capacity for chimeric contributions.

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

Eri Kawakami, Akinori Tokunaga, Manabu Ozawa, Reiko Sakamoto and Nobuaki Yoshida

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.

5. DOK7 gene therapy benefits mouse models of diseases characterized by defects in the neuromuscular junction

Sumimasa Arimura[1], Takashi Okada[2], Tohru Tezuka[1], Tomoko Chiyo[2], Yuko Kasahara[2], Toshiro Yoshimura[3], Masakatsu Motomura[4], Nobuaki Yoshida[5], David Beeson[6], Shin'ichi Takeda[2], Yuji Yamanashi[1]: [1]Division of Genetics, The Institute of Medical Science, The University of Tokyo. [2]De-

partment of Molecular Therapy, National Institute of Neuroscience, National Center of Neurology and Psychiatry. [3]Department of Occupational Therapy, Nagasaki University School of Health Sciences. [4]Department of Electrical and Electronics Engineering, Faculty of Engineering, Nagasaki Institute of Applied Science. [5]Laboratory of Developmental Genetics, The Institute of Medical Science, The University of Tokyo. [6]Neurosciences Group, Weatherall Institute of Molecular Medicine, University of Oxford.

The neuromuscular junction (NMJ) is the synapse between a motor neuron and skeletal muscle. Defects in NMJ transmission cause muscle weakness, termed myasthenia. The muscle protein Dok-7 is essential for activation of the receptor kinase MuSK, which governs NMJ formation, and DOK7 mutations underlie familial limb-girdle myasthenia (DOK7 myasthenia), a neuromuscular disease characterized by small NMJs. Here, we show in a mouse model of DOK7 myasthenia that therapeutic administration of an adeno-associated virus (AAV) vector encoding the human DOK7 gene resulted in an enlargement of NMJs and substantial increases in muscle strength and life span. When applied to model mice of another neuromuscular disorder, autosomal dominant Emery-Dreifuss muscular dystrophy, DOK7 gene therapy likewise resulted in enlargement of NMJs as well as positive effects on motor activity and life span. These results suggest that therapies aimed at enlarging the NMJ may be useful for a range of neuromuscular disorders.

6. Runx2-I isoform contributes to fetal bone formation even in the absence of specific N-terminal amino acids

Hideaki Okura[7,8], Shintaro Sato[7], Sari Kishikawa[7], Satoshi Kaneto[7], Tomoki Nakashima[9], Nobuaki Yoshida, Hiroshi Takayanagi[10] and Hiroshi Kiyono[7,8]: [7]Division of Mucosal Immunology, Department of Microbiology and Immunology, Institute of Medical Science, [8]Department of Medical Genome Science, Graduate School of Frontier Science, The University of Tokyo. [9]Department of Cell Signaling, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University. [10]Department of Immunology, Graduate School of Medicine and Faculty of Medicine, The University of Tokyo.

The Runt-related transcription factor 2 (Runx2) gene encodes the transcription factor Runx2, which is the master regulator of osteoblast development; insufficiency of this protein causes disorders of bone development such as cleidocranial dysplasia. Runx2 has two isoforms, Runx2-II and Runx2-I, and production of each isoform is controlled by a unique promoter: a distal promoter (P1) and a proximal promoter (P2), respectively. Although several studies have focused on differences and similarities between the two Runx2 isoforms, their individual roles in bone formation have not yet been determined conclusively, partly because a Runx2-I-targeted mouse model is not available. In this study, we established a novel Runx2-manipulated mouse model in which the first ATG of Runx2-I was replaced with TGA (a stop codon), and a neomycin-resistant gene (neo) cassette was inserted at the first intron of Runx2-I. Homozygous Runx2-Ineo/neo mice showed severely reduced expression of Runx2-I, whereas Runx2-II expression was largely retained. Runx2-Ineo/neo mice showed neonatal lethality, and in these mice, intramembranous ossification was more severely defective than endochondral ossification, presumably because of the greater involvement of Runx2-I, compared with that of Runx2-II in intramembranous ossification. Interestingly, the depletion of neo rescued the above-described phenotypes, indicating that the isoform-specific N-terminal region of Runx2-I is not functionally essential for bone development. Taken together, our results provide a novel clue leading to a better understanding of the roles of Runx2 isoforms in osteoblast development.

7. Mammalian-Specific Sequences in *Pou3f2* Contribute to Maternal Behavior

Makoto Nasu[11], Saori Yada[11], Atsushi Igarashi[11], Den'etsu Sutoo[11,12], Kayo Akiyama[12], Meguru Ito[11], Nobuaki Yoshida and Shintaroh Ueda[11]: [11]Department of Biological Sciences, Graduate School of Science, The University of Tokyo. [12]Institute of Medical Science, University of Tsukuba.

Various mutations have occurred during evolution among orthologs, genes in different species that diverged from a common ancestral gene by speciation. Here, we report the remarkable deterioration of a characteristic mammalian maternal behavior, pup retrieval, in nonmammalized mice, in which the transcription factor *Pou3f2* was replaced with the *Xenopus* ortholog lacking all of the homopolymeric amino acid repeats of mammalian POU3F2. Most of the pups born to the nonmammalized mice died within days after birth, depending on the dam genotype alone. Quantitative immunohistochemical analysis revealed decreases in the rate-limiting enzymes of dopamine and serotonin synthesis in various brain structures. Similar results were obtained in knock-in mice in which all of the homopolymeric amino acid repeats of mammalian POU3F2 were removed. Pup retrieval behavior in mammals is thus strongly related to monoamine neurotransmitter levels via the acquisition of homopolymeric amino acid repeats during

mammalian evolution.

8. Lnk prevents inflammatory CD8+ T-cell proliferation and contributes to intestinal homeostasis

Hiroko Katayama[13], Taizo Mori[13], Yoichi Seki[13], Masaki Anraku[13], Masanori Iseki[13], Masashi Ikutani[14], Yukiko Iwasaki[13], Nobuaki Yoshida, Kiyoshi Takatsu[14,15], and Satoshi Takaki[13]: [13]Department of Immune Regulation, Research Institute, National Center for Global Health and Medicine. [14]Department of Immunobiology and Pharmacological Genetics, Graduate School of Medicine and Pharmaceutical Science for Research, University of Toyama. [15]Toyama Prefectural Institute for Pharmaceutical Research.

The intracellular adaptor Lnk (also known as SH2B3) regulates cytokine signals that control lymphohematopoiesis, and Lnk−/− mice have expanded B-cell, megakaryocytes and hematopoietic stem-cell populations. Moreover, mutations in the LNK gene are found in patients with myeloproliferative disease, whereas LNK polymorphisms have recently been associated with inflammatory and autoimmune diseases, including celiac disease. Here, we describe a previously-unrecognized function of Lnk in the control of inflammatory CD8+ T-cell proliferation and in intestinal homeostasis. Mature T cells from newly-generated Lnk-Venus reporter mice had low but substantial expression of Lnk, whereas Lnk expression was down-regulated during homeostatic T-cell proliferation under lymphopenic conditions. The numbers of CD44hi IFN-γ +CD8+ effector or memory T cells were found to be increased in Lnk−/− mice, which also exhibited shortening of villi in the small intestine. Lnk−/− CD8+ T cells survived longer in response to stimulation with interleukin-15 (IL-15) and proliferated even in non-lymphopenic hosts. Transfer of Lnk−/− CD8+ T cells together with wild-type CD4+ T cells into Rag2-deficient mice recapitulated a sign of villous abnormality. Our results reveal a link between Lnk and immune cell-mediated intestinal tissue destruction.

Publications

1. Kawakami, E., Tokunaga, A., Ozawa, M., Sakamoto, R. and Yoshida, N.: The histone demethylase Fbxl11 plays an essential role in embryonic development by repressing cell-cycle regulators. Mech. Develop., in press
2. Arimura, S., Okada, T., Tezuka, T., Chiyo, T., Kasahara, Y., Yoshimura, T., Motomura, M., Yoshida, N., Beeson, D., Takeda, S. and Yamanashi, Y.: DOK7 Gene Therapy Benefits Mouse Models of Diseases Characterized by Defects in the Neuromuscular Junction. Science, 345: 1505-1508, 2014
3. Okura, H., Sato, S., Kishikawa, S., Kaneto, S., Nakashima, T., Yoshida, N., Takayanagi, H. and Kiyono, H.: Runx2-I Isoform Contributes to Fetal Bone Formation Even in the Absence of Specific N-Terminnal Amino Acids. PLoS ONE, 9: e108294, 2014
4. Ozawa, M., Kawakami, E., Sakamoto, R., Shibasaki, T., Goto, A. and Yoshida, N.: Development of FGF2-dependent pluripotent stem cells showing naïve state characteristics from murine preimplantation inner cell mass. Stem Cell Res., 13: 75-87, 2014
5. Nasu, M., Yada, S., Igarashi, A., Den'etsu Sutoo, Akiyama, K., Ito, M., Yoshida, N. and Ueda, S.: Mammalian-Specific Sequences in Pou3f2 Contribute to Maternal Behaviour. Genome Biol Evol., 6: 1145-1156, 2014
6. Ichise, H., Ichise, T., Sasanuma, H. and Yoshida, N.: The Cd6 gene as a permissive locus for targeted transgenesis in the mouse. Genesis, 52: 440-450, 2014
7. Katayama H., Mori. T., Seki, Y., Anraku, M., Iseki, M., Ikutani, M., Iwasaki, Y., Yoshida, N., Takatsu, K. and Takaki, S.: Lnk prevents inflammatory CD8+ T-cell proliferation and contributes to intestinal homeostasis. Eur. J. Immunol., 44: 1622-1632, 2014
8. Ichise, T., Yoshida, N. and Ichise, H.: FGF2-induced Ras/Erk MAPK signalling maintains lymphatic endothelial cell identity by up-regulating endothelial cell-specific gene expression and suppressing TGFb signalling via Smad2. J. Cell Sci., 127: 845-857, 2014
9. Liu, X., Pasula, S., Song, H., Tessneer, K.L., Dong, Y., Hahn, S., Yago, T., Brophy, M.L., Chang, B., Cai, X., Wu, H., McManus, J., Ichise, H., Georgescu, C., Wren, J.D., Griffin, C., Xia, L., Srinivasan, R.S., and Chen, H. Temporal and spatial regulation of epsin abundance and VEGFR3 signaling are required for lymphatic valve formation and function. Sci Signal. 7:ra97, 2014

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.

Potential role of myeloid cell/eosinophil-derived IL-17 in LPS-induced endotoxin shock.

Shimura E[1], Shibui A[2], Narushima S, Nambu A, Yamaguchi S, Akitsu A[3], Leonard WJ[4], Iwakura Y[3], Matsumoto K[5], Suto H[1], Okumura K[1], Sudo K[6], Nakae S: [1]Atopy Research Center, Juntendo University, [2]Department of Medical Genomics, Graduate School of Frontier Sciences, The University of Tokyo, [3]Division of Experimental Animal Immunology, Tokyo University of Science, [4]Laboratory of Molecular Immunology, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD, USA, [5]Department of Allergy and Immunology, National Research Institute for Child Health and Development, [6]Animal Research Center, Tokyo Medical University

IL-17RA is a shared receptor subunit for several cytokines of the IL-17 family, including IL-17A, IL-17C, IL-17E (also called IL-25) and IL-17F. It has been shown that mice deficient in IL-17RA are more susceptible to sepsis than wild-type mice, suggesting that IL-17RA is important for host defense against sepsis. However, it is unclear which ligands for IL-17RA, such as IL-17A, IL-17C, IL-17 E/IL-25 and/or IL-17F, are involved in the pathogenesis of sepsis. Therefore, we examined IL-17A, IL-17E/IL-25 and IL-17F for possible involvement in LPS-induced endotoxin shock. IL-17A-deficient mice, but not IL-25- or IL-17F-deficient mice, were resistant to LPS-induced endotoxin shock, as compared with wild-type mice. Nevertheless, studies using IL-6-deficient, IL-21Rα-deficient and Rag-2-deficient mice, revealed that neither IL-6 and IL-21, both of which are important for Th17 cell differentiation, nor Th17 cells were essential for the development of LPS-induced endotoxin shock, suggesting that IL-17A-producing cells other than Th17 cells were important in the setting. In this connection, IL-17A was produced by macrophages, DCs and eosinophils after LPS injection. Taken together, these findings indicate that IL-17A, but not IL-17F or IL-25, is crucial for LPS-induced endotoxin shock. In addition, macrophages, DCs and eosinophils, but not Th17 cells or γδ T cells, may be sources of IL-17A during LPS-induced endotoxin shock.

Basophil-derived interleukin-4 controls the function of natural helper cells, a member of ILC2s, in lung inflammation.

Motomura Y[1,2], Morita H[6], Moro K[3,5,7], Nakae S, Artis D[8], Endo TA[4], Kuroki Y[4], Ohara O[4], Koyasu S[3], Kubo M[1,2]: [1]Division of Molecular Pathology, Research Institute for Biomedical Science, Tokyo University of Science, [2]Laboratory for Cytokine

Regulation, [3]Laboratory for Immune Cell System, [4]Laboratory for Integrative Genomics, RIKEN Center for Integrative Medical Sciences (IMS), [5]Division of Immunobiology, Department of Medical Life Science, Graduate School of Medical Life Science, Yokohama City University, [6]Department of Allergy and Immunology, National Research Institute for Child Health and Development, [7]Precursory Research for Embryonic Science and Technology (PRESTO), Japan Science and Technology Agency, [8]Department of Microbiology, Institute for Immunology, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, USA

Allergic asthma is an inflammatory disease characterized by lung eosinophilia controlled by type 2 cytokines. Cysteine proteases are potent triggers of allergic inflammation by causing barrier disruption in lung epithelial cells inducing the elevation of interleukin-5 (IL-5) and IL-13 from natural helper (NH) cells, a member of ILC2s, which leads to lung eosinophilia. In this study, we found that basophils play a crucial role in NH cell-mediated eosinophilic inflammation induced by protease allergens. Conditional deletion of basophils caused a resolution of the papain-induced eosinophilia and mucus production. Resolution of eosinophilia was also observed in mice lacking IL-4 specifically in basophils, indicating that basophil-derived IL-4 enhanced expression of the chemokine CCL11, as well as IL-5, IL-9, and IL-13 in NH cells, thus attracting eosinophils. These results demonstrate that IL-4 from basophils has an important role in the NH-derived cytokine and chemokine expression, subsequently leading to protease allergen-induced airway inflammation.

Publications

1. Nakajima, S., Kitoh, A., Egawa, G., Natsuaki, Y., Nakamizo, S., Sagita-Moniaga, C., Otsuka, A., Honda, T., Hanakawa, S., Amano, W., Iwakura, Y., Nakae, S., Kubo, M., Miyachi, Y. and Kabashima, K. IL-17A as an inducer for Th2 immune responses in murine atopic dermatitis models. J Invest Dermatol. 134: 2122-30, 2014.
2. Unno, H., Futamura, K., Morita, H., Kojima, R., Arae, K., Nakae, S., Ida, H., Saito, H., Matsumoto, K., and Matsuda, A. Silica and Double-Stranded RNA Synergistically Induce Bronchial Epithelial Apoptosis and Airway Inflammation. Am J Respir Cell Mol Biol. 51: 344-53, 2014.
3. Motomura, Y., Morita, H., Moro, K., Nakae, S., Artis, D., Endo, T.A., Kuroki, Y., Ohara, O., Koyasu, S., and Kubo, M. Basophil-Derived Interleukin-4 Controls the Function of Natural Helper Cells, a Member of ILC2s, in Lung Inflammation. Immunity. 40: 758-71, 2014.
4. Shimura, E., Shibui, A., Narushima, S., Nambu, A., Yamaguchi, S., Akitsu, A., Leonard, W.J., Iwakura, Y., Matsumoto, K., Suto, H., Okumura, K., Sudo, K., and Nakae, S. Potential role of myeloid cell/eosinophil-derived IL-17 in LPS-induced endotoxin shock. Biochem Biophys Res Commun. 453: 1-6, 2014.
5. Osada, Y., Yamada, S., Nakae, S., Sudo, K., and Kanazawa, T. Reciprocal effects of Schistosoma mansoni infection on spontaneous autoimmune arthritis in IL-1 receptor antagonist-deficient mice. Parasitol Int. 64: 13-17, 2014.

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) family, 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 by Genome Engineering.

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

Atsuo Kanno[1,2], Yuji Motoi[1], 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. Next, the therapeutic effect of anti-TLR7 Ab was evaluated in *Unc93b1*$^{D34A/D34A}$ mice that cause thrombocytopenia, splenomegaly and chronic active hepatitis due to TLR7 hyper-responsiveness. Surprisingly, the platelet counts in mice were 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.

To investigate the target of anti-TLR7 Ab, we examined the distribution of endogenous TLR7. Unexpectedly, TLR7 highly expressed on cell surface of bone-marrow derived macrophages and splenic dendritic cells. Cell surface TLR7 dependently express Unc93B1 because of no detection on splenic dendritic cells in *Unc93b1*$^{3d/3d}$ mice that harbour a loss-of-function mutation. To further study cell surface TLR7, TLR7 and Unc93B1 were expressed in a fibroblast cell line NIH3T3. Anti-TLR7 Ab detected TLR7 on cell surface of these cell lines and was internalized in endolysosome for 24hr after incubation. These results demonstrate that TLR7 is internalized in cell surface TLR7-dependent manner and promising target for therapeutic intervention in autoimmune diseases (Kanno et al., *Nat. Commun*. 2015). On basis of these results, we are working to establish anti-human TLR7 Ab for blocking human

TLR7 responses. Moreover, we generated human TLR7 transgenic (huTLR7 Tg) mice. HuTLR7 Tg mice will be evaluated the effects of anti-human TLR7 Ab *in vivo*.

On the other hands, human TLR8 is likely to nearly recognize TLR7 ligands. Thus, in case of human disease, the anti-human TLR8 Ab that inhibits human TLR8 responses will need for clinical application. For this reason, we are also working to obtain such anti-human TLR8 Ab and making human TLR8 transgenic mice.

2. Characterization of cleaved forms of TLR7 and TLR9

Atsuo Kanno[1], Chikako Yamamoto[2], Yuji Motoi[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. Now we are trying to confirm the relation between proteolytic cleavage of TLR9 and its response.

To understand physiologic function of proteolytic cleavage in nucleic acid sensing-TLRs, we generated TLR9 knock-in (TLR9 KI) mice having the mutation of cleavage sites. The endogenous TLR9 in these mice was not cleaved in bone-marrow derived dendritic cells and macrophage. Furthermore, the condition of full-length TLR9 was not respond to TLR9 ligands stimulation. These results demonstrate *in vivo* that the functional TLR9 is the cleaved TLR9. We will further study the physiologic meaning at cleavage sites *in vivo*.

3. Roles for Unc93 homolog B1-dependent TLR 7/9 balance in vivo

Atsuo Kanno[1], Ryutaro Fukui[1], Shin-Ichiroh Saitoh[1], Takuma Shibata[1,2], Yuji Motoi[1], 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.

Nucleic acid sensing Toll-like receptor 7 (TLR7) and TLR9 recognize microbial RNA and DNA, respectively. These TLRs potentially recognize self-derived nucleic acid and have been shown to have a role in autoimmune diseases. For maintenance of homeostasis, it is important to keep the responsiveness of the nucleic acid-sensing TLRs under the tight control.

Unc93 homolog B1 (Unc93B1) has been reported to be indispensable for TLR7/9 responses in vitro. We have previously found that the alanine substitution for the 34th aspartic acid (D34A) of Unc93B1 enhanced TLR7 response but downregulated TLR9 response. These results suggest that Unc93B1 reciprocally regulate between TLR7 and TLR9 responses, and the TLR7/TLR9 balance is biased towards TLR9 in the steady state.

To further clarify a role for Unc93B1-dependent TLR7/TLR9 balance *in vivo*, we started to generate DEL/AAA knock-in (*Unc93b1*$^{KI/KI}$) mice harboring the mutations from the aspartic acid to leucine around D34 locus in the Unc93B1 gene that showed more accelerated phenotypes than D34A mutant

mice. These knock-in mice were also designed that Unc93B1 knock-out mice were able to be generated by Cre-Lox recombination system. Therefore, we will be able to investigate the role of Unc93B1 *in vivo* for TLR7 and TLR9 responses. *Unc93b1*$^{KI/KI}$ mice and knock-out mice were succeeded in germ line transmission. *Unc93b1*$^{KI/KI}$ mice cause thrombocytopenia and died 50% at 28 weeks. We will further study to search the phenotype *in vivo*.

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

Takuma Shibata[1,2], Yuji Motoi[1], Atsuo Kanno[1], Ryutaroh Fukui[1], Shin-ichiroh Saitoh[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. After checking the function of candidate genes *in vitro* using knock-out cell lines, we also 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*.

Publications

Kanno A, Tanimura N, Ishizaki M, Ohko K, Motoi Y, Onji M, Fukui R, Shimozato T, Yamamoto K, Shibata T, Sano S, Sugahara-Tobinai A, Takai T, Ohto U, Shimizu T, Saitoh SI, and Miyake K. Targeting cell surface TLR7 for therapeutic intervention in autoimmune diseases. *Nat. Commun.* (2015)