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
Associate Professor	Hirotake Ichise D.V.M., Ph.D.		准教授	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. *Foxc2CreERT2* knock-in mice mark stage-specific *Foxc2*-expressing cells during mouse organogenesis

Mohammed Badrul Amin[1], Naoyuki Miura[1], Mohammad Khaja Mafij Uddin[1], Mohammod Johirul Islam[1], Nobuaki Yoshida, Sachiko Iseki[2], Tsutomu Kume[3], Paul Trainor[4,5], Hirotomo Saitsu[1], Kazushi Aoto[6]: [1]Department of Biochemistry, Hamamatsu University School of Medicine. [2]Section of Molecular Craniofacial Embryology, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University. [3]Erin Lambers, Feinberg Cardiovascular Research Institute, Department of Medicine, Northwestern University School of Medicine. [4]Stowers Institute for Medical Research. [5]Department of Anatomy and Cell Biology, University of Kansas Medical Center.[6]Department of Biochemistry, Hamamatsu University School of Medicine.

Foxc2, a member of the winged helix transcription factor family, is essential for eye, calvarial bone, cardiovascular and kidney development in mice. Nevertheless, how *Foxc2*-expressing cells and their descendent cells contribute to the development of these tissues and organs has not been elucidated. Here, we generated a *Foxc2* knock-in (*Foxc2CreERT2*) mouse, in which administration of estrogen receptor antagonist tamoxifen induces nuclear translocation of Cre recombinase in *Foxc2*-expressing cells. By crossing with *ROSA-LacZ* re-

porter mice (*Foxc2CreERT2; R26R*), the fate of Foxc2 positive (*Foxc2*+) cells was analyzed through *LacZ* staining at various embryonic stages. We found *Foxc2*+ cell descendants in the supraoccipital and exoccipital bone in E18.5 embryos, when tamoxifen was administered at embryonic day (E) 8.5. Furthermore, *Foxc2*+ descendant cranial neural crest cells at E8-10 were restricted to the corneal mesenchyme, while *Foxc2*+ cell derived cardiac neural crest cells at E6-12 were found in the aorta, pulmonary trunk and valves, and endocardial cushions. *Foxc2*+ cell descendant contributions to the glomerular podocytes in the kidney were also observed following E6.5 Tamoxifen treatment. Our results are consistent previous reports of *Foxc2* expression during early embryogenesis and the *Foxc2CreERT2* mouse provides a tool to investigate spatiotemporal roles of *Foxc2* and contributions of *Foxc2*+ expressing cells during mouse embryogenesis.

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

Hiroshi Kuribayashi[7], Asano Tsuhako[7], Mio Kikuchi, Nobuaki Yoshida, Hideto Koso[7], Sumiko Watanabe[8]: [7]Division of Molecular and Developmental Biology, Institute of Medical Science, University of Tokyo. [8]Division of Molecular and Developmental Biology, Institute of Medical Science, University of Tokyo.

5'TG3'-interacting factors (TGIFs) function as transcriptional repressors. Defects in TGIFs cause severe abnormalities in the developing brain and face. We found that Tgif2 was highly expressed in the mouse retina at early stages of development and examined its role in retinal development. Knockdown of Tgif2 in retinal explants at E14 using shRNA (sh-Tgif2) resulted in a decreased number of rod photoreceptors, whereas the number of cone photoreceptors increased without perturbation of cell proliferation and apoptosis. Concomitantly, the expression levels of photoreceptor-related genes were decreased in sh-Tgif2-introduced retinal explants. To examine the in vivo effects of Tgif2 overexpression, we generated Tgif2 conditional knock-in mice (Tgif2cKI). Although retinal cell differentiation, based on the relative proportions of retinal subtypes in the mature retina, was not affected, we observed abnormal localization of cone photoreceptor cell nuclei in the outer nuclear layer of the Tgif2cKI retina. However, electrical retinography suggest that cones in Tgif2cKI were functionally equivalent to those of wild mice. Our study revealed that Tgif2 participates in photoreceptor cell differentiation in the early stages of retinal development and regulates proper subretinal localization of the cone photoreceptors.

3. Cell-Type-Specific Alternative Splicing Governs Cell Fate in the Developing Cerebral Cortex

Xiaochang Zhang[9], Ming Hui Chen[10], Xuebing Wu[11], Andrew Kodani[12], Jean Fan[13], Ryan Doan[14], Manabu Ozawa, Jacqueline Ma[15], Nobuaki Yoshida, Jeremy F. Reiter[12], Douglas L. Black[16], Peter V. Kharchenko[13], Phillip A. Sharp[17], Christopher A. Walsh[18]: [9]Division of Genetics and Genomics, Manton Center for Orphan Disease Research, Howard Hughes Medical Institute, Departments of Neurology and Pediatrics, Harvard Medical School. [10]Division of Genetics and Genomics, Manton Center for Orphan Disease Research, Howard Hughes Medical Institute, Boston Children's Hospital. Department of Cardiology, Boston Children's Hospital and Harvard Medical School. [11]David H. Koch Institute for Integrative Cancer Research, Department of Biology, Computational and Systems Biology Graduate Program, Massachusetts Institute of Whitehead Institute for Biomedical Research. [12]Department of Biochemistry and Biophysics, Cardiovascular Research Institute, University of California, San Francisco. [13]Department of Biomedical Informatics, Harvard Medical School ; Harvard Stem Cell Institute. [14]Division of Genetics and Genomics, Manton Center for Orphan Disease Research, Howard Hughes Medical Institute, Boston Children's Hospital. Departments of Neurology and Pediatrics, Harvard Medical School. [15]Division of Genetics and Genomics, Manton Center for Orphan Disease Research, Howard Hughes Medical Institute, Boston Children's Hospital. [16]Department of Microbiology, Immunology, and Molecular Genetics, University of California, Los Angeles. [17]David H. Koch Institute for Integrative Cancer Research, Department of Biology, Computational and Systems Biology Graduate Program, Massachusetts Institute of Technology. [18]Division of Genetics and Genomics, Manton Center for Orphan Disease Research, Howard Hughes Medical Institute, Boston Children's Hospital ; Departments of Neurology and Pediatrics, Harvard Medical School; Harvard Stem Cell Institute ; Broad Institute of MIT and Harvard.

Alternative splicing is prevalent in the mammalian brain. To interrogate the functional role of alternative splicing in neural development, we analyzed purified neural progenitor cells (NPCs) and neurons from developing cerebral cortices, revealing hundreds of differentially spliced exons that preferentially alter key protein domains-especially in cytoskeletal proteins-and can harbor disease-causing mutations. We show that Ptbp1 and Rbfox proteins antagonistically govern the NPC-to-neuron transi-

tion by regulating neuron-specific exons. Whereas Ptbp1 maintains apical progenitors partly through suppressing a poison exon of Flna in NPCs, Rbfox proteins promote neuronal differentiation by switching Ninein from a centrosomal splice form in NPCs to a non-centrosomal isoform in neurons. We further uncover an intronic human mutation within a PTBP1-binding site that disrupts normal skipping of the FLNA poison exon in NPCs and causes a brain-specific malformation. Our study indicates that dynamic control of alternative splicing governs cell fate in cerebral cortical development.

4. Phospholipase Cγ2 Is Required for Luminal Expansion of the Epididymal Duct during Postnatal Development in Mice.

Hirotake Ichise, Taeko Ichise, Nobuaki Yoshida

Phospholipase Cγ2 (PLCγ2)-deficient mice exhibit misconnections of blood and lymphatic vessels, and male infertility. However, the cell type responsible for vascular partitioning and the mechanism for male infertility remain unknown. Accordingly, we generated a mouse line that conditionally expresses endogenous *Plcg2* in a Cre/loxP recombination-dependent manner, and found that Tie2-Cre- or Pf4-Cre-driven reactivation of *Plcg2* rescues PLCγ2-deficient mice from the vascular phenotype. By contrast, male mice rescued from the vascular phenotype exhibited epididymal sperm granulomas. As judged from immunostaining, PLCγ2 was expressed in clear cells in the epididymis. PLCγ2 deficiency did not compromise differentiation of epididymal epithelial cells, including clear cells, and tube formation at postnatal week 2. However, luminal expansion of the epididymal duct was impaired during the prepubertal period, regardless of epithelial cell polarity and tube architecture. These results suggest that PLCγ2-deficient clear cells cause impaired luminal expansion, stenosis of the epididymal duct, attenuation of luminal flow, and subsequent sperm granulomas. Clear cell-mediated luminal expansion is also supported by the observation that PLCγ2-deficient males were rescued from infertility by epididymal epithelium-specific reactivation of *Plcg2*, although the edematous and hemorrhagic phenotype associated with PLCγ2 deficiency also caused spontaneous epididymal sperm granulomas in aging males. Collectively, our findings demonstrate that PLCγ2 in clear cells plays an essential role in luminal expansion of the epididymis during the prepubertal period in mice, and reveal an unexpected link between PLCγ2, clear cells, and epididymal development.

5. Establishment of a tamoxifen-inducible Cre-driver mouse strain for widespread and temporal genetic modification in adult mice.

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

Temporal genetic modification of mice using the ligand-inducible Cre/loxP system is an important technique that allows the bypass of embryonic lethal phenotypes and access to adult phenotypes. In this study, we generated a tamoxifen-inducible Cre-driver mouse strain for the purpose of widespread and temporal Cre recombination. The new line, named CM32, expresses the GFPneo-fusion gene in a wide variety of tissues before FLP recombination and tamoxifen-inducible Cre after FLP recombination. Using FLP-recombined CM32 mice (CM32Δ mice) and Cre reporter mouse lines, we evaluated the efficiency of Cre recombination with and without tamoxifen administration to adult mice, and found tamoxifen-dependent induction of Cre recombination in a variety of adult tissues. In addition, we demonstrated that conditional activation of an oncogene could be achieved in adults using CM32Δ mice. CM32Δ;T26 mice, which harbored a Cre recombination-driven, SV40 large T antigen-expressing transgene, were viable and fertile. No overt phenotype was found in the mice up to 3 months after birth. Although they displayed pineoblastomas (pinealoblastomas) and/or thymic enlargement due to background Cre recombination by 6 months after birth, they developed epidermal hyperplasia when administered tamoxifen. Collectively, our results suggest that the CM32Δ transgenic mouse line can be applied to the assessment of adult phenotypes in mice with loxP-flanked transgenes.

6. Regulation of gene expression in the bovine blastocyst by colony stimulating factor 2.

Manabu Ozawa, Miki Sakatani[20], Kyle B. Dobbs[21,22], Jasmine Kannampuzha-Francis[22], Peter J. Hansen[22]: [20]Kyushu-Okinawa Agricultural Research Center, National Agriculture and Food Research Organization. [21]Thermo Fisher Scientific. [22]Dept. of Animal Sciences, D.H. Barron Reproductive and Perinatal Biology Research Program and Genetics Institute, University of Florida.

BACKGROUND: Colony stimulating factor 2 can have multiple effects on the function of the preimplantation embryo that include increased potential to develop to the blastocyst stage, reduced apoptosis, and enhanced ability of inner cell mass (ICM) to remain pluripotent after culture. The objective of the current experiment was to identify genes regulated by CSF2 in the ICM and trophectoderm (TE) of the bovine blastocyst with the goal of identifying

possible molecular pathways by which CSF2 increases developmental competence for survival. Embryos were produced in vitro and cultured from Day 6 to 8 in serum-free medium containing 10 ng/ml recombinant bovine CSF2 or vehicle. Blastocysts were harvested at Day 8 and ICM separated from TE by magnetic-activated cell sorting. RNA was purified and used to prepare amplified cDNA, which was then subjected to high-throughput sequencing using the SOLiD 4.0 system. Three pools of amplified cDNA were analyzed per treatment.

RESULTS: The number of genes whose expression was regulated by CSF2, using $P<0.05$ and >1.5-fold difference as cut-offs, was 945 in the ICM (242 upregulated by CSF2 and 703 downregulated) and 886 in the TE (401 upregulated by CSF2 and 485 downregulated). Only 49 genes were regulated in a similar manner by CSF2 in both cell types. The three significant annotation clusters in which genes regulated by ICM were overrepresented were related to membrane signaling. Genes downregulated by CSF2 in ICM were overrepresented in several pathways including those for ERK and AKT signaling. The only significant annotation cluster containing an overrepresentation of genes regulated by CSF2 in TE was for secreted or extracellular proteins. In addition, genes downregulated in TE were overrepresented in TGFβ and Nanog pathways.

CONCLUSIONS: Differentiation of the blastocyst is such that, by Day 8 after fertilization, the ICM and TE respond differently to CSF2. Analysis of the genes regulated by CSF2 in ICM and TE are suggestive that CSF2 reinforces developmental fate and function of both cell lineages.

KEYWORDS: Blastocyst; Bovine; Colony stimulating factor-2; Inner cell mass; Transcriptome; Trophectoderm

7. The Histone Demethylase FBXL10 Regulates the Proliferation of Spermatogonia and Ensures Long-Term Sustainable Spermatogenesis in Mice.

Manabu Ozawa, Tsuyoshi Fukuda, Reiko Sakamoto, Hiroaki Honda[23], Nobuaki Yoshida: [23]Department of Disease Model, Research Institute of Radiation Biology and Medicine, Hiroshima University.

The F-box and leucine-rich repeat protein 10 (*Fbxl 10*) gene encodes a protein that catalyzes demethylation of H3K4 and H3K36. In this study, we show the important roles of FBXL10 as a histone demethylase in sustainable sperm production using mice in which the JmjC domain of *Fbxl10* was deleted (*Fbxl* $10^{DeltaJ/DeltaJ}$). In histological analysis, testis sections from 10-wk-old $Fbxl10^{DeltaJ/DeltaJ}$ mice appeared normal. On the other hand, testes from 7-mo-old *Fbxl* $10^{DeltaJ/DeltaJ}$ mice contained a greater ratio of seminiferous tubules exhibiting degeneration of spermatogenesis. Further analysis using an in vitro spermatogonia culture system, that is, germline stem cells (GSCs), revealed that $Fbxl10^{DeltaJ/DeltaJ}$ GSCs expressed a significantly higher level of *P21* and *P19* mRNA, cyclin-dependent kinase inhibitors and also known as cellular senescence markers, than wild-type (WT) GSCs. Furthermore, the ratio of $Fbxl10^{DeltaJ/DeltaJ}$ GSCs in G0/G1 phase was higher and the ratios in S and G2/M phases were lower than the corresponding ratios of WT GSCs, and the doubling speed of *Fbxl* $10^{DeltaJ/DeltaJ}$ GSCs was significantly slower than that of WT GSCs. In addition to these in vitro results, an in vivo study indicated that recovery of spermatogenesis after a transient reduction in the number of testicular germ cells by busulfan treatment was significantly slower in $Fbxl10^{DeltaJ/DeltaJ}$ mice than in WT mice. These data suggest that *Fbxl10* plays important roles in long-term sustainable spermatogenesis via regulating cell cycle.

Publications

1. Amin, M., Miura, N., Uddin, M., Islam, M., Yoshida, N., SachikoIsekiS., Tsutomu Kume, T., Trainor, P., Hirotomo, S., Aoto, K. Foxc2creERT2 knock-in mice mark stage-specific Foxc2-expressing cells during mouse organogenesis. Congenit. Anomal, in press
2. Kuribayashi, H., Tsuhako, A., Kikuchi, M., Yoshida, N., Koso, H. and Watanabe, S. Role of transcription factor Tgif2 in photoreceptor differentiation in the mouse retina. Exp. Eye Res., in press
3. Zhang, X., Chen, H., Wu. X., Kodani, A., Fan, J., Doan, R., Ozawa, M., Ma, J., Yoshida, N., Reiter. J., Black, D., Kharchenko, P., Sharp and P., Walsh, C. Cell type-specific alternative splicing governs cell fate in the developing cerebral cortex. Cell, 166: 1147-1162, 2016
4. Ozawa M, Sakatani M, Dobbs KB, Kannampuzha-Francis J, Hansen PJ. Regulation of gene expression in the bovine blastocyst by colony stimulating factor 2. BMC Research Notes, 9: 250, 2016
5. Ozawa M, Fukuda T, Sakamoto R, Honda H, Yoshida N. The Histone Demethylase FBXL10 Regulates the Proliferation of Spermatogonia and Ensures Long-Term Sustainable Spermatogenesis in Mice. Biology of Reproduction, 94(4): 92, 2016
6. Ichise H, Ichise T, Yoshida N.Phospholipase Cγ2

Is Required for Luminal Expansion of the Epididymal Duct during Postnatal Development in Mice. PLoS ONE, 11(3): e0150521, 2016
7. Ichise H, Hori A, Shiozawa S, Kondo S, Kanegae Y, Saito I, Ichise T, Yoshida N. Establishment of a tamoxifen-inducible Cre-driver mouse strain for widespread and temporal genetic modification in adult mice. Exp Anim. 15-0126, 2016

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.

IL-25, IL-33 and TSLP receptor are not critical for development of experimental murine malaria

Shibui, A.[1,2], Takamori, A.[2], Tolba, M.E.[1,3], Nambu, A.[2], Shimura, E.[2,4], Yamaguchi, S.[2], Sanjoba, C.[4], Suto, H.[5], Sudo, K.[6], Okumura, K.[5], Sugano, S.[1], Morita, H.[7], Saito, H.[7], Matsumoto, K.[7] Nakae, S.[2,8]: [1]Department of Medical Genomics, Graduate School of Frontier Sciences, The University of Tokyo, Chiba, [2]Laboratory of Systems Biology, Center for Experimental Medicine and Systems Biology, The Institute of Medical Science, The University of Tokyo, Tokyo, [3]Department of Parasitology, Assiut University, Assiut, [4]Department of Molecular Immunology, School of Agricultural and Life Sciences, The University of Tokyo, Tokyo, [5]Atopy Research Center, Juntendo University, Tokyo, [6]Animal Research Center, Tokyo Medical University, Tokyo, [7]Department of Allergy and Immunology, National Research Institute for Child Health and Development, Tokyo, [8]Precursory Research for Embryonic Science and Technology (PRESTO), Japan Science and Technology Agency, Saitama,

IL-25, IL-33 and TSLP, which are produced predominantly by epithelial cells, can induce production of Th2-type cytokines such as IL-4, IL-5 and/or IL-13 by various types of cells, suggesting their involvement in induction of Th2-type cytokine-associated immune responses. It is known that Th2-type cytokines contribute to host defense against malaria parasite infection in mice. However, the roles of IL-25, IL-33 and TSLP in malaria parasite infection remain unclear. Thus, to elucidate this, we infected wild-type, IL-25$^{-/-}$, IL-33$^{-/-}$ and TSLP receptor (TSLPR)$^{-/-}$ mice with *Plasmodium berghei* (*P. berghei*) ANKA, a murine malaria strain. The expression levels of IL-25, IL-33 and TSLP mRNA were changed in the brain, liver, lung and spleen of wild-type mice after infection, suggesting that these cytokines are involved in host defense against *P. berghei* ANKA. However, the incidence of parasitemia and survival in the mutant mice were comparable to in the wild-type mice. These findings indicate that IL-25, IL-33 and TSLP are not critical for host defense against *P. berghei* ANKA.

TSLP receptor is not essential for house dust mite-induced allergic rhinitis in mice

Nakanishi, W.[1,2], Hiraishi, Y.[1,3], Yamaguchi, S.[1], Takamori, A.[1], Morita, H.[4], Matsumoto, K.[4], Saito, H.[4], Sudo, K.[5], Yamasoba, T.[2], Nakae, S.[1,6]: [1]Laboratory of Systems Biology, Center for Experimental Medicine and Systems Biology, The Institute of Medical Science, The University of Tokyo, Tokyo, [2]Department of Otolaryngology Head and Neck

Surgery, [3]Department of Respiratory Medicine, Graduate School of Medicine, The University of Tokyo, Tokyo, [4]Department of Allergy and Clinical Immunology, National Research Institute for Child Health and Development, Tokyo, [5]Animal Research Center, Tokyo Medical University, Tokyo, [6]Precursory Research for Embryonic Science and Technology, Japan Science and Technology Agency, Saitama,

TSLP induces Th2 cytokine production by Th2 cells and various other types of cells, thereby contributing to Th2-type immune responses and development of allergic disorders. We found that house dust mite (HDM) extract induced TSLP production by nasal epithelial cells, suggesting that TSLP may be involved in development of HDM-induced allergic rhinitis (AR). To investigate that possibility in greater detail, wild-type and TSLP receptor-deficient ($TSLPR^{-/-}$) mice on the C57BL/6J background were repeatedly treated intranasally with HDM extract. The frequency of sneezing, numbers of eosinophils and goblet cells, thickness of submucosal layers, serum levels of total IgE and HDM-specific IgG1, and levels of IL-4, IL-5 and IL-13 in the culture supernatants of HDM-stimulated LN cells were comparable in the two mouse strains. Those findings indicate that, in mice, TSLPR is not crucial for development of HDM-induced AR.

TIM-3 is not essential for development of airway inflammation induced by house dust mite antigens

Hiraishi, Y.[1,2,3], Nambu, A.[1], Shibui, A.[1,4], Nakanishi, W.[1,5], Yamaguchi, S.[1], Iikura, M.[6] McKenzie, A,N.[7], Matsumoto, K.[8], Sudo, K.[9], Yamasoba, T.[5], Nagase, T.[2], Nakae, S.[1,10]: [1]Laboratory of Systems Biology, Center for Experimental Medicine and Systems Biology, The Institute of Medical Science, The University of Tokyo, Tokyo, [2]Department of Respiratory Medicine, and [5]Department of Otolaryngology Head and Neck Surgery, Graduate School of Medicine, The University of Tokyo, Tokyo, [3]Department of Endoscopy, Respiratory Endoscopy Division, National Cancer Center Hospital, Tokyo, [4]Department of Medical Genomics, Graduate School of Frontier Sciences, The University of Tokyo, Chiba, [6]Department of Respiratory Medicine, National Center for Global Health and Medicine, Tokyo, [7]Medical Research Council Laboratory of Molecular Biology, Cambridge, [8]Department of Allergy and Immunology, National Research Institute for Child Health and Development, Tokyo, [9]Animal Research Center, Tokyo Medical University, Tokyo, [10]Precursory Research for Embryonic Science and Technology (PRESTO), Japan Science and Technology Agency, Saitama,

T cell immunoglobulin domain and mucin domain-containing molecule 3 (TIM-3), which is preferentially expressed on Th1 cells rather than Th2 cells, is considered to be a negative regulator of Th 1 cell function. This suggests that TIM-3 indirectly enhances Th2-type immune responses by suppressing Th1 cell function. To investigate TIM-3's possible involvement in Th2-type acute and chronic airway inflammation, wild-type and TIM-3-deficient ($TIM\text{-}3^{-/-}$) mice were sensitized and challenged with a house dust mite (HDM) extract. Airway inflammation and the number of inflammatory cells in bronchoalveolar lavage fluids (BALFs) in the mice were determined by histological analysis and with a hemocytometer, respectively. Expression of mRNA in the lungs was determined by quantitative PCR, while the levels of cytokines in the BALFs and IgE in sera were determined by ELISA. Despite constitutive expression of TIM-3 mRNA in the lungs, the number of eosinophils in bronchoalveolar lavage fluids (BALFs) and the score of pulmonary inflammation were comparable between wild-type and $TIM\text{-}3^{-/-}$ mice during both acute and chronic HDM-induced airway inflammation. On the other hand, the number of lymphocytes in the BALFs of $TIM\text{-}3^{-/-}$ mice was significantly increased compared with wild-type mice during HDM-induced chronic, but not acute, airway inflammation, while the levels of Th2 cytokines in the BALFs and HDM-specific IgG1 and IgG2a and total IgE in the sera were comparable in both groups. Our findings indicate that, in mice, TIM-3 is not essential for development of HDM-induced acute or chronic allergic airway inflammation, although it appears to be involved in reduced lymphocyte recruitment during HDM-induced chronic allergic airway inflammation.

Publications

1. Kudo F, Ikutani M, Seki Y, Otsubo T, Kawamura YI, Dohi T, Oshima K, Hattori M, Nakae S, Takatsu K, Takaki S. Interferon-γ constrains cytokine production of group 2 innate lymphoid cells. Immunology. 147(1): 21-9, 2016.
2. Akimoto M, Hayashi JI, Nakae S, Saito H, Takenaga K. Interleukin-33 enhances programmed oncosis of ST2L-positive low-metastatic cells in the tumour microenvironment of lung cancer. Cell Death Dis. 7: e2057, 2016.
3. Oh JE, Kim BC, Chang DH, Kwon M, Lee SY, Kang D, Kim JY, Hwang I, Yu JW, Nakae S,

Lee HK Dysbiosis-induced IL-33 contributes to impaired antiviral immunity in the genital mucosa. Proc Natl Acad Sci USA. 113(6): E762-71, 2016.
4. Shibui A, Takamori A, Tolba EMT, Nambu A, Shimura E, Yamaguchi S, Sanjoba C, Suto H, Sudo K, Okumura K, Sugano S, Morita H, Saito H, Matsumoto K & Nakae S. IL-25, IL-33 and TSLP receptor are not critical for development of experimental murine malaria. Biochem Biophys Rep. 5: 191-5, 2016.
5. Aoki R, Kawamura T, Goshima F, Ogawa Y, Nakae S, Moriishi K, Nakao A, Shimada S. The alarmin IL-33 derived from HSV-2-infected keratinocytes triggers mast cell-mediated antiviral innate immunity. J Invest Dermatol. 136(6): 1290-2, 2016.
6. Shimura S, Takai T, Iida H, Maruyama N, Ochi H, Kamijo S, Nishioka I, Hara M, Matsuda A, Saito H, Nakae S, Ogawa H, Okumura K, Ikeda S. Epicutaneous Allergic Sensitization by Cooperation between Allergen Protease Activity and Mechanical Skin Barrier Damage in Mice. J Invest Dermatol. 136(7): 1408-17, 2016.
7. Furusawa JI, Mizoguchi I, Chiba Y, Hisada M, Kobayashi F, Yoshida H, Nakae S, Tsuchida A, Matsumoto T, Ema H, Mizuguchi J, Yoshimoto T. Promotion of Expansion and Differentiation of Hematopoietic Stem Cells by Interleukin-27 into Myeloid Progenitors to Control Infection in Emergency Myelopoiesis. PLoS Pathog. 12(3): e 100507, 2016.
8. Kamijo S, Suzuki M, Hara M, Shimura S, Ochi H, Maruyama N, Matsuda A, Saito H, Nakae S, Suto H, Ichikawa S, Ikeda S, Ogawa H, Okumura K, Takai T, Subcutaneous Allergic Sensitization to Protease Allergen Is Dependent on Mast Cells but Not IL-33: Distinct Mechanisms between Subcutaneous and Intranasal Routes. J Immunol. 196(9): 3559-69, 2016.
9. Takeda T, Unno H, Morita H, Futamura K, Emi-Sugie M, Arae K, Shoda T, Okada N, Igarashi A, Inoue E, Kitazawa H, Nakae S, Saito H, Matsumoto K, Matsuda A. Platelets constitutively express IL-33 protein and modulate eosinophilic airway inflammation. J Allergy Clin Immunol. 138(5): 1395-1403. e6, 2016.
10. Yang Y, Andersson P, Hosaka K, Zhang Y, Cao R, Iwamoto H, Yang X, Nakamura M, Wang J, Zhuang R, Morikawa H, Xue Y, Braun H, Beyaert R, Samani N, Nakae S, Hams E, Dissing S, Fallon PG, Langer R, Cao Y. The PDGF-BB-SOX7 axis-modulated IL-33 in pericytes and stromal cells promotes metastasis through tumour-associated macrophages. Nat Commun. 7: 11385, 2016.
11. Hiraishi Y, Nambu A, Shibui A, Nakanishi W, Yamaguchi S, Morita H, Iikura M, McKenzie AN, Matsumoto K, Sudo K, Yamasoba T, Nagase T, Nakae S. TIM-3 is not essential for development of airway inflammation induced by house dust mite antigens. Allergol Int. 65(4): 459-65, 2016.
12. Nakanishi W, Hiraishi Y, Yamaguchi S, Takamori A, Morita H, Matsumoto K, Saito H, Sudo K, Yamasoba T and Nakae S. TSLP receptor is not essential for house dust mite-induced allergic rhinitis in mice Biochem Biophys Rep. 7: 119-23, 2016.
13. Waseda M, Arimura S, Shimura E, Nakae S, Yamanashi Y. Loss of Dok-1 and Dok-2 in mice causes severe experimental colitis accompanied by reduced expression of IL-17A and IL-22. Biochem Biophys Res Commun. 478(1): 135-42, 2016.
14. Siede J, Fröhlich A, Datsi A, Hegazy AN, Varga DV, Holecska V, Saito H, Nakae S, Löhning M. IL-33 Receptor-Expressing Regulatory T Cells Are Highly Activated, Th2 Biased and Suppress CD4 T Cell Proliferation through IL-10 and TGF-β Release. PLoS One. 11(8): e0161507, 2016.
15. Morita H, Saito H, Matsumoto K, Nakae S. Regulatory roles of mast cells in immune responses. Semin Immunopathol. 38(5): 623-9, 2016.

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[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, TOKYO, Japan.

TLR7 senses microbial-derived RNA in endolysosome, but can also erroneously respond to self-derived RNA. In fact, it has been reported that TLR 7-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

Ryutaro Fukui[2], Yusuke Murakami[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, TOKYO, 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, TOKYO, Japan.

We are trying functional cloning using CRISPR/CAS9 based lentiviral Knock-Out Library to comprehensively identify regulatory molecules associating with TLR responses. After 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 ROSA 26 locus to reveal the physiological function of novel TLR associating molecules *in vivo*.

Publications

Zhang Z, Ohto U, Shibata T, Krayukhina E, Taoka M, Yamauchi Y, Tanji H, Isobe T, Uchiyama S, Miyake K, and Shimizu T "Structural analysis reveals that Toll-like receptor 7 is a dual receptor for guanosine and single-stranded RNA" Immunity. 45(4): 737-748, 2016

Miyake K, Shibata T, Ohto U, Shimizu T "Emerging roles of the processing of nucleic acids and Toll-like receptors in innate immune responses to nucleic acids. " J Leukoc Biol. 101(1): 135-142, 2017

Maekawa S, Ohto U, Shibata T, Miyake K, Shimizu T "Crystal structure of NOD2 and its implications in human disease" Nat. Commun. 7, 11813, 2016

Tanji H, Ohto U, Motoi Y, Shibata T, Miyake K, Shimizu T. "Autoinhibition and relief mechanism by the proteolytic processing of Toll-like receptor 8" Proc Natl Acad Sci U S A. 113(11), 3012-3017, 2016

Pelka K, Shibata T, Miyake K, Latz E. "Nucleic acid-sensing TLRs and autoimmunity: novel insights from structural and cell biology." Immunol Rev. 269(1): 60-75, 2016

Shibata T, Ohto U, Nomura S, Kibata K, Motoi Y, Zhang Y, Murakami Y, Fukui R, Ishimoto T, Sano S, Ito T, Shimizu T, and Miyake K "Guanosine and its modified derivatives are endogenous ligands for TLR7" International Immunology. 28 (5), 211-22, 2016