Center for Experimental Medicine and Systems Biology

Laboratory of Developmental Genetics
発生工学研究分野

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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. Role of lysine acetyltransferases CBP and p300 in tumorigenesis

Taeko Ichise, Nobuaki Yoshida and Hirotake Ichise

CREB binding protein (CBP) and p300 are highly related lysine acetyltransferases that contribute to transcriptional regulation. These proteins co-localize with transcription factors on transcriptional regulatory regions of genes, change protein-DNA interaction, and activate the transcriptional machinery. Inhibiting CBP/p300 is a potential anti-cancer therapy because it co-activates pro-oncogenic transcription factors that accelerate cell proliferation during tumorigenesis. By contrast, acetyltransferase-inactivating mutations of CBP and p300 are common in human lymphomas and epithelial tumors, suggesting that CBP/p300 may have a tumor suppressor activity in particular types of cell. To find out whether or how loss-of-function mutations in CBP/p300 contribute to tumorigenesis, we generated genetically engineered mouse models and found that reduced CBP/p300 expression accelerates an oncogene-induced hyperplastic phenotype and promotes tumor formation *in vivo*. We also studied the molecular mechanism of CBP/p300 expression level-dependent regulation of cell proliferation.

2. The RNA binding protein Ptbp1 is essential for humoral immune response

Hiroki Sasanuma and Nobuaki Yoshida

The RNA binding protein Ptbp1 binds to the pyrimidine-rich sequence of the target RNA and controls gene expression via post-transcriptional regulation such as alternative splicing. Recently, we found that Ptbp1 is highly expressed in B lymphocytes. To clarify its role in B cell development and function, we have generated B cell-specific Ptbp1 deficient (P1BKO) mice. B cell development in the bone marrow, spleen and peritoneal cavity in P1BKO mice was almost normal, and no significant changes was observed in the number of B cells as compared with the control mice. These data indicate that Ptbp1 is dispensable for B cell development. However, P1BKO mice had significantly lower levels of all Ig isotypes in the serum compared to control mice. To investigate the impact of Ptbp1 deficiency during immune response *in vivo*, we immunized P1BKO mice with T cell-independent (TI) antigen NP-Ficoll and T cell-dependent (TD) antigen NP-CGG. As a result, we found that B cell-specific Ptbp1 deficiency results in severe defect in antigen-specific antibody production both TI- and TD- immune responses. In other words, B cell-specific Ptbp1 deficiency causes immunodeficiency due to defect in antibody production. This immunodeficiency was accompanied by impaired antigen-specific B cell proliferation, plasma cell generation and germinal center formation. These results demonstrate that Ptbp1 is essential for humoral immune response. Additional work is needed to elucidate the more detailed mechanism of immunodeficiency caused by B cell-specific Ptbp1 deficiency.

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.

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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], Ryutaro Fukui[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. 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

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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.

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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*.

Publications

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Center for Experimental Medicine and Systems Biology

Laboratory of Reproductive Systems Biology
生殖システム研究分野

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In the "post-genome project era," genetically modified animals play a key role in basic molecular biological investigations and act as models of human disease. Our laboratory studies the mechanisms underlying the mammalian reproductive system in gene-manipulated mice. We are the first group in the world to generate transgenic mice expressing GFP throughout the body (Green mice). We also established the ES cells that give green fluorescent spermatozoa to trace their movement and acrosome reaction during fertilization. Another tool invented in our laboratory is the placenta-specific gene manipulation system using lentiviral (LV) vectors. Using these techniques, we are trying to elucidate the mechanism underlying gametogenesis, fertilization, implantation, and placentation. Our recent interest is using the CRISPR/Cas9 system as a genome-editing tool. The combination of GWAS studies with genome editing will pave the way to understand and control human fertility problems.

1. TCTE1 is a conserved component of the dynein regulatory complex and is required for motility and metabolism in mouse spermatozoa.

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Flagella and cilia are critical cellular organelles that provide a means for cells to sense and progress through their environment. The central component of flagella and cilia is the axoneme, which comprises the "9 + 2" microtubule arrangement, dynein arms, radial spokes, and the nexin-dynein regulatory complex (*N*-DRC). Failure to properly assemble components of the axoneme leads to defective flagella and in humans leads to a collection of diseases referred to as ciliopathies. Ciliopathies can manifest as severe syndromic diseases that affect lung and kidney function, central nervous system development, bone formation, visceral organ or-

ganization, and reproduction. T-Complex-Associated-Testis-Expressed 1 (TCTE1) is an evolutionarily conserved axonemal protein present from *Chlamydomonas* (DRC5) to mammals that localizes to the *N*-DRC. Here, we show that mouse TCTE1 is testis-enriched in its expression, with its mRNA appearing in early round spermatids and protein localized to the flagellum. TCTE1 is 498 aa in length with a leucine rich repeat domain at the C terminus and is present in eukaryotes containing a flagellum. Knockout of *Tcte1* results in male sterility because *Tcte1*-null spermatozoa show aberrant motility. Although the axoneme is structurally normal in *Tcte1* mutant spermatozoa, *Tcte1*-null sperm demonstrate a significant decrease of ATP, which is used by dynein motors to generate the bending force of the flagellum. These data provide a link to defining the molecular intricacies required for axoneme function, sperm motility, and male fertility.

2. Human Globozoospermia-Related Gene Spata 16 Is Required for Sperm Formation Revealed by CRISPR/Cas9-Mediated Mouse Models.

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A recent genetic analysis of infertile globozoospermic patients identified causative mutations in three genes: a protein interacting with C kinase 1 (*PICK1*), dpy 19-like 2 (*DPY19L2*), and spermatogenesis associated 16 (*SPATA16*). Although mouse models have clarified the physiological functions of *Pick1* and *Dpy19l2* during spermatogenesis, *Spata16* remains to be determined. Globozoospermic patients carried a homozygous point mutation in *SPATA16* at 848G→A/R283Q. We generated CRISPR/Cas9-mediated mutant mice with the same amino acid substitution in the fourth exon of *Spata16* to analyze the mutation site at R284Q, which corresponded with R283Q of mutated human SPATA16. We found that the point mutation in *Spata16* was not essential for male fertility; however, deletion of the fourth exon of *Spata16* resulted in infertile male mice due to spermiogenic arrest but not globozoospermia. This study demonstrates that *Spata16* is indispensable for male fertility in mice, as well as in humans, as revealed by CRISPR/Cas9-mediated mouse models.

3. WNT regulation of embryonic development likely involves pathways independent of nuclear CTNNB1.

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The bovine was used to examine the potential for WNT signaling to affect the preimplantation embryo. Expression of seven key genes involved in canonical WNT signaling declined to a nadir at the morula or blastocyst stage. Expression of 80 genes associated with WNT signaling in the morula and inner cell mass (ICM) and trophectoderm (TE) of the blastocyst was also evaluated. Many genes associated with WNT signaling were characterized by low transcript abundance. Seven genes were different between ICM and TE, and all of them were overexpressed in TE as compared to ICM, including WNT6, FZD1, FZD7, LRP6, PORCN, APC and SFRP1 Immunoreactive CTNNB1 was localized primarily to the plasma membrane at all stages examined from the 2-cell to blastocyst stages of development. Strikingly, neither CTNNB1 nor non-phospho (i.e., active) CTNNB1 was observed in the nucleus of blastomeres at any stage of development even after the addition of WNT activators to culture. In contrast, CTNNB1 associated with the plasma membrane was increased by activators of WNT signaling. The planar cell polarity pathway (PCP) could be activated in the embryo as indicated by an experiment demonstrating an increase in phospho-JNK in the nucleus of blastocysts treated with the non-canonical WNT11. Furthermore, WNT11 improved development to the blastocyst stage. In conclusion, canonical WNT signaling is attenuated in the preimplantation bovine embryo but WNT can activate the PCP component JNK. Thus, regulation of embryonic development by WNT is likely to involve activation of pathways independent of nuclear actions of CTNNB1.

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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

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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.

Role of interleukin-25 in development of spontaneous arthritis in interleukin-1 receptor antagonist-deficient mice

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Interleukin (IL)-25, which is a member of the IL-17 family of cytokines, induces production of such Th2 cytokines as IL-4, IL-5, IL-9 and/or IL-13 by various types of cells, including Th2 cells, Th9 cells and group 2 innate lymphoid cells (ILC2). On the other hand, IL-25 can suppress Th1- and Th17-associated immune responses by enhancing Th2-type immune responses. Supporting this, IL-25 is known to suppress development of experimental autoimmune encephalitis, which is an IL-17-mediated autoimmune disease in mice. However, the role of IL-25 in development of IL-17-mediated arthritis is not fully understood. Therefore, we investigated this using IL-1 receptor antagonist-deficient (IL-1Ra$^{-/-}$) mice, which spontaneously develop IL-17-dependent arthritis. However, development of spontaneous arthritis (incidence rate, disease severity, proliferation of synovial cells, infiltration of PMNs, and bone erosion in joints) and differentiation of Th17 cells in draining lymph nodes in IL-25$^{-/-}$ IL-1Ra$^{-/-}$ mice were similar to in control IL-25$^{+/+}$ IL-1Ra$^{-/-}$ mice. These observations indicate that IL-25 does not exert any inhibitory and/or pathogenic effect on development of IL-17-mediated spontaneous arthritis in IL-1Ra$^{-/-}$ mice.

IL-25 enhances Th17 cell-mediated contact dermatitis by promoting IL-1β production by dermal dendritic cells

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As well as thymic stromal lymphopoietin (TSLP) and interleukin-33 (IL-33), IL-25 is known to induce Th2 cytokine production by various cell types—including Th2 cells, Th9 cells, invariant NKT cells and group 2 innate lymphoid cells—involved in Th2-type immune responses. Since both Th2-type and Th17-type cells/cytokines are crucial for contact hypersensitivity (CHS), IL-25 may contribute to this by enhancing Th2-type immune responses. However, the precise role of IL-25 in the pathogenesis of CHS is poorly understood. In contrast to TSLP, we found that IL-25 was not essential for skin DC migration or hapten-specific Th cell differentiation in the sensitization phase of CHS. Unexpectedly, mast cell- and non-immune cell-derived IL-25 was important for hapten-specific Th17 cell-, rather than Th2 cell-, mediated inflammation in the elicitation phase of CHS by enhancing Th17-related, but not Th2-related, cytokines in the skin. In particular, IL-1β produced by dermal dendritic cells in response to IL-25 was crucial for hapten-specific Th17 cell activation, contributing to induction of local inflammation in the elicitation phase of CHS. Our results identify a novel IL-25 inflammatory pathway involved in induction of Th17, but not Th2, cell-mediated CHS.

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