Center for Experimental Medicine and Systems Biology

Laboratory of Molecular Pathogenesis
システム疾患モデル研究センター分子病態研究分野

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Recent development of transgenic techniques has made it possible to directly analyze the functions of a particular gene in a living animal. These techniques have also made it possible to produce various animal disease models as well as tools to analyze them. Immune disorders and infectious diseases are our major concerns, and we are attempting to produce transgenic mouse models for these diseases.

1. Studies on rheumatoid arthritis models

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Rheumatoid arthritis (RA) is one of the most serious medical problems worldwide with approximately 1% of the people in the world affected. The disease is autoimmune in nature and characterized by chronic inflammation of the synovial tissues in multiple joints that leads to joint destruction. High levels of inflammatory cytokine expression in the joints are a characteristic of the disease, although the pathogenesis has not been elucidated completely. We have been studying the pathogenesis of the disease using two arthritis models that we originally developed. One is HTLV-I transgenic mice (Iwakura et al., *Science*, 1991) and the other is IL-1 receptor antagonist-deficient mice (Horai et al., *J. Exp. Med*., 2000). Both of these models develop autoimmunity and chronic inflammatory arthropathy closely resembling RA in humans.

To identify genes involved in the pathogenesis of arthritis, we analyzed the gene expression profiles of these animal models by using high-density oligonucleotide arrays, and identified genes in the RA-related gene locus which are overexpressed in the affected joints (Fujikado et al., *Arthritis Res. Ther*., 2006). Furthermore, we have also searched for pathogenesis-related genes by analyzing the effect of genetic backgrounds on arthritis development. BALB/cA strain mice were highly susceptible to develop arthritis in these models, whereas C57BL/6J strain mice were resistant. Since genetic factors have been suggested to be involved in the development of arthritis, we performed linkage-analysis using BALB/c-HTLV-I transgenic mouse backcross progenies into C57BL/6J, and identified several RA-related gene loci that affected the strain specificity of arthritis susceptibility. We are now analyzing the roles of these genes in the pathogenesis of arthritis and autoimmunity by generating the knockout mice of these genes.

Among these genes, we are now analyzing

the functions of two cytokine receptors and four novel genes, "*Tora*" and "*Kusa*", which encode cytoplasmic proteins, and "*Shimi*", which encodes transmenbrane protein, and "*Mura1*", which encodes secreted protein. Because the physiological roles of these genes have not been reported, we generated gene-targeted mice for these genes. Both *Tora*$^{-/-}$, *Kusa*$^{-/-}$, *Shimi*$^{-/-}$ and *Mura1*$^{-/-}$ mice were fertile, were born at the expected mendelian ratios, and showed no obvious phenotypic abnormalities. However, we found that *Mura1*$^{-/-}$ mice were highly susceptible to experimental induction of arthritis by immunizing with type 2 collagen. We are now analyzing the function of these genes.

2. Studies on CXCR4 in the development of arthritis

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Chemokines and their receptors are potential therapeutic targets in RA. Among these, several studies suggested the involvement of CXC chemokine 4 (CXCR4) and its ligand CXC ligand 12 (SDF-1) in RA pathogenesis. However, the role of these molecules in T-cell function is not known completely because of embryonic lethality of *Cxcr4*- and *Cxcl12*-deficient mice. To investigate the roles of CXCR4 in the development of arthritis, we generated T cell-specific *Cxcr4*-deficient mice using the Cre-loxP system.

The incidence, but not the severity, of CIA was significantly reduced in *Cxcr4*$^{flox/flox}$*/Lck-Cre* mice compared with *Cxcr4*$^{+/+}$*/Lck-Cre* mice. We found that the expression of CXCR4 was enhanced in activated T cells, and the migration of *Cxcr4*-deficient T cells toward SDF-1 was severely impaired. However, antibody production, cellular proliferative response, and cytokine production on treatment with type II collagen (IIC) were normal in these knockout mice, suggesting that CXCR4 is not involved in T-helper functions. Interestingly, the proportion of CXCR4-expressing T cells was much increased in affected joints compared with that in draining lymph nodes in CIA-induced mice, and distribution of *Cxcr4*$^{flox/flox}$*/Lck-Cre* mouse-derived T cells into affected joints was suppressed compared with that in *Cxcr4*$^{+/+}$*/Lck-Cre* T cells.

These results indicate that CXCR4 expression in T cells is important for the development of CIA, by recruiting activated T cells toward inflammatory sites, and suggest that CXCR4 is a good target for the treatment of RA in humans.

3. TNF, but not IL-6 and IL-17, is crucial for the development of T cell-independent psoriasis-like dermatitis in *Il1rn*$^{-/-}$ Mice

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IL-1 is a proinflammatory cytokine, consisting of two molecular species, IL-1α and IL-1β, and IL-1 receptor antagonist (IL-1Ra; gene symbol: *Il1rn*) is the endogenous suppressor. *Il1rn*$^{-/-}$ mice spontaneously develop autoimmune diseases, such as arthritis and aortitis, and a dermatitis that histologically resembles human psoriasis. The pathogenic mechanisms underlying this dermatitis, however, remain to be elucidated. Here, we demonstrated that the production of inflammatory cytokines and chemokines was enhanced at the site of inflammation. The development of dermatitis was completely suppressed in *Tnfsf1a*$^{-/-}$, but not in *Il6*$^{-/-}$ mice, similarly as that observed in arthritis and aortitis. However, IL-17 deficiency did not affect the development of dermatitis at all, making clear contrast to that of arthritis and aortitis. Different from arthritis and aortitis, adoptive transfer of *Il1rn*$^{-/-}$ T cells did not induce dermatitis in the recipient SCID mice and skin lesions developed in *Il1rn*$^{-/-}$ SCID mice, indicating that T cells are not involved in the development of skin lesions. In support for this, bone marrow cell (BMC) transplantation experiments showed that TNF produced by skin residential cells, but not BMC-derived cells, was important for the development of dermatitis. Furthermore, we showed that IL-1 directly enhanced TNF and chemokine expression in keratinocytes. These observations suggest that excess IL-1 signaling directly activates keratinocytes to produce TNF and chemokines, resulting in the development of psoriasis-like skin lesions without the involvement of autoimmunity in *Il1rn*$^{-/-}$ mice.

4. IL-1 plays an important role in the bone metabolism under physiological conditions

Young-Mi Lee, Noriyuki Fujikado, Hiroko Manaka, Hisataka Yasuda, and Yoichiro Iwakura

It is well known that IL-1 is involved in bone resorption under pathological conditions. The role of this cytokine in bone remodeling under physiological conditions, however, remains obscure. In this study, we addressed the role of IL-1 in physiological bone metabolism through analyses of IL-1α-deficient (KO), IL-1β KO, and IL-1α/β double KO mice which were housed under SPF conditions. The femur mineral density, trabecular bone mass and cortical thickness significantly increased in all KO mice compared with wild type (WT) mice. The number of osteoclasts in trabecular bones decreased, suggesting that IL-1 regulates bone metabolism through regulation of osteoclast formation. When differentiation of bone marrow (BM) cells into osteoclasts was induced by parathyroid hormone (PTH) in cocultures of osteoblasts and BM cells from WT and IL-1α/β KO mice, IL-1α/β KO BM cell cocultures failed to undergo efficient osteoclast-like multinucleated cell (OCL) differentiation, although high levels of receptor activator of NF-κB ligand (RANKL) was induced. In contrast, efficient OCL differentiation was observed in IL-1α/β KO osteoblast/WT BM cell cocultures, in which high levels of IL-1α/β and low levels of RANKL were produced. Addition of IL-1α to IL-1α/β KO BM-derived macrophage cultures markedly enhanced OCL differentiation induced by soluble RANKL, and the downstream molecules of RANK including JNK, ERK and c-Fos were less activated in the absence of IL-1 upon treatment with RANKL. Taken together, these results indicate that IL-1 directly activates RANK signaling other than inducing RANKL to promote osteoclastogenesis and plays an important role in physiological bone metabolism.

5. DCIR as an inhibitory regulator in the immune system and bone metabolism

Tomonori Kaifu, Rikio Yabe, Guangyu Ma, Takumi Maruhashi, Akimasa Seno, and Yoiciro Iwakura

Stress responses against environmental stimulations have to eventually fade out. Persistent immune activation results in undesirable tissue damage and causes irreversible destruction; therefore, negative regulatory mechanisms in the immune system is critically important for maintaining the homeostasis of a living body. Dendritic cell immunoreceptor (DCIR) is a member of C-type lectin receptors, which is highly expressed in DCs. Interestingly, DCIR contains immunoreceptor tyrosine-based inhibitory motif in the cytoplasmic region, suggesting negative regulatory roles of this molecule in the immune system. Indeed, a loss-of-function mutation of DCIR results in spontaneous development of autoimmune-like diseases, such as enthesitis and sialadentis. We also found that DCIR-deficient mice developed exacerbated experimental allergic encephalomyelitis, a mouse model for multiple sclerosis, due to an increased DC infiltration in the spinal cord. DCIR-deficient mice also developed ankylosing spondylitis, a disease associated with cartilaginous proliferation in joints. We are now analyzing possible involvement of immune responses in the pathogenesis. Given that DCIR deficient mice are prone to autoimmune diseases and bone-related disorders, DCIR may be a potential therapeutic target for these diseases. We are now trying to develop possible ways to control DCIR activity.

6. Studies on C-type lectins in the host defense against fungal infection

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The C-type lectin is one of pattern recognition receptors (PRRs) with a lectin-like carbohydrate recognition domain in their extracellular carboxyterminal domains. They bind sugar chains in a calcium dependent manner. Some C-type lectins have endogenous ligands, while other C-type lectins bind pathogen-associated molecular patterns. Some of them recognize both as their lignads. The cytoplasmic tails of the C-type lectins are diverse and contain several conserved motifs that are important for antigen uptake or potential signaling. Although the C-type lectins

in NK cells are relatively well studied, the roles of these molecules in DCs or macrophages remain to be elucidated. Dectin-1 has an ITAM which is well known for transducing activation signals in T cells and B cells, but it is not known if ITAMs in DCs are also functional. Thus, we have generated Dectin-1 and Dectin-2 deficient mice to study the roles of these lectins in DCs.

We have shown that Dectin-1 is a specific receptor for β-glucans, one of the fungal cell wall components, to produce cytokines. On the other hand, Dectin-2 recognizes α-mannans, another fungal cell wall component, and Dectin-2-deficient DCs had virtually no fungal α-mannan-induced cytokine production. Dectin-2 signaling induced cytokines through a FcRγ and Syk-CARD9-NF-κB-dependent pathway without involvement of MAPKs. *In vivo*, Dectin-1 deficient mice were more susceptible to *Pneumocystis carinii* infection, but not *Candida albicans* infection, compared to wild-type mice. On the other hand, Dectin-2 deficient mice were more susceptible to candida infection. Interestingly, we found that the cytokine production including IL-1β and IL-23 induced by yeast form *C. albicans* was completely abosished by the mutation of Dectin-2, but that by hyphae depended only partially. Both yeasts and hyphae preferentially induced Th17 differentiation, in which Dectin-2, but not Dectin-1, was mainly involved. Because IL-17A-, but not IL-17F-, deficient mice were highly susceptible to systemic candida infection, it was suggested that Dectin-2 plays an important role in host defense against *C. albicans* by inducing Th17 differentiation. Thus, Dectin-1 and Dectin-2 play important roles in DCs as a PRR to activate innate immune responses.

7. Roles of IL-17A and IL-17F in host defense against bacteria, inflammatory colitis, and intestinal polyp formation

Ce Tang, Shigeru Kakuta, Motohiko Kadoki, Tomonori Kamiya, Yamato Sasaki, Liu Yang, and Yoichiro Iwakura

IL-17A is a cytokine produced by Th17 cells and plays important roles in the development of allergic and autoimmune diseases. IL-17A also plays important roles in host defense against bacteria and fungi. We showed that IL-17F, the highest homologous member to IL-17A in the IL-17 family, plays only marginal roles in the development of allergic or autoimmune diseases, but is equally important with IL-17A in the host defense against mucoepithelial bacteria, such as *Staphylococcus* and *Citrobacter*. Although several lines of evidence suggest that IL-17A is not involved in the host defense against extracelluar bacteria such as *Helicobacterium* or intracellular bacteria such as *Mycobacterium*, which induce robust Th1-, but not Th17-, type immune response, the roles of IL-17F in the host defense against these bacteria remain to be elucidated. Thus, we are now analyzing the roles of IL-17F in *Helicobacterium* and *Mycobacteium* infection models. We are also analyzing the role of IL-17F in $CD45RB^{high}CD4$ T cell-induced colitis model.

8. Generation of AIDS disease models and analysis of the pathogenesis using animal models

Motohiko Kadoki, Byung-Il Choi, Takuya Tada, and Yoichiro Iwakura

In the course of the development of AIDS, bacterial infection causes deleterious effects on the progression of the disease; bacterial lipopolysaccharides (LPS) in the circulation activate immune cells, resulting in the acceleration of HIV replication. However, the precise HIV activation mechanisms in infected hosts remain largely unknown. Previously, we generated transgenic mice carrying the HIV-1 genome and showed that LPS induces the activation of HIV-1 in splenocytes through the induction of TNF and IL-1, although similarly induced IFN-γ and IL-6 are not involved. In this study, we analyzed the mechanisms of HIV-1 activation in macrophages using these HIV-1 transgenic mice, because macrophages are one of the major reservoirs of HIV-1. In contrast to splenocytes, direct TLR4 signaling rather than TLR-induced proinflammatory cytokines was responsible for the LPS-induced activation of HIV-1 in macrophages, because the time course of HIV-1 activation was earlier than that observed in splenocytes and TNF neutralization did not inhibit the activation. p38 MAPK and NF-κB activation, but neither ERK nor JNK activation, were required for the activation, because only inhibitors for p38 MAPK and NF-κB suppressed activation of HIV-1. Furthermore, we showed that MyD88, rather than TRIF, was required as an adaptor molecule for this activation using $MyD88^{-/-}$ mice and Dynasore, a specific inhibitor for TRIF, and siRNAs specific for *MyD88* and *Trif*. These observations suggest that suppression of these molecules, which are involved in the TLR4-MyD 88 pathway and the downstream p38 MAPK and NF-κB pathways, should be beneficial to prevent development of AIDS in HIV-1 infected people.

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Center for Experimental Medicine and Systems Biology

Laboratory of Developmental Genetics
システム疾患モデル研究センター　発生工学研究分野

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Gene targeting technology has revealed many aspects of gene functions in vivo. Knock out mice offer the opportunities of not only analyzing the complex gene functions in vivo, but also presenting various human disease models, where new therapeutic approaches can be explored. To allow more detailed dissection of gene function, we introduce a point mutation or disrupt genes in certain lineages (or stages) using Cre-loxP system, a method of conditional gene targeting. In the process of analyzing knock out mice, we have isolated spontaneous mutant mice which develop chylous ascites and edematous limbs. In order to understand the mechanism of lymphatic development and functions in more detail, we are also generating various knock-out/knock-in mouse lines including a conditional knock out mouse. In addition, we focus on analysis of neural development, aiming to understand the molecular mechanism of the maintenance of stemness and neural differentiation and to advance towards cell therapy of the damaged or degenerating nervous system. For this purpose, we are generating several conditional knock out mouse lines.

1. Fbxl10/Kdm2b deficiency accelerates neural progenitor cell death and leads to exencephaly.

Tsuyoshi Fukuda, Akinori Tokunaga, Reiko Sakamoto and Nobuaki Yoshida.

Histone methylation is the important transcription regulatory system that affects mammalian development and cell differentiation. Alterations in epigenetic gene regulation are associated with disease. Fbxl10 (F-box and leucine-rich repeat protein 10) is a JmjC domain-containing histone demethylase. Although Fbxl 10 has been implicated in cell cycle regulation, cell death, senescence, and tumorigenesis, these functions are controversial and its physiological function is unclear. To determine the in vivo function of Fbxl10, in this study, we generated a homozygous mutation in the mouse Fbxl10 gene. About half of Fbxl10-deficient mice exhibit failure of neural tube closure, resulting in exencephaly and die shortly after birth. Fbxl10 deficiency also causes retinal coloboma and a curled tail with low penetrances. Fbxl10 mRNA is specifically expressed in the cranial neural folds at E8.5 embryos, and apoptosis increased in the neuroepithelium and mesenchyme of Fbxl10-deficient E9.5 embryos, consistent with neural tube defects found in Fbxl10-deficient mice. Depletion of Fbxl10 induced the increased expression of p19ARF, an inducer of apoptosis, in E8.5 embryos and mouse embryonic fibroblast cells. In addition, the number of mitotic neural progenitor cells is significantly increased in the mutant E14.5 brain. Our findings suggest that the

Fbxl10 gene makes important contributions to embryonic neural development by regulating cell proliferation and cell death in mice.

2. Nucleoredoxin sustains Wnt/β-catenin signaling by retaining a pool of inactive dishevelled protein.

Yosuke Funato[1], Takeshi Terabayashi[1], Reiko Sakamoto, Daisuke Okuzaki[2], Hirotake Ichise, Hiroshi Nojima[2], Nobuaki Yoshida and Hiroaki Miki[1]: [1]Laboratory of Intracellular Signaling, Institute for Protein Research, Osaka University. [2]Department of Molecular Genetics, Research Institute for Microbial Diseases, Osaka University.

Overexpression of Dishevelled (Dvl), an essential component of the Wnt signaling pathway, is frequently associated with tumors, and thus the Dvl protein level must be tightly controlled to sustain Wnt signaling without causing tumors. Kelch-like 12 (KLHL12) targets Dvl for ubiquitination and degradation, suggesting its potential importance in avoiding aberrant Dvl overexpression. However, the regulatory mechanism of the KLHL12 activity remained elusive. We show here that nucleoredoxin (NRX) determines the Dvl protein level, which is revealed by analyses on NRX(−/−) mice showing skeletal and cardiovascular defects. Consistent with the previously reported Dvl-inhibiting function of NRX, Wnt/β-catenin signaling is hyperactivated in NRX(−/−) osteoblasts. However, the signal activity is suppressed in cardiac cells, where KLHL12 is highly expressed. Biochemical analyses reveal that Dvl is rapidly degraded by accelerated ubiquitination in NRX(−/−) mouse embryonic fibroblasts, and they fail to activate Wnt/β-catenin signaling in response to Wnt ligands. Moreover, experiments utilizing purified proteins show that NRX expels KLHL12 from Dvl and inhibits ubiquitination. These findings reveal an unexpected function of NRX, retaining a pool of inactive Dvl for robust activation of Wnt/β-catenin signaling upon Wnt stimulation.

3. Nucleoredoxin Negatively Regulates Toll-like Receptor 4 Signaling via Recruitment of Flightless-I to Myeloid Differentiation Primary Response Gene (88)*

Tatsuya Hayashi[3],Yosuke Funato[3] ,Takeshi Terabayashi[3], Akifumi Morinaka[3], Reiko Sakamoto, Hirotake Ichise, Hiroyuki Fukuda[4], Nobuaki Yoshida and Hiroaki Miki[3].: [3]Institute for Protein Research, Osaka University. [4]THERAVALUES CORPORATION.

We previously characterized nucleoredoxin (NRX) as a negative regulator of the Wnt signaling pathway through Dishevelled (Dvl). We perform a comprehensive search for other NRX-interacting proteins and identify Flightless-I (Fli-I) as a novel NRX-binding partner. Fli-I binds to NRX and other related proteins, such as Rod-derived cone viability factor (RdCVF), whereas Dvl binds only to NRX. Endogenous NRX and Fli-I *in vivo* interactions are confirmed. Both NRX and RdCVF link Fli-I with myeloid differentiation primary response gene (88) (MyD88), an important adaptor protein for innate immune response. NRX and RdCVF also potentiate the negative effect of Fli-I upon lipopolysaccharide-induced activation of NF-κB through the Toll-like receptor 4/MyD88 pathway. Embryonic fibroblasts derived from *NRX* gene-targeted mice show aberrant NF-κB activation upon lipopolysaccharide stimulation. These results suggest that the NRX subfamily of proteins forms a link between MyD88 and Fli-I to mediate negative regulation of the Toll-like receptor 4/MyD88 pathway.

4. H-, N- and Kras cooperatively regulate lymphatic vessel growth by modulating VEGFR3 expression in lymphatic endothelial cells in mice.

Taeko Ichise, Nobuaki Yoshida and Hirotake Ichise.

Mammalian Ras, which is encoded by three independent genes, has been thought to be a versatile component of intracellular signalling. However, when, where and how Ras signalling plays essential roles in development and whether the three Ras genes have overlapping functions in particular cells remain unclear. Here, we show that the three Ras proteins dose-dependently regulate lymphatic vessel growth in mice. We find that lymphatic vessel hypoplasia is a common phenotype in Ras compound knockout mice and that overexpressed normal Ras in an endothelial cell lineage selectively causes lymphatic vessel hyperplasia in vivo. Overexpression of normal Ras in lymphatic endothelial cells leads to sustained MAPK activation, cellular viability and enhanced endothelial network formation under serum-depleted culture conditions in vitro, and knockdown of endogenous Ras in lymphatic endothelial cells impairs cell proliferation, MAPK activation, cell migration and endothelial network formation. Ras overexpression and knockdown result in up- and downregulation of vascular endothelial growth factor receptor (VEGFR) 3 expression, respectively, in lymphatic endothelial cells in vi-

tro. The close link between Ras and VEGFR3 in vitro is consistent with the result that Ras knockout and transgenic alleles are genetic modifiers in lymphatic vessel hypoplasia caused by Vegfr3 haploinsufficiency. Our findings demonstrate a cooperative function of the three Ras proteins in normal development, and also provide a novel aspect of VEGFR3 signalling modulated by Ras in lymphangiogenesis.

5. An FGF4-FRS2α-Cdx2 Axis in Trophoblast Stem Cells Induces Bmp4 to Regulate Proper Growth of Early Mouse Embryos.

Michiko Murohashi[5,6], Takahisa Nakamura[7], Satoshi Tanaka[8], Taeko Ichise, Nobuaki Yoshida, Tadashi Yamamoto[7], Masabumi Shibuya[5], Joseph Schlessinger[8] and Noriko Gotoh[5,6]: [5]Division of Genetics, University of Tokyo. [6]Division of Systems Biomedical Technology, University of Tokyo. [7]Division of Oncology, University of Tokyo. [8]Department of Pharmacology, Yale University School of Medicine.

A variety of stem cells are controlled by the actions of multiple growth factors in vitro. However, it remains largely unclear how growth factors control the proliferation and differentiation of stem cells in vivo. Here, we describe a novel paracrine mechanism for regulating a stem cell niche in early mammalian embryos, which involves communication between the inner cell mass (ICM) and the trophectoderm, from which embryonic stem (ES) cells and trophoblast stem (TS) cells can be derived, respectively. It is known that ES cells produce fibroblast growth factor (FGF) 4 and that TS cells produce bone morphogenetic protein (Bmp) 4. We provide evidence that FRS2α mediates activation of the extracellular signal-regulated progein kinase (ERK) pathway to enhance expression of transcription factor Cdx2 in TS cells in response to FGF4. Cdx 2 in turn binds to an FGF4-responsive enhancer element of the promoter region of *Bmp4*, leading to production and secretion of Bmp4. Moreover, exogenous Bmp4 is able to rescue the defective growth of *Frs2*α-null ICM. These findings suggest an important role of Cdx2 for production of Bmp4 in TS cells to promote the proper growth of early mouse embryos.

6. Analysis of the role of Polypyrimidine tract-binding protein, a RNA binging protein in embryonic ES cells and neural development

Akinori Tokunaga, Satona Ohno, Takayuki Shibasaki, Shinya Masaki, Reiko Sakamoto, Nobuaki Yoshida

(1) Role of PTB in cell cycle regulation.

Polypyrimidine tract-binding protein (PTB) is a widely expressed RNA binding protein (RBP) with multiple roles in RNA processing, including the splicing of alternative exons, mRNA stability, mRNA localization, and internal ribosome entry site-dependent translation of RNA. Although it has been reported that increased expression of PTB is correlated with cancer cell growth, the role of PTB in mammalian development is still unclear.

We have shown that a homozygous mutation in the mouse Ptb gene causes embryonic lethality shortly after implantation. We also established Ptb(−/−) embryonic stem (ES) cell lines and found that these mutant cells exhibited severe defects in cell proliferation without aberrant differentiation in vitro or in vivo. To reveal a cause of proliferation defect in $ptb^{-/-}$ ES cells, we analyzed the cell cycle progression of $ptb^{-/-}$ ES cells by flow cytometry. Cell cycle analysis and a cell synchronization assay revealed that Ptb(−/−) ES cells have a prolonged G (2)/M phase. Thus, our data indicate that PTB is essential for early mouse development and ES cell proliferation.

For more detailed analysis, we focused on the regulation of IRES-dependent translation, which is one of the functions of PTB involved in M phase regulation. We revealed that the IRES activity of CDK11^{p58}, which is one of the M phase regulators, is higher in $ptb^{-/-}$ ES cell than that in $ptb^{+/+}$ ES cells. Furthermore, we found that PTB binds to CDK11^{p58} IRES directly and represses the IRES-dependent translation of CDK 11^{p58} in ES cells. These results suggest the importance of PTB in the progression and termination of M phase through the regulation of IRES-dependent translation and the regulation of IRES-dependent translation in ES cells is different from that in differentiated cells. Our finding would contribute to further understanding of the cell cycle regulation in ES cells.

(2) Analysis of PTB conditional knockout mouse.

PTB preferentially expressed in neural stem cells (NSCs) in the central nervous system. Although RBPs are indispensable for the normal functions and cell migration of neurons, little is known about the role of RBPs in neural stem cells (NSCs). In vitro functional analysis of PTB in vertebrate neural cell have revealed that PTB has important roles on many alternative splicing events during neural cell differentiation. But it is still unknown whether self-renew and/or cell fate of NSCs are regulated by PTB in vivo. To explore the role of PTB in the early develop-

ment of mouse brain, we inactivated the gene by employing a Nestin promoter-driven Cre-mediated conditional gene targeting system. We found that most mutant mice die by 10 weeks and almost all mutant mice developed a characteristic dome-like appearance of their heads. Histological analyses of PTB mutant brains revealed that these mice develop severe hydrocephalus. The cell polarity and adherens junction (AJ) of the apical ventricular surface in dorsal cortex were lost in a pacthy distribuion at E 15.5. By E16.5, Tbr2-positive neural progenitor cells and neurons were observed in ventricular zone (VZ) of all AJ-negative spots, which leads depletion of VZ by E18.5. Thus postnatal maturation of ependymal cells with ciliary tufts from radial glia cells (RGCs) was disturbed, which may compromises cerebrospinal fluid dynamics and results in hydrocephalus. Our findings suggest that PTB is important for self-renew and/or cell fate of RGCs, through involving in maintenance of cell polarity and AJ in the dorsal neuroepithelium of lateral ventricles.

(3) Analysis of nPTB conditional knockout mouse.

Neural polypyrimidine-tract-binding protein (nPTB), which is identified as a homologue of PTB, is one of RNA binding protein involved in the heterogeneous nuclear ribonucleoprotein (hnRNP) family. It is common knowledge that many genes are regulated by alternative splicing during neural development, and previous studies suggested that alternative splicing could contribute to the gene expression and functional diversity of isoforms. nPTB is expressed predominantly in the nervous system, muscle and testis. During neural development, expression of PTB is decreased along with differentiation of neural stem cells into neurons. In contrast, expression level of nPTB that was repressed by PTB is accordingly increased. Thus, tissue-specific RNA binding protein, nPTB may play an important role in neural development. But, functional differences between PTB/nPTB and meaning of developmental stage associated expression change from PTB to nPTB remain to be elucidated. In our study, we are generating nPTB conditional knockout mice to shed light on a role of nPTB in vivo. And now we are also analyzing the function of PTB/nPTB in vitro by using knockdown experiment via a formation of embryoid body (EB) from ES cells.

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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.

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Immunocompetent cells express multiple Toll-like receptors (TLRs) which play sentinel roles in detecting microbial products during infection. As one microbe loads multiple TLR ligands, orchestrated TLR activations are indispensable for inducing appropriate innate/adaptive immune responses. Meanwhile, molecular mechanisms balancing those responses remain unclear. Recent reports have suggested that TLR associating molecules or post-translational TLR protein modification were involved in controlling TLR responses. To correctly understand coordinated TLR responses, we search for novel TLR accessory molecules controlling multiple TLRs and post-translational modification in TLRs affecting TLR responses. Our goal is to elucidate molecular regulatory mechanism in TLR responses through genetically engineered mice.

1. Roles of PRAT4A (PRotein Associated with Toll-like receptor 4) in immune modulation.

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

TLRs induce complex inflammatory responses that functions to protect the host from infection. But in an excessive or persistent inflammatory responses, hosts suffer the disadvantage of septic shock, delayed tissue repair and autoimmune diseases. To control the extent and duration of inflammation by TLRs, hosts evolved the multiple regulatory mechanisms, called tolerance.

We have previously reported that a novel TLR associating protein 'PRotein Associated with TLR4 (PRAT4A)'. Endoplasmic reticulum PRAT4A controls maturation and intracellular trafficking of multiple TLRs with another TLR specific co-chaperone gp96. PRAT4A deficient Macrophages/Dendritic cells (DCs), with abnormal TLR distribution, showed impaired immune responses to TLR2/4/7/9 ligands, except to TLR 3 ligand. Interestingly, PRAT4A deficient immune cells remained specific immune responses induced by TLR2/4 ligands which were distinct from typical inflammatory responses. As mRNA level of PRAT4A significantly decreased after stimulation by various TLR ligands in physiological state, PRAT4A deficiency presumably mimicked the tolerant state. These facts raised the possibility that PRAT4A play a central role in tolerance mechanism suppressing excessive inflammation by TLRs. Overexpression studies may be helpful in evaluating the tolerant effect of PRAT4A.

Furthermore, using established anti-PRAT4A monoclonal antibodies, we found secreted endogenous PRAT4A which was detected in the normal mice serum and the culture supernatant of Bone Marrow (BM) derived Macrophages by immunoprecipitation assay. Since PRAT4A deficient mice showed high neonatal lethality and severe dwarfism, there is another possibility that PRAT4A is not only a TLR associating chaper-

one molecule but also a modulator of homeostasis.

To further address PRAT4A function, we have tried to construct Cre/loxP conditional transgenic mice by targeting ROSA26 locus which enables us to analyze cell type-specific overexpression studies *in vitro* and *in vivo*. Now we confirm germline transmission of the targeted allele and start mating with various types of Cre mice, including CAG-Cre, Tie-2 Cre and LysM-Cre mice, to establish conventional or conditional PRAT4A transgenic lines.

2. Finding a novel tetraspanin protein involved in the negative regulation of multiple TLR responses.

Takuma Shibata[1,2], Xiaobing Li[1], Nobuaki Yoshida[2] and Kensuke Miyake[1,2]

Multiple TLRs work in concert to sense a pathogen and mount defense responses. Little is known, however, about a mechanism coordinating multiple TLRs responses.

Under the screening of TLR2 regulating molecules, we found a novel tetraspanin protein which co-precipitated with TLR2 in immunoprecipitation assay. Knock down of this gene in murine macrophage cell line RAW264.7 resulted in enhanced cytokine production by various TLR ligands, including TLR2 ligand. This finding suggested the negative regulatory function of this novel tetraspanin protein in multiple TLR responses.

Conditional knock out and Conditional transgenic mice using ROSA26 locus of this novel tetraspanin protein have been generated to allow rigorous assessment of its function. Further study is under way to reveal a role of this gene in immune responses.

3. Characterization of cleaved form in TLR7 and TLR9

Atsuo Kanno[1], Masahiro Onji[1], Takuma Shibata[1,2], Yuji Motoi[1], Ryutaro Fukui[1], Shin-Ichiroh Saitoh[1], Nobuaki Yoshida[2] and Kensuke Miyake[1,2]

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 ectodomain of TLR7/9 was cleaved in endolysosome. It seemed that ectodomain cleavage in TLR7/9 represents another strategy to restrict receptor activation in endolysosome.

Recently, we originally identified cleavage sites in TLR7 and TLR9. To clarify the meaning of ectodomain cleavage in TLR7/9, we are trying to construct knock-in mice which load TLR 7/9 without cleavage site. So far, germline transmission was confirmed.

4. Roles for Unc93 homolog B1-dependent TLR7/9 balance in vivo

Atsuo Kanno[1], Ryutaro Fukui[1], Shin-Ichiroh Saitoh[1], Takuma Shibata[1,2], Yuji Motoi[1], Nobuaki Yoshida[2] and Kensuke Miyake[1,2]

Nucleic acid sensing Toll-like receptor 7 (TLR 7) 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. It is important to keep the responsiveness of the nucleic acid-sensing TLRs under the tight control.

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

To clarify a role for Unc93B1-dependent TLR 7/TLR9 balance *in vivo*, we started to generat knock-in mice harboring the mutations around D34 in the Unc93B1 gene which showed accelerated phenotypes of D34A mutant. So far, we have got Homologous Recombinants for this knock in mice.

5. Identification of regulatory molecules for innate immune responses and constructing genetically altered mice.

Takuma Shibata[1,2], Yuji Motoi[1], Natsuko Yamakawa[1], Xiaobing Li[1], Yusuke Murakami[1], Shin-ichiroh Saitoh[1], Mabel Chan[1], He Zhao[1], Masahiro Onji[1], Atsuo Kanno[1], Ryutaroh Fukui[1], Nobuaki Yoshida[2] and Kensuke Miyake[1,2]

Except above projects, we tried functional clonings and co-immunoprecipitation studies to comprehensively identify regulatory molecules associated with innate immune responses. And after simple screenings in cell lines, we constructed conditional knock out mice to reveal the physiological function *in vivo*.

So far, we found more than 20 candidate genes, and started to construct conditional knock out mice. We finished constructing targeting vector in 13 genes and getting homologous recombinants, using B6 background JM8.A3 ES cell line, in 11/13 genes. Now we have got chimeric mice in 8/13 genes. Germline transmission was confirmed in 5/13 genes.

Publications

No publications

Center for Experimental Medicine and Systems Biology

Laboratory of Cancer Signaling
発癌機構研究分野

Professor Tadashi Yamamoto, Ph.D

教 授 理学博士 山 本 雅

Protein-tyrosine kinases are important not only for the development of malignant tumors but also for the regulation of growth and function of normal cells. We are interested in characterizing signal transduction downstream of protein-tyrosine kinases that are relevant to cancer development and to neuronal function.

Roles of Fyn kinase signaling in the brain development

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During the course of Fyn substrate screening, we have identified Protocadherin17 (PCDH17) as a potential target of the Fyn tyrosine kinase. Although several protocadherin members are involved in cancer progression, its physiological roles are largely unknown. To analyze physiological roles of specific protocadherin members *in vivo*, we addressed the role of PCDH17. PCDH17 was specifically expressed in the basal ganglia including striatum, globus pallidus, and substantia nigra. Developmentally, PCDH17 was highly expressed at the stage of synaptogenesis. Furthermore, PCDH17 and its family member, PCDH10 exhibited totally complementary expression patterns in the basal ganglia. At synapses, PCDH17 was localized in perisynaptic regions and showed calcium-dependent homophilic binding, but not heterophilic binding to PCDH10. To elucidate the function of PCDH17 *in vivo*, we generated PCDH17 knockout mice. With these mice, we are now studying the roles of PCDH17 in the basal ganglia circuitry using morphological, electrophysiological and behavioral approaches.

Expression levels of cadherin family proteins have been implicated in the progression of various cancers. Generally, tumors expressing lower levels of cadherin tended to exhibit higher invasive potentials. Recent reports suggested that silencing of PCDH17 or its closely related family member PCDH10 is involved in cancer malignancy. Therefore, we would like to examine whether mice lacking expression of PCDH17 are prone to develop tumors and/or whether deficiency of PCDH17 could promote tumor progression by introducing *pcdh17* homo-deficiency into cancer-prone mice harboring oncogenically mutated K-*ras*.

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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.

1. The role of mast cells in cardiac allograft in mice

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It has been hypothesized that mast cells promote rejection and adverse remodeling in cardiac allografts. By contrast, it has been reported that mast cells can contribute to peripheral tolerance to skin allografts in mice and may also enhance cardiac allograft survival in rats. We used mast cell-deficient C57BL/6-*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice and the corresponding wild type mice to investigate the possible contributions of mast cells to acute or chronic cardiac allograft rejection. In the acute rejection model, donor heart VCAM-1 expression was significantly lower in C57BL/6-*Kit*$^{W\text{-}sh/W\text{-}sh}$ than in wild type recipients, however, there were no significant differences in acute rejection scores, graft survival, inflammatory cells, or cytokine expression. In the chronic rejection model, the number of mast cells per mm^2 of allograft tissue was significantly increased 52 days post-transplantation in allografts transplanted into C57BL/6-*Kit*$^{+/+}$ but not C57BL/6-*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice; however, there were no substantial differences in Graft Coronary Artery Disease (GCAD), graft inflammatory cells, or levels of graft tissue expression of cytokines or adhesion molecules. Cardiac allografts undergoing chronic rejection in wild type C57BL/6-*Kit*$^{+/+}$ mice exhibit increased numbers of mast cells, but acute or chronic cardiac allograft rejection can develop in C57BL/6-*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice even though these recipients lack mast cells. These findings indicate that recipient mast cells are not required for acute or chronic cardiac allograft rejection in the models examined.

2. The role of IL-17 in cardiac allograft in mice

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Although interleukin-17 (IL-17) has been reported to participate in the pathogenesis of infectious, autoimmune and allergic disorders, the precise role in allograft rejection remains uncertain. This study illustrates that IL-17 contributes to the pathogenesis of chronic allograft rejection. Utilizing a murine heterotopic heart transplant model system, IL-17-deficient recipient mice had decreased allograft inflammatory cell recruitment, decreased IL-6, MCP-1, and KC production, and reduced graft coronary artery disease (GCAD). Intragraft γδ T cells appear to be the predominant source of IL-17 production. Therefore, IL-17 neutralization may provide a potential target for novel therapeutic treatment for cardiac allograft rejection.

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