Center for Experimental Medicine

Laboratory of Cell Biology
細胞機能研究分野

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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 autoinflammatory cytokine expression in the joints are characteristics 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 the HTLV-I transgenic mice (Iwakura et al., *Science*, 1991) and the other is IL-1 receptor antagonist-deficient (*Il1rn*$^{-/-}$) mice (Horai et al., *J. Exp. Med*., 2000). Both of these models develop autoimmunity and chronic inflammaroty 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. We extracted 554 genes which expression significantly changed in both models. Then, each of these commonly changed genes was mapped into the whole genome in a scale of the 1-megabase pairs. We found that the transcriptome map of these genes formed clusters including the major histocompatibility complex class I and class II genes, complement genes, and chemokine genes, which are well known to be involved in the pathogenesis of RA at the effector phase. By searching for such clusters, we could detect genes with marginal expression changes, including several genes whose involvement in the arthritis pathogenesis has previously not known. We have also searched for pathogenesis-related genes by analyzing the genetic background dependency of the arthritis development. We have identified several genes which were involved in the BALB/c strain-specific development of arthritis in these model mice. We are now analyzing the roles of these genes in the pathogenesis of arthritis and autoimmunity by generating knock-

out mice of these genes. This year, we have published *Dcir*$^{-/-}$ mice which spontaneously develop sialadenitis and enthesitis in *Nature Medicine* (Fujikado et al., 2008).

2. Studies on the roles of IL-17 family genes in inflammation and host defense against bacteria

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IL-17A is a proinflammatory cytokine that activates T cells and other immune cells to produce a variety of cytokines, chemokines, and cell adhesion molecules. This cytokine is augmented in the sera and/or tissues of patients with contact dermatitis, asthma, and rheumatoid arthritis. We previously demonstrated that IL-17 A is involved in the development of autoimmune arthritis, contact, delayed, and airway hypersensitivity, and experimental autoimmune encephalomyelitis in mice (Nakae et al., *Immunity*, 2002; Nakae et al., *PNAS*, 2003, Nakae et al., *J. Immunol*., 2003; Komiyama et al., *J. Immunol*., 2006). Recently, it was shown that IL-17A is produced by a newly discovered $CD4^+$ T cell subset, Th17. Now, many diseases that were thought to be induced by Th1 or Th2 are proven to be induced by Th17. Thus, we are analyzing the roles of IL-17A and its family molecules in inflammatory diseases and host defense against infection.

IL-17F is an IL-17 family member and has the highest amino acid sequence homology to IL-17 A. Although IL-17F is also produced by Th17 cells and binds the same receptor, the functional roles of this molecule remain largely unknown. To distinguish the roles of IL-17A and IL-17F, in allergy, autoimmunity, and infection, we generated *Il17a*$^{-/-}$, *Il17f*$^{-/-}$ and *Il17a/f*$^{-/-}$ mice and demonstrated that IL-17A, but not IL-17F, is involved in the development of delayed-type hypersensitivity and encephalomyelitis. Similarly, IL-17A played a greater role than IL-17F in the development of collagen-induced arthritis and autoimmune arthritis in *Il1rn*$^{-/-}$ mice. In contrast, both IL-17F and IL-17A were involved in host defense against mucoepithelial infection by *Staphylococcus aureus* and *Citrobacter rodentium*. IL-17A was produced mainly in T cells, whereas IL-17F was produced in T cells, innate immune cells, and epithelial cells. Although only IL-17A efficiently induced cytokines in macrophages, both cytokines activated epithelial innate immune responses. These observations indicate that IL-17A and IL-17F have overlapping yet distinct roles in host immune and defense mechanisms.

3. The role of cytokines in the neuro-immuno-endocrine system

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Proinflammatory cytokines, including IL-1, exert pleiotropic effects on the neuro-immuno-endocrine system. Previously, we showed that mice with knockout of the gene encoding *Il1 rn*$^{-/-}$ mice have a lean phenotype. This year, to analyze the mechanisms leading to this lean phenotype, *Il1rn*$^{-/-}$ mice were fed a high-fat diet following weaning. Energy expenditure, body temperature, heart rate, blood parameters, urinary catecholamines and adipose tissue were analyzed. *Il1rn*$^{-/-}$ mice exhibited resistance to obesity induced by a high-fat diet; this resistance was associated with increased energy expenditure and a decreased respiratory quotient, indicating that the ratio of fat:carbohydrate metabolism in *Il1rn*$^{-/-}$ mice is greater than in controls. Activity level in *Il1rn*$^{-/-}$ mice was significantly decreased and body temperature was significantly increased, compared with wild-type (WT) mice. Inguinal white adipose tissues in *Il1 rn*$^{-/-}$ mice express increased levels of Ucp1 and mitochondrial respiratory chain genes compared with WT mice. Histological analysis of adipose tissue in *Il1rn*$^{-/-}$ mice revealed that brown adipose tissue is hyperactive and inguinal white adipose tissue contains smaller cells, which exhibit the distinctive multilocular appearance of brown adipocytes. Urinary epinephrine and norepinephrine excretion in *Il1rn*$^{-/-}$ mice was significantly increased compared with WT mice, suggesting that *Il1rn*$^{-/-}$ mice have increased sympathetic tone. Consistent with this, heart rate in *Il1rn*$^{-/-}$ mice was also significantly increased. These observations indicate that *Il1 rn*$^{-/-}$ mice have increased energy expenditure, fat: carbohydrate oxidation ratio, body temperature, heart rate and catecholamine production. All of these observations are consistent with an enhanced sympathetic tone.

4. Characterization of mice deficient in Melanocortin receptor 2 on a B6/BALB/c mix background

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We previously reported that Melanocortin 2 receptor ($Mc2r^{-/-}$) deficient mice on B6 N5 generations exhibit macroscopically detectable adrenal glands with markedly atrophied zona fasciculata (zF) and lack of detectable levels of corticosterone, and reduced serum concentrations of aldosterone and epinephrine. All $Mc2r^{-/-}$ mice on B6/N8 background die within 2 days after birth, while about half of the $Mc2r^{-/-}$ mice on B6/Balbc mix background survived to adulthood. Both male and female $Mc2r^{-/-}$ mice were fertile, suggesting that normal development and function of reproductive organs. $Mc2r^{-/-}$ mice delivered from $Mc2r^{-/-}$ dams failed to survive due to lung failure, suggesting that fetal or maternal corticosterone is essential for lung maturation. $Mc2r^{-/-}$ mice failed to activate the hypothalamic-pituitary-adrenal axis in response to both immune and non-immune stimuli. $Mc2r^{-/-}$ mice maintained glomerular structure and achieved electrolyte homeostasis by the activation of the renin-angiotensin-aldosterone system under low aldosterone and undetectable levels of corticosterone.

5. Studies on 2', 5'-oligoadenylate synthetase and inflammation related genes

Shigeru Kakuta and Yoichiro Iwakura

2', 5'-oligoadenylate synthetase (OAS) is one of IFN-inducible proteins and is known to be involved in the major antiviral mechanism through activation of RNase L. In mice, the OAS family consists of 12 genes with a well conserved motif. However, the physiological role and functional difference among these genes are not elucidated completely. In this study, to elucidate the roles of OAS family genes, we generated each of Oas1a-, Oas1c-, Oas2-, Oas1a/Oas2-, and Oas1-3 gene cluster (OASC)-deficient mice by targeted gene disruptions.

Oas1a/Oas2 deficient (flox) mice have two loxP sites inserted into the flanking regions of the Oas gene cluster. A large size deletion (~170 kb) between loxP sites was created by a Cre-mediated recombination. OASC-deficient mice were generated by injecting a Cre expression plasmid, pCAG-Cre, into 1-cell embryos derived from Oas1a/Oas2-deficient (flox) mice. OASC mice were born in a Mendelian ratio, fertile, and appeared healthy under SPF conditions. The thymus appeared normal, in contrast to RNaseL-deficient mice in which the thymus becomes hyperplastic because of the deficiency of thymocyte apoptosis. As OASC-deficient mice lack 10 conventional OAS genes, but still express 2 OASL genes, which encode enzymatically inactive and functionally unknown, these observations suggest that OASL may compensate the OAS 1-3 functions in the 2-5A system, or RNaseL could be activated in an OAS independent manner.

On the other hand, it is reported that RNaseL-deficient mice were resistant to the induction of IFN expression in response to intracellular dsRNA. They showed that activation of RNaseL by OAS produced small RNA cleavage products from self-RNA which initiated IFN production. We found that Oas1a- and OASC-, but not Oas2-, deficient mice were hypersensitive against intracellular dsRNA and dsDNA stimulation. These results indicate that Oas1a has a unique function in OAS family molecules, and acts as a novel negative regulator of IFN expression signaling pathway in a RNaseL-independent manner.

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

Motohiko Kadoki, Takuya Tada and Yoichiro Iwakura

Studies of AIDS pathogenesis and development of therapeutic drugs and vaccines have been hampered by the lack of appropriate small animal models, because HIV-1 cannot infect and replicate in small animals like mice due to the difference of the structure of some host factors necessary for HIV-1 replication. As primate models are difficult to handle, expensive and require expensive facilities, genetically not well characterized, often infected with pathogens, and ethically less acceptable, we are trying to generate mouse models for AIDS. We are taking two approaches to generate AIDS models, one is HIV-1 carrier models which carry the HIV-1 genome as a transgene (Iwakura et al., *AIDS*, 1992) and the other is HIV-1 susceptible models in which all the host factors involved in the species barrier are humanized.

We previously showed that HIV-1 expression is efficiently activated in the spleen of HIV-1

transgenic mice by bacterial lipoplysaccharide (LPS) (Tanaka et al., *AIDS*, 200; 2003). This year, we analyzed the molecular mechanism of the HIV-1 activation in macrophages of HIV-I transgenic mice. We found that, in contrast to spleen cells in which HIV-1 expression was induced by cytokines such as TNF-α or IL-1, HIV-1 was directly activated by TLR-mediated signaling. We are now analyzing the mechanisms. We are also trying to identify host factors that are involved in the nuclear localization of pre-integration complex, that consists one of species-specific host range barriers.

7. Gene trap screening of membrane proteins involved in the regulation of pluripotent stem cell differentiation

Hiroaki Okae and Yoichiro Iwakura

The pluripotent stem cells can give rise to all types of cells seen in the adulthood. Although the cell fate is determined by cell-cell interactions, the molecular mechanisms are not fully understood. Thus, we tried to identify membrane proteins which regulate pluripotent stem cell differentiation.

Our strategy was a combination of both signal sequence trap and in vitro differentiation of ES cells. The signal sequence trap is a method to specifically disrupt genes that have signal sequences, using signal sequence-specific β-galactosidase expression in the trap vector. Then, the trapped clones were induced to differentiate into extraembryonic cells by the forced expression of specific transcription factors to examine the effects of the gene disruption on the differentiation.

By using these methods, 26 genes that were highly expressed in ES cells and encoded membrane bound proteins were disrupted in ES cells. Mutant mice of the 16 genes among these trapped genes have been already reported, and 13 of them are embryonic lethal. Three trapped genes were novel and two mutant mouse lines carrying one of these three mutations showed lethal phenotypes. In addition, 4 genes were involved in the regulation of pluripotent stem cell differentiation. These data indicate that the combination of signal sequence trap and *in vitro* differentiation of ES cells is a powerful method to enrich genes that are required for pluripotent stem cell differentiation and embryonic development.

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Center for Experimental Medicine

Laboratory of Gene Expression & Regulation
遺伝子機能研究分野

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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. ES cells, which are used for gene targeting, are the only stem cells being cultured in vitro. To elucidate the molecular mechanism regulating self-renewal of pluripotent ES cells, we have tried to identify a factor(s) cooperating with Oct-3/4, the critical transcription factor for maintaining undifferentiated state of ES cells.

1. An Improved Transgenic Mouse Line Expressing Temperature-Sensitive SV40 tsA 58 T Antigen Based on Cre/loxP Recombination System

Takashi Yamaguchi, Taeko Ichise, Osamu Iwata, Akiko Hori, Tomomi Adachi, Masaru Nakamura, Nobuaki Yoshida and Hirotake Ichise

As an indispensable component of the vascular system, endothelial cells (ECs) have pivotal roles in development and in health and disease. Their properties have been studied by a combination of *in vitro* analyses of human primary ECs and *in vivo* analyses of genetically modified mice exhibiting vascular phenotypes. Human primary ECs are well-established resources and are suitable for studying signal transduction and cellular physiology *in vitro.* However, it has been still difficult to control their gene expression strictly by current overexpression and knockdown procedures. In addition, they are not representative of all types of ECs in different developmental stages and vascular beds. On the other hand, many genetically modified mice have accelerated the understanding of genetic mechanisms of endothelial development and functions. However, further analyses of vascular phenotypes *in vivo* have been hampered by the complicated relationship between ECs and non-ECs such as mural, hematopoietic, and mesenchymal fibroblast cells, even though a conditional genetic modification such as endothelium-specific knockouts can provide a partial solution to this problem. Therefore, the isolation and

maintenance of murine endothelial cells from different times and places is important for dissecting molecular and cellular mechanisms of endothelial development and function. In order to overcome the difficulties, we generated a transgenic mouse line expressing the SV40 tsA58 large T antigen (tsA58T Ag) under the control of a binary expression system based on Cre/loxP recombination, and crossed the mice with Tie2-Cre transgenic mice. Several organs (the brain, heart, lung, liver, and uterus) were obtained from the double-transgenic mice, dissected, and dissociated with enzymatic digestion. Dispersed cell suspensions were plated onto gelatin-coated plastic dishes. tsA58T Ag-positive endothelial cells in primary cultures of a variety of organs proliferated continuously at 33°C without undergoing cell senescence. The resulting cell populations consisted of blood vascular and lymphatic endothelial cells, which could be separated by immunosorting. Even when cultured for two months, the cells maintained endothelial cell properties as assessed by the expression of endothelium-specific markers, intracellular signaling via vascular endothelial growth factor receptor (VEGFR) -2 and -3 and physiological characteristics. In addition, lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1) expression in liver sinusoidal endothelial cells *in vivo* was retained *in vitro*, suggesting that an organ-specific endothelial characteristic was also maintained. These results show that our transgenic system is useful for culturing murine endothelial cells and will provide an accessible method and applications for endothelial cell biology.

2. Role of polypyrimidine tract binding protein in cell cycle regulation

Satona Ohno, Mitsuharu Sato, Masaki Shibayama and Nobuaki Yoshida

Polypyrimidine tract-binding potein (PTB) is a member of the heterogeneous nuclear ribonucleoprotein particle family that binds to pyrimidine-rich sequences of RNAs. PTB is a multifunctional protein that has been implicated in many aspects of RNA regulations, including alternative splicing, polyadenylation, stabilization, subcellular localization, and internal ribosome entry site (IRES)-dependent translation of cellular and viral RNA. Our group has shown that mouse embryonic stem (ES) cells with a homozygous disruption of *ptb* ($ptb^{-/-}$) represent severe delay in cell proliferation.

To reveal a cause of proliferation defect in $ptb^{-/-}$ ES cells, we analyzed the cell cycle progression of $ptb^{-/-}$ ES cells by flow cytometry. We found that M phase is prolonged in $ptb^{-/-}$ ES cells. Then we focused on the regulation of IRES-dependent translation, which is one of the functions of PTB involved in M phase regulation. We showed that the IRES activity of CDK 11^{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 CDK11^{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. We also found that 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.

3. Analysis of Fbxl10 function in mouse development

Tsuyoshi Fukuda, Reiko Sakamoto, Mitsuharu sato and Nobuaki Yoshida

Histone methylation has important roles in regulating transcription, genome integrity and epigenetic inheritance. Historically, methylated histone lysine residue has considered stable modification because of its thermodynamic stability. Recent study, however, demonstrated that methylation of histone lysine is removed by jumonji C (JmjC)-domain-containing protein.

Fbxl10 is a paralog of the first identified JmjC-domain-containing histone demethylase, Fbxl11, which targets histone 3 lysine 36 for demethylation. Although histone demethylase activity and the target genes of Fbxl10 have been identified so far, very little is known regarding the physiologic functions of Fbxl10.

To investigate biological functions of Fbxl10, we disrupted Fbxl10 gene in mouse embryonic stem cells and generated Fbxl10 knockout mice. Fbxl10 null mice show prominent abnormalities during embryonic stage and die shortly after birth with moderate penetration rate. These results indicated that Fbxl10 has important roles in early mouse development. Physiological roles of Fbxl10 in mouse embryogenesis are currently under investigation.

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