Center for Experimental Medicine

Laboratory of Cell Biology
ヒト疾患モデル研究センター細胞機能研究分野

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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: human T cell leukemia virus type I (HTLV-I) transgenic (Tg) mouse model and IL-1 receptor antagonist (IL-1Ra)-deficient (KO) mouse model

Shinobu Saijo, Noriyuki Fujikado, Keisuke Seki, Soo-hyun Chung and Yoichiro Iwakura

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 autoantibodies in the serum and augmentation of proinflammatory 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 Tg mouse model (Iwakura et al., Science, 1991) and the other is IL-1Ra KO (Horai et al., J. Exp. Med., 2000). Both of these models develop autoimmunity and chronic inflammatory arthropathy closely resembling RA in humans.

To elucidate the pathogenesis of arthritis and autoimmunity, gene expression profile in the joints was analyzed using DNA microarrays (Affymetric Inc.) containing approximately 36,000 full-length mouse genes and EST clusters from UniGene database. The gene expression profiles of the two models correlated well. We have identified 554 genes that showed significantly different expression levels in the inflamed joints in both models. Each of these commonly changed genes was mapped into the whole genome in a scale of the one megabase pairs. We found that the transcriptome map of these genes did not distribute evenly on the chromosome but formed clusters. These gene clusters identified include the major histocompatibility complex (MHC) 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 effecter phase. Moreover, by the search of such clusters, we could detect the augmentation of schlafen family and LLR family, whose function have not been known yet in arthritis. To examine the roles for these genes in the development of arthritis, we produced gene targeted mice of these genes and found that one

of them was resistant to collagen induced arthritis, and the other one was more sensitive than the wild-type mice. Thus, generation of gene targeted mice of the genes identified by microarray analysis of these arthritic models is useful to identify genes important for the development of arthritis.

2. Involvement of tumor necrosis factor-alpha in the development of T cell-dependent aortitis in interleukin-1Ra-deficient mice

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IL-1Ra KO mice on the BALB/c background spontaneously develop inflammatory arthropathy that resembles rheumatoid arthritis in humans. These mice also frequently develop aortitis at the root of the aorta, but the mechanism underlying the development of this disease has not been completely elucidated. Using IL-1Ra KO (backcrossed 8 generations to the BALB/c background) and wild-type mice, we studied the histopathology and examined the immunologic mechanisms involved in the development of aortic inflammation by cell transplantation experiments. Half of the IL-1Ra KO mice developed aortitis at the root of the aorta, with massive infiltration of macrophages and monocytes and loss of elastic lamellae in the aortic media. Left ventricular hypertrophy and mild aortic stenosis were also shown by transthoracic echocardiography. Transplantation of T cells from IL -1Ra KO mice induced aortitis in recipient nu/nu mice. Bone marrow cell transplants from IL-1 Ra KO mice also induced aortitis in irradiated wild-type recipient mice. Furthermore, tumor necrosis factor (TNF)-α deficiency completely suppressed the development of aortitis in IL-1Ra KO mice, whereas IL-6 deficiency did not affect pathology. These observations suggest that IL-1 Ra deficiency in T cells activates them excessively, resulting in the development of aortitis in IL-1Ra KO mice in a TNF-α-dependent manner.

3. Abnormal T cell activation caused by the imbalance of the IL-1/IL-1R antagonist system is responsible for the development of experimental autoimmune encephalomyelitis

Taizo Matsuki, Susumu Nakae, Katsuko Sudo, Reiko Horai and Yoichiro Iwakura

IL-1 is a pro-inflammatory cytokine that plays an important role in inflammation and host responses to infection. We have previously shown that imbalances in the IL-1 and IL-1Ra system cause the development of inflammatory diseases. To explore the role of the IL-1/IL-1Ra system in autoimmune diseases, we analyzed myelin oligodendrocyte glycoprotein (MOG)-induced experimental autoimmune encephalomyelitis (EAE) in mice bearing targeted disruptions of the IL-1α, IL-1β, IL-1α and IL-1β (IL-1α /β) or IL-1Ra genes. IL-1α/β double-deficient mice exhibited significant resistance to EAE induction with a significant reduction in disease severity, while IL-1α KO or IL-1β KO mice developed EAE in a manner similar to wild-type mice. IL-1Ra KO mice also developed MOG-induced EAE normally with pertussis toxin (PTx) administration. In contrast to wild-type mice, however, these mice were highly susceptible to EAE induction in the absence of PTx administration. We found that both IFN-γ and IL-17 production and proliferation were reduced in IL-1α/β KO T cells upon stimulation with MOG, while IFN-γ, IL-17 and TNF-α production and proliferation were enhanced in IL-1Ra KO T cells. These observations suggest that the IL-1/ IL-1Ra system is crucial for auto-antigen-specific T cell induction and contributes to the development of EAE.

4. Role of IL-1 in the Delayed-type hypersensitivity response

Aya Nambu, Susumu Nakae and Yoichiro Iwakura

IL-1 is a potent proinflammatory cytokine that is involved in many inflammatory and autoimmune diseases. Since high levels of IL-1 expression were observed in delayed-type hypersensitivity (DTH) responses, we analyzed the role of IL-1 in DTH responses. We found that DTH responses against methyl (m)-BSA were significantly suppressed in IL-1β KO and IL-1α/β KO mice, with marked suppression of leukocyte infiltration in the DTH footpads. While, IL-1Ra KO mice showed significantly exacerbated DTH responses, and IL-1α KO mice showed comparable responses to wild-type mice. Lymph node cells from mBSA-sensitized IL-1β KO, IL-1α/β KO and IL-1 type I receptor (IL-1RI) KO mice showed significantly reduced proliferative responses against mBSA, while these from IL-1Ra

KO mice showed significantly augmented proliferative responses. DTH responses in wild-type mice adoptively transferred with $CD4^+$ T cells from mBSA-sensitized IL-1α/β KO mice were reduced, while those in mice transferred from IL -Ra KO mice were increased. DTH responses were suppressed in IL-1RI KO mice transferred with mBSA-sensitized $CD4^+$ T cells from wild-type mice, while the responses were normal in IL-1α/β KO mice. Proliferative responses of mBSA-sensitized $CD4^+$ T cells co-cultured with DCs from IL-1RI KO mice were decreased, while these of IL-1α/β KO mice were comparable to those of wild-type mice. DTH responses in TNF-α KO mice were also suppressed; the magnitude of the suppression in IL-1α/β/TNF-α KO mice, however, was similar to that observed in IL-1α/β KO mice. These observations indicate that IL-1 possesses dual functions during the DTH response. IL-1β is necessary for the efficient priming of T cells. In addition, $CD4^+$ T cell-derived IL-1 plays an important role in the activation of DCs during the elicitation phase, resulting in the production of TNF-α, that activate allergen-specific T cells.

5. The role of IL-17 in the development of autoimmune and inflammatory diseases

Yutaka Komiyama, Harumichi Ishigame, Taizo Matsuki, Susumu Nakae, Reiko Horai, Shigeru Kakuta and Yoichiro Iwakura

IL-17 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 inflammatory diseases, such as contact dermatitis, asthma, and rheumatoid arthritis. We previously demonstrated that IL-17 is involved in the development of autoimmune arthritis and contact, delayed, and airway hypersensitivity in mice. However, the role of IL-17 in the development of autoimmune and inflammatory diseases was not fully understood. In this study, we examined the role of IL-17 in the development of EAE a mouse model for multiple sclerosis (MS).

We found that the development of EAE was significantly suppressed in IL-17 KO mice. We also demonstrated that the IL-1/IL-1Ra system contributes to the development of EAE. Moreover, the development of arthritis and aortitis, which spontaneously develop in IL-1Ra KO mice, was significantly suppressed by the deficiency of IL-17. These data indicate that IL-17 plays a crucial role in the development of EAE, arthritis, and aortitis, downstream of IL-1 signaling.

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

Shigeru Kakuta, Reiko Ichikawa, Mari Shibukawa, Satoe Azechi, Megumi Matsuda, Hayato Kotaki, and Yoichiro Iwakura

The 2',5'-oligoadenylate synthetase (OAS) is one of IFN-induced proteins and plays an important role in the host defense mechanisms upon viral infection. The OAS genes form a well conserved family. In humans, there are four classes of 2-5OAS genes, short (OAS1), middle (OAS2), and long (OAS3) form and one OAS-like protein (OASL). Whereas in mice, we previously showed that the OAS1 gene is multiplicated into 10 genes and the OASL gene is duplicated. The short form 2-5OAS has a set of the essential motifs for the 2-5OAS enzyme activity, OAS2 has two and OAS3 has three catalytic units. OASL has a single OAS unit and two consecutive ubiquitin-like sequences in the carboxyl -terminal, but lacks 2-5OAS activity. We previously generated *Oas1a* KO, *Oas1c* KO, *Oas2* KO and *Oas1a/Oas2* KO mice. These homozygous KO mice were born normally and were fertile. This year, we generated *Oas1s*, *Oas2* and *Oas3* gene cluster-deficient (*OASC* KO) mice. *OASC* KO mice also showed normal development and they were fertile under SPF conditions. These OAS family gene deficient mice should be useful for the analysis of OAS functions in viral infection, apoptosis and inflammation. We also generated several gene targeted mice of the genes that are augmented in arthritic joints, and are now analyzing roles of these genes in allergic response, infection, and tumorigenesis.

7. Involvement of CRH- and IL-6-dependent proopiomelanocortin induction in the anterior pituitary during hypothalamic-pituitary-adrenal axis activation by IL-1α

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IL-1α/β and IL-6 are endogenous modulator of hypothalamo-pituitary-adrenal axis (HPAA) and are thought to play key roles in immune-neuroendocrine interactions during inflammation. Here, we show IL-1α induced a normal HPAA activation in *IL-1*α/β KO and *IL-6* KO mice at 1 h; however, at 6 h HPAA activation was reduced relative to WT mice, indicating a role for endogenous IL-1α/β and IL-6 in pro-

longed HPAA activation. We found that the induction of *proopiomelanocortin* (*POMC*) transcript in the anterior pituitary (AP) at 6 h in response to IL-1α was reduced in *IL-1α/β* KO and *IL-6* KO mice, as well as in *CRH* KO mice, suggesting IL-1α/β, IL-6, and CRH are all required for *POMC* induction. The induction of *CRH* transcript in the paraventricular nucleus (PVN) at 6 h and plasma IL-6 levels, in response to IL-1α, were reduced in *IL-1α/β* KO KO mice. Since IL-1α-induced activation of STAT3 in the AP was also suppressed in *IL-6* KO mice, we suggest that plasma IL-6 is first induced by IL-1α, and IL-6 activates STAT3 in the AP, leading to the induction of *POMC* in concert with *CRH.* Our results suggest a role for IL-1α/β in the induction of *POMC* in the AP through the induction of two independent pathways, *CRH* and IL-6.

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

Motohiko Kadoki, Kuniaki Yabe, and Yoichiro Iwakura

Studies of AIDS pathogenesis and development of therapeutic drugs for AIDS have been hampered by the lack of appropriate animal models for AIDS. To circumvent the difficulties, we are trying to generate mouse models for AIDS. It is known that the structure of some host factors necessary for HIV-1 infection and replication such as the receptor molecule is different in mice causing HIV-1 difficult to replicate in murine cells. We are taking two approaches to generate the AIDS models, one is HIV-1 carrier models which carry the HIV-1 genome as a transgene and the other is HIV-1 susceptible models in which all the host factors involved in the species barrier are humanized.

We have produced a line of transgenic mice that carry the HIV-1 genome deficient in the *pol* gene (Iwakura et al., *AIDS*, 1992). In this model, HIV-1 genome can be activated *in vivo* via lipopolysaccharide (LPS) administration through induction of IL-1α/β and TNF-α, and high concentrations of viral particles are released into the blood. Since cells carrying the HIV-1 genome could produce HIV-1 particles, it was suggested that the replication in murine cells is mainly blocked before the integration process. We found that nuclear localization process of preintegration complex of HIV-1 is defective in murine cells due to a dysfunction of integrase in murine cells. We are now analyzing the host factors that are involved in the nuclear translocation process.

9. Studies on the role of cell-cell interactions in the early development of mouse embryos

Deug-chan Lee, Seiji Takashima, Kenjiro Adachi, Hiroaki Okae, and Yoichiro Iwakura

Successful production of cloned animals by nuclear transplantation has demonstrated that differentiated somatic cells can be reprogrammed into undifferentiated embryonic cells that can form whole animal body. Moreover, it was reported that a subset of somatic stem cells including haematopoietic stem cells and neural stem cells could differentiate into cells of all the embryonic three germ layers when they formed chimeras with normal blastocysts, suggesting that some types of somatic stem cells potentially maintain pluripotency and can be reprogrammed by some stimulation. We are trying to elucidate the molecular mechanisms involved in establishment of undifferentiated state of embryonic stem cells, regulation of differentiation and reprogramming of lineage-specific somatic stem cells.

Since the extrinsic cues are important for the regulation of cell fates in normal development, we are analyzing genes that are involved in cell-cell communication and cell adhesion. We screened genes which have putative signal sequences or transmembrane domains by in silico subtraction, and by the siRNA-based functional screening, we identified putative membrane associated genes which are essential for murine preimplantation development. We found that siRNAs against some of these genes caused developmental arrest of preimpleantation mouse embryos and suppression of ES cell self-renewal. We are also trying to construct hybridoma library against ES cell surface antigens and gene trap ES cell library against ES cell surface glycoproteins. The identification of cell surface molecules important for inducing and maintaining undifferentiated state of early embryonic stem cells should be useful in future tissue regeneration medicine.

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Center for Experimental Medicine

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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. Disruption of *Sept6*, a fusion partner gene of *Mixed Lineage Leukemia (MLL)*, does not affect the ontogeny, leukemogenesis induced by *MLL-SEPT6*, or the phenotype induced by the loss of *Sept4*.

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Septins are evolutionarily conserved GTP-binding proteins that can heteropolymerize into filaments. Recent studies have revealed that septins are involved in not only diverse normal cellular processes but also the pathogenesis of various diseases, including cancer. SEPT6 is ubiquitously expressed in tissues and one of the fusion partner genes of *MLL* in the 11q23 translocations implicated in acute leukemia. However,

the roles of this septin in vivo remain elusive. We have developed *Sept6*-deficient mice that exhibited neither gross abnormalities, changes in cytokinesis, nor spontaneous malignancy. *Sept6* deficiency did not cause any quantitative changes in any of the septins evaluated in this study, nor did it cause any additional changes in the *Sept4*-deficient mice. Even the depletion of Sept11, a close homolog of Sept6, did not affect the *Sept6*-null cells in vitro, thus implying a high degree of redundancy in the septin system. Furthermore, a loss of *Sept6* did not alter the phenotype of myeloproliferative disease induced by *MLL-SEPT6*, thus suggesting that Sept6 does not function as a tumor suppressor. To our knowledge, this is the first report demonstrating that a disruption of the translocation partner gene of *MLL* in 11q23 translocation does not contribute to leukemogenesis by the *MLL* fusion gene.

2. Phospholipase C-δ1 and -δ3 Are Essential in Trophoblast for Placental Development.

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Phosphoinositide-specific phospholipase C (PLC) is a key enzyme in phosphoinositide turnover and is involved in a variety of physiological functions. We analyzed *PLC δ1* knockout mice and found that PLCδ1 is required for the maintenance of skin homeostasis. However, there were no remarkable abnormalities except hair loss and runting in *PLC δ1* knockout mice, even though PLCδ1 is broadly distributed. Here, we report that mice lacking both *PLC δ1* and *PLC δ3* died at embryonic day 11.5 (E11.5) to E 13.5. *PLC δ1/PLC δ3* double-knockout mice exhibited severe disruption of the normal labyrinth architecture in the placenta and decreased placental vascularization, as well as abnormal proliferation and apoptosis of trophoblasts in the labyrinth area. Furthermore, *PLC δ1/PLC δ3* double-knockout embryos supplied with a normal placenta by the tetraploid aggregation method survived beyond E14.5, clearly indicating that the embryonic lethality is caused by a defect in trophoblasts. On the basis of these results, we conclude that PLCδ1 and PLCδ3 are essential in trophoblasts for placental development.

3. Infertility with Defective Spermiogenesis in Mice Lacking AF5q31, the Target of Chromosomal Translocation in Human Infant Leukemia.

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AF5q31 (also called *MCEF*) was identified by its involvement in chromosomal translocation with the gene *MLL* (mixed lineage leukemia), which is associated with infant acute lymphoblastic leukemia. Several potential roles have been proposed for AF5q31 and other family genes, but the specific requirements of AF5q31 during development remain unclear. Here, we show that AF5q31 is essential for spermatogenesis. Although most AF5q31-deficient mice died in utero and neonatally with impaired embryonic development and shrunken alveoli, respectively, 13% of AF5q31-deficient mice thrived as wild-type mice did. However, the male mice were sterile with azoospermia. Histological examinations revealed the arrest of germ cell development at the stage of spermiogenesis, and virtually no spermatozoa were seen in the epididymis. AF5q31 was found to be preferentially expressed in Sertoli cells. Furthermore, mutant mice displayed severely impaired expression of protamine 1, protamine 2, and transition protein 2, which are indispensable to compact the haploid genome within the sperm head, and an increase of apoptotic cells in seminiferous tubules. Thus, AF5q31 seems to function as a transcriptional regulator in testicular somatic cells and is essential for male germ cell differentiation and survival. These results may have clinical implications in the understanding of human male infertility.

4. IRF-7 is the master regulator of type-I interferon dependent immune responses.

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The type-I interferon (IFN-α/β) response is critical to immunity against viruses and can be triggered in many cell types by cytosolic detection of viral infection, or in differentiated plasmacytoid dendritic cells by the Toll-like receptor 9 (TLR9) subfamily, which generates signals via the adaptor MyD88 to elicit robust IFN induction1, 2, 3, 4. Using mice deficient in the *Irf7* gene (*Irf7-/-* mice), we show that the transcription factor IRF-7 is essential for the induction of IFN-α/β)) genes via the virus-activated, MyD88-independent pathway and the TLR-activated, MyD88-dependent pathway. Viral induction of MyD88-independent IFN-α/β genes is severely impaired in *Irf7-/-* fibroblasts. Consistently, *Irf7-/-* mice are more vulnerable than *Myd88-/-* mice to viral infection, and this correlates with a marked decrease in serum IFN levels, indicating the importance of the IRF-7-dependent induction of systemic IFN responses for innate antiviral immunity. Furthermore, robust induction of IFN production by activation of the TLR9 subfamily in plasmacytoid dendritic cells is entirely dependent on IRF-7, and this MyD88-IRF-7 pathway governs the induction of CD8+ T-cell responses. Thus, all elements of IFN responses, whether the systemic production of IFN in innate immunity or the local action of IFN from plasmacytoid dendritic cells in adaptive immunity, are under the control of IRF-7.

5. Exacerbation of Granuloma Formation in IL-1 Receptor Antagonist-Deficient Mice with Impaired Dendritic Cell Maturation Associated with Th2 Cytokine Production.

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Dendritic cell (DC) maturation at the site of inflammation and migration into draining lymph nodes is fundamental to initiate Ag-specific immune responses. Although several proinflammatory cytokines, including IL-1, are known to promote DC maturation in vitro, their contributions to DC activation and migration within peripheral inflamed tissue compartments are not yet fully understood. We show here that endogenous IL-1 receptor antagonist (IL-1ra) controls the activation state of liver-recruited DCs and their migration in a *Propionibacterium acnes*-induced murine granulomatous liver disease model. After *P. acnes* treatment, formation of portal tract-associated lymphoid tissue was conversely impaired in IL-1ra-deficient mice. IL-1ra-deficient mice developed hepatic granulomas within 3 days after *P. acnes* administration and showed a more pronounced granuloma formation than wild-type mice. Although sinusoidal granulomas contained numerous CD11c+ DCs at day 7, expressions of CCR7, IL-12p40 by these Dcs were dramatically decreased in IL-1ra-deficient mice, suggesting aberrant DC maturation and sinusoid portal migration in the absence of endogenous IL-1ra. This was accompanied with enhanced intrahepatic Th2 cytokine production and severe hepatocellular damage. Thus, hepatocyte-derived IL-1ra may control optimal activation and migration of inflammatory DCs within the liver and thereby determine the local immune responses in granulomatous liver disease.

6. Essential role of Flk-1 (VEGF receptor 2) tyrosine residue 1173 in vasculogenesis in mice.

Yoshiko Sakurai[26], Kaori Ohgimoto[26], Yuki Kataoka, Nobuaki Yoshida and Masabumi Shibuya[26]: [26]Divisions of Genetics, Institute of Medical Science, University of Tokyo.

Flk-1 (human counterpart, KDR) tyrosine kinase, which is one of the two VEGF receptors, is crucial for vascular development. Recently, we showed that, among tyrosine residues of KDR, tyrosine residues 1175 (Y1175, corresponding to Y1173 in murine Flk-1) and Y1214 (Y1212 in Flk-1) are autophosphorylated in response to VEGF, and that Y1175 is important for VEGF-dependent phospholipase Cγ/PKC/mitogen-activated protein kinase activation leading to DNA synthesis in cultured endothelial cells. However, the importance of these tyrosine residues in Flk-1/KDR *in vivo* is not yet known. To examine the role of these Flk-1 tyrosine residues

in vivo, we generated knock-in mice substituting Y1173 and Y1212 of the *Flk-1* gene with phenylalanine, respectively. As a result, *Flk-11173F* homozygous mice died between embryonic days 8.5 and 9.5 without any organized blood vessels or yolk sac blood islands, and hematopoietic progenitors were severely reduced, similar to the case of *Flk-1* null mice. In contrast, *Flk-11212 F* homozygous mice were viable and fertile. These results suggest that the signaling via Y 1173 of Flk-1 is essential for endothelial and hematopoietic development during embryogenesis.

7. Conditional control of gene expression for the analysis of lymphatic vascular system in mice.

Taeko Ichise, Hirotake Ichise, Seiji Shiozawa, Takashi Yamaguchi, Yuhko Chujo-Ayabe, Akiko Hori, Jun-ichi Matsuo, Osamu Iwata, Nobuaki Yoshida.

The lymphatic vascular system is thought to be responsible for edematous condition in patients, especially in those suffering from lymphedema. Recent studies show that lymphangiogenesis, as well as angiogenesis, also plays important roles on tumor metastasis. However, it has been still difficult to understand the lymphatic development because the analysis of lymphangiogenesis *in vivo* is often hindered by deleterious impairments other than a lymphatic vascular defect.

In order to overcome this problem, we are trying to utilize Cre/loxP binary system *in vivo* for the analysis of lymphatic vascular system. We have generated transgenic mouse lines expressing lacZ reporter gene and those expressing Cre recombinase gene under the transcriptional regulation of exogenous mouse vegfr-3 promoter. In VEGFR-3-lacZ transgenic mouse lines, lacZ gene was expressed in lymphatic endothelial cells of some tissues. The expression of Cre recombinase gene in VEGFR3-Cre transgenic mouse lines was verified with lymphatic endothelial cell-specific recombination of floxed ROSA26 loci. Using these Cre transgenic mouse strains, we have been able to regulate tissue-specific activation of floxed transgenes harboring cDNAs which encode angiogenic / lymphangiogenic factors. Some of these double transgenic mouse strains are postnatally lethal due to cardiovascular defects.

8. Analysis of the regulation during separation of the blood and lymphatic vascular networks in mice.

Hirotake Ichise, Taeko Ichise, Yoshihiro Hayata, Kaori Yamanaka, Nobuaki Yoshida

We have maintained and analyzed a novel spontaneous mutant mouse strain developing chylous ascites and lymphedema due to lymphatic abnormality. In the homozygous mutant mice, the blood flow is found not only in blood vessels but also in lymphatic vessels of intestine and a part of skin. The peripheral capillary-lacteal shunt at the intestinal villi is observed in homozygous mutant mice. It is thought to be cause for blood flow observed in lymphatics of the homozygous mutant mice. In our immunohistochemical study, VEGFR-3, one of the receptor tyrosine kinase regulating lymphangiogenesis, is expressed intensely in the intestinal lymphatic endothelial cells of the wild-type mice, but not in those of the homozygous mutant mice. The intestinal lymphatic vascular structure of the homozygous mutant mice is immature and dilated. In addition to the lymphatic defect, the intestinal vascular sturucture of blood vessels is also impaired in them. The candidate gene for this mutation is responsible for both angiogenesis and lymphangiogenesis on late stage of embryogenesis, and is thought to regulate them in tissue-specific manner.

Using the intersubspecific mapping between CAST/Ei strain and the mutant strain in 129/SvEv background, we have mapped the candidate locus to approximately 1Mb region, which contained 4 known genes and 3 unknown genes. We are trying to identify the candidate gene and the mutation within these genes through a transgenic rescue experiment and sequence analysis. We have also established germ-line-competent embryonic stem cell lines from blastocysts homozygous for the mutation. The mutation-bearing ES cells are useful for chimera analysis of the mutation in the vasculature and transgenic rescue of the mutation.

9. Pluripotency of mouse embryonic stem cells.

Masaki Shibayama, Takashi Osaka, Mio Furutani, Mitsuharu Sato and Nobuaki Yoshida

Stem cells are defined by their ability to give rise to various mature progeny while maintaining the capacity to self-renew. Mouse embryonic stem cells (ESCs) are used as a powerful tool to study gene functions through gene targeting in mice. However, the nature of ESC itself is largely unknown. Despite the findings showing the importance of some transcription factors, such as Oct-3/4, Stat3 and Nanog, in ESCs, we still do not know how pluripotency is established.

1) Analysis of PTB function in ESCs and mouse development.

To obtain a clue to understand ESC specific gene regulation, we focused on the promoter region of Rex-1 gene. Rex-1 is one of the marker genes for undifferentiated ESCs and rapidly down regulated by retinoic acid treatment. Previous study has shown that Rex-1 is regulated by Oct-3/4 and unidentified DNA binding activity, Rox-1. We tried to identify Rox-1 by protein purification and found PTB (polypyrimidine tract binding protein) as a protein which shows specific binding to Rox-1 site.

While PTB was originally identified as a member of hnRNP complex, subsequent studies showed multiple functions of PTB in regulation of translation and transcription. Although the expression of PTB is ubiquitous and detected in early stage of mouse development, we did not know the role of PTB for stem cell function.

To study the requirement for PTB during mouse ontogeny, we disrupted PTB gene in mouse and found homozygous mutant was lethal at the stage of implantation. The blastocyst of homozygous mutant shows normal morphology, but no ES cell line could be established from the mutant. These results suggest the pivotal role of PTB for the maintenance of pluripotent stem cells in embryos.

In order to explore further the role of PTB in stem cells, we disrupted the PTB gene in ESCs. PTB null cells were viable and formed apparently normal colonies, but they displayed severe defects in proliferation. In addition, the induction of developmental markers, such as Fgf-5 and GATA-1, were defective in PTB null ESCs. These results demonstrate that PTB is essential for maintaining ESCs.

2) Early transposon (Etn) and pluripotency of ESCs.

One idea to explain how ESCs acquire pluripotency is that there are a small number of key factors that govern the stemness in ESCs. Based on this idea, many researchers have been looking for a factor that is specific in undifferentiated ESCs. We took the same way in PTB analysis described above. However, we still do not know what establishes pluripotency in ESCs. All we know is that stem cells show miscellaneous gene expression pattern which would be a sign of pluripotency. In other words, many genes are in "ready-to-go" state for transcription in stem cells. Maintaining stem cell genes in this state is the point that should be elucidated.

During a screening for a gene that is down regulated by retinoic acid treatment in ESCs, we found one of the retrotransposons, ETn1, was specifically expressed in pluripotent cells. ETn1 is located in every chromosome and there are about 400 copies per cells. DNase I hypersensitivity assay showed that genomes near Etn1 sequence is highly sensitive to DNase I in undifferentiated ESCs. This means chromatin structure in ETn surroundings is loose and euchromatic. We hypothesized that the machinery actively transcribes ETn is critical for maintaining many genes in "ready-to-go" state.

We started to narrow the promoter region which is essential for ETn transcription and identified DNA binding activity which was specific in undifferentiated ESCs. We are trying to identify the factor that regulates ETn expression to elucidate the mechanism which establishes pluripotency in ESCs.

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Stem cells are generally defined as clonogenic cells capable of both self-renewal and multilineage differentiation. Because of these unique properties, stem cells offer the novel and exciting possibility of organ reconstitution in place of transplanted or artificial organs in the treatment of organ failure. In addition, stem cells are considered as ideal target cells for gene therapy. The goal of this laboratory is to provide new insights into stem cell biology as well as approaches to therapeutic intervention for various organ failures.

1. The mechanism of hematopoietic stem cell self-renewal and commitment.

a) Selective activation of STAT5 unveils its role in stem cell self-renewal in normal and leukemic hematopoiesis

Although the concept of a leukemic stem cell system has recently been well accepted, its nature and the underlying molecular mechanisms remain obscure. Constitutive activation of signal transducers and activators of transcription 3 (STAT3) and STAT5 is frequently detected in various hematopoietic tumors. To evaluate their role in normal and leukemic stem cells, we took advantage of constitutively active STAT mutants to activate STAT signaling selectively in hematopoietic stem cells (HSCs). Activation of STAT5 in CD34− c-Kit+ Sca-1+ lineage marker− (CD 34−KSL) HSCs led to a drastic expansion of multipotential progenitors and promoted HSC self-renewal ex vivo. In sharp contrast, STAT3 was demonstrated to be dispensable for the HSC maintenance in vivo, and its activation facilitated lineage commitment of HSCs in vitro. In a mouse model of myeloproliferative disease (MPD), sustained STAT5 activation in CD34− KSL HSCs but not in CD34+KSL multipotential progenitors induced fatal MPD, indicating that the capacity of STAT5 to promote self-renewal of hematopoietic stem cells is crucial to MPD development. Our findings collectively establish a specific role for STAT5 in self-renewal of normal as well as leukemic stem cells.

b) Endomucin, a CD34-like sialomucin, marks hematopoietic stem cells throughout development

To detect as yet unidentified cell-surface molecules specific to hematopoietic stem cells (HSCs), a modified signal sequence trap was successfully applied to mouse bone marrow (BM) $CD34^{-}c\text{-}Kit^{+}Sca\text{-}1^{+}lineage^{-}$ ($CD34^{-}KSL$) HSCs. One of the identified molecules, Endomucin, is an endothelial sialomucin closely related

to CD34. High-level expression of Endomucin was confined to the BM KSL hematopoietic stem and progenitor cells and, importantly, long-term repopulating (LTR)-HSCs were exclusively present in the Endomucin$^+$CD34$^-$KSL population. Notably, in the yolk sac, Endomucin expression separated multipotential hematopoietic cells from committed erythroid progenitors in the cell fraction positive for CD41, an early embryonic hematopoietic marker. Furthermore, developing HSCs in the intraembryonic aorta-gonad-mesonephros (AGM) region were highly enriched in the CD45$^-$CD41$^+$Endomucin$^+$ fraction at day 10.5 of gestation (E10.5) and in the CD 45$^+$CD41$^+$Endomucin$^+$ fraction at E11.5. Detailed analyses of these fractions uncovered a drastic change in their BM repopulating capacity as well as *in vitro* cytokine responsiveness within this narrow time window. Our findings establish Endomucin as a novel cell-surface marker for LTR-HSCs throughout development and provide a powerful tool in understanding HSC ontogeny.

2. Stem/progenitor cells in hepato-biliary system

a) Prospective isolation of multipotent pancreatic progenitors using flow-cytometric cell sorting

Previously, we have identified hepatic stem cells in the fetal liver that can differentiate into hepatocytes and cholangiocytes. Using the same approach, we attempted to a prospective isolation of mouse adult liver stem cells. It has been shown that adult liver has a tremendous regenerative activity, however, it remains elusive whether hepatic stem cells are present and play a role in regeneration in adult life. Using the FACS cell sorting system, we purified and cultured the CD45$^-$Ter119$^-$c-Kit$^-$Sca1$^-$CD49f$^+$ cell fraction. These cells proliferated well and differentiated to CK19$^+$ endothelial cells after 7-9 days of culture, suggesting that adult hepatic stem/progenitor cells were enriched in this cell fraction.

b) Mechanism of fetal liver differentiation and maturation

Fetal hepatocytes have little metabolic functions and acquire gene expression in several genes during development. The mature-hepatocyte specific gene expression is regulated by transcription factors such as hepatocyte nuclear factor and C/EBPs. In addition, we recently found that expression of DNA methyltransferases (dnmt 3a and 3b) was decreased during fetal and adult liver development, suggesting that epigenetic changes between fetal and adult hepatocytes contribute the regulation of mature liver-specific gene expression. Based on these findings, we are currently analyzing fetal liver-specific dnmt 3a and 3b knockout mice using alpha-fetoprotein promoter Cre transgenic mice.

3. Integrin signaling in various cells: platelets, mast cells, and hematopoietic stem cells

Integrin is an essential adhesion receptor for cell and organ function. αIIbβ3 expressed in platelets is a key integrin for hemostasis and thrombosis. Ligand (i.e., fibrinogen, fibronectin) binding to αIIbβ3 in platelets and megakaryocytes is tightly regulated by "inside-out" signals that regulate integrin conformational changes and clustering. In turn, ligand binding triggers "outside-in" signals that are required for efficient platelet adhesion and irreversible aggregation. We, in collaboration with Dr. Sanford Shattil's group at the University of California, San Diego, had demonstrated the different roles by protein kinase C (PKC) subtype (PKCβ and PKCθ) in outside-in signaling from αIIbβ3. Furthermore, in collaboration with Dr. Jiro Kitaura at the IMS, University of Tokyo, we had showed that mast cells express integrin αIIbβ3 for adhesive function as well as platelets. The current focus is on elucidating the signals from integrin αIIbβ3 and α4β1 in hematopoietic stem cells for maintenance of stemness and self-renewal in the bone marrow niche.

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