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: 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, Soo-hyun Chung, Kazusuke Shimamori, Keiko Yamabe, 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 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 found 1,467 genes that were differentially expressed from the normal control mice by greater than threefold in one of these animal models. The gene expression profiles of the two models correlated well. We extracted 554 genes whose expression significantly changed in both models, assuming that pathogenically important genes at the effector phase would change 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 did not distribute evenly on the chromosome but formed clusters. These identified gene clusters include 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. The activation of these gene clusters suggests that antigen presentation and lymphocyte chemotaxis are important for the development of ar-

thritis. Moreover, by searching for such clusters, we could detect genes with marginal expression changes. These gene clusters include schlafen and membrane-spanning four-domains subfamily A genes whose function in arthritis has not yet been determined. Thus, by combining two etiologically different RA models, we succeeded in efficiently extracting genes functioning in the development of arthritis at the effector phase. Furthermore, we demonstrated that identification of gene clusters by transcriptome mapping is a useful way to find potentially pathogenic genes among genes whose expression change is only marginal.

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. We are now analyzing the function of the genes that are activated in the arthritic joints by generating gene targeted mice of these genes.

2. Analysis of the roles of IL-17 family genes in the immune system

Harumichi Ishigame, Yutaka Komiyama, Yuko Tanahashi, Susumu Nakae, Taizo Matsuki, Aya Nambu, Shigeru Kakuta, Katsuko Sudo, 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 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. Recently, it was shown that IL-17 is produced by a newly discovered $CD4^+$ T cell subset, Th17. Now, many diseases that were though to be induced by Th1 or Th2 are proven to be induced by Th17. Thus, we are analyzing the roles of IL-17 and its family molecules in inflammatory diseases and host defense mechanism against infection.

As the expression of IL-17 is augmented in multiple sclerosis (MS), we examined the involvement of this cytokine in these diseases using IL-$17^{-/-}$ murine disease models. We found that the development of experimental autoimmune encephalomyelitis (EAE), the rodent model of MS, was significantly suppressed in IL-$17^{-/-}$ mice; these animals exhibited a delayed onset, a reduced maximum severity scores, ameliorated histological changes, and early recovery. T cell sensitization against myelin oligodendrocyte glycoprotein was reduced in IL-$17^{-/-}$ mice upon sensitization. The major producer of IL-17 upon treatement with MOG was $CD4^+$ T cells rather than $CD8^+$ T cells, and adoptive transfer of IL-$17^{-/-}$ $CD4^+$ T cells inefficiently induced EAE in recipient mice. Notably, IL-17-producing T cells were increased in IFN-$\gamma^{-/-}$ cells, while IFN-γ-producing cells were increased in IL-$17^{-/-}$ cells, suggesting that IL-17 and IFN-γ mutually regulate IFN-γ and IL-17 production. These observations indicate that IL-17 rather than IFN-γ plays a crucial role in the development of EAE.

3. Dectin-1 is required for host defense against *Pneumocystis carinii*, but not Candida albicans

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Dectin-1 is a C-type lectin involved in the recognition of β-glucans found in cell walls of fungi. We generated Dectin1-deficient mice to examine the importance of Dectin-1 in defense against pathogenic fungi. *In vitro*, β-glucan-induced cytokine production from wild-type dendritic cells (DCs) and macrophages was abolished in *Dectin1*$^{-/-}$ cells. *In vivo*, compared to wild-type mice, *Dectin1*$^{-/-}$ mice were more susceptible to pneumocystis infection even though cytokine production was normal. However, pneumocystis-infected *Dectin1*$^{-/-}$ macrophages did demonstrate defective production of reactive oxygen. In contrast those results, wild-type and *Dectin1*$^{-/-}$ mice were equally susceptible to candida infection. Thus, Dectin-1 is required for immune responses to some fungal infection, as protective immunity to pneumocystis,

but not candida, requires Dectin-1 for production of anti-fungal reactive oxygen.

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

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Proinflammatory cytokines including IL-1 and IL-6 exert pleiotropic effects on the neuro-immuno-endocrine system. Previously, we showed that IL-1 receptor antagonist deficient (IL-1Ra$^{-/-}$) mice show a lean phenotype due to an abnormal lipid metabolism. On the contrary, it was reported that IL-6$^{-/-}$ mice exhibit obesity after 6 months of age. This study sought to assess the roles of IL-1 and IL-6 in body weight homeostasis. We generated mice deficient in IL-6 and IL-1Ra (IL-6$^{-/-}$IL-1Ra$^{-/-}$), and IL-6, IL-1α, and IL-1β (IL-6$^{-/-}$IL-1$^{-/-}$). IL-6$^{-/-}$IL-1Ra$^{-/-}$ mice exhibited a lean phenotype, similarly to IL-1 Ra$^{-/-}$mice. On the other hand, IL-6$^{-/-}$IL-1$^{-/-}$ mice became obese as early as 10 weeks of age, while IL-1$^{-/-}$ mice and IL-6$^{-/-}$ mice were normal at this age. The daily food intake was significantly higher in IL-6$^{-/-}$IL-1$^{-/-}$ mice than in IL-6$^{-/-}$IL-1$^{+/-}$ mice, while energy expenditure was comparable in these two strains. Acute anorexia induced by peripheral administration of IL -1 was significantly suppressed in IL-6$^{-/-}$IL-1$^{-/-}$ mice, but not in IL-1$^{-/-}$ mice or IL-6$^{-/-}$ mice, as compared to WT mice. These results indicate that IL-1 and IL-6 are both involved in the regulation of body fat in a redundant manner in young mice. We are now analyzing the roles of IL-1 in the central nervous system, because IL-1 is induced in the brain upon exposure to stress and affects various neuronal functions such as appetite, sleep and emotion. IL-1$^{-/-}$ and IL1 Ra$^{-/-}$ mice should be useful to analyze the functions of these cytokines in the neuronal system.

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

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

2', 5'-oligoadenylate synthetase (OAS) is one of IFN-inducible proteins and involved in the antiviral mechanism through activation of RNaseL. In humans, there are four classes of OAS genes, short (OAS1), middle (OAS2), and long (OAS3) form genes and one OAS-like protein (OASL) gene. On the other hand, in mice, we previously showed that the OAS1 gene is multiplicated into 8 genes and the OASL gene is duplicated. The short form OAS has a set of the essential motifs for the OAS 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 OAS activity. The physiological roles and functional difference among these genes, however, 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 with targeted gene disruptions.

Oas1a/Oas2 deficient (flox) mice have two *lox*P sites inserted into the flanking regions of the Oas gene cluster. A large size deletion (~170kb) between *lox*P 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. We are now analyzing the effects of these gene deficiencies on the host defense against viral infection and various inflammatory responses including Concanavalin A-induced hepatitis. We are also analyzing the roles of genes that are activated in the joints of rheumatoid arthritis models in allergic responses and tumor development.

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

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Studies of AIDS pathogenesis and development of therapeutic drugs for AIDS have been hampered by the lack of appropriate small animal models for AIDS, because human immunodeficiency virus type-1 (HIV-1) cannot infect and replicate in mice because the structure of some host factors necessary for HIV-1 replication is different between mice and humans. As primate models are difficult to handle, expensive and require expensive facilities, genetically not well characterized, often infected with pathogens and sometimes dangerous, and ethically less permissible, we are trying to generate mouse models for AIDS. 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 (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.

Mouse cells do not support HIV-1 replication because of host range barriers at steps including virus entry, transcription, RNA splicing, polyprotein processing, assembly, and release. The exact mechanisms for the suppression, however, are not completely understood. To elucidate further the barriers against HIV-1 replication in mouse cells, we analyzed the replication of the virus in lymphocytes from human CD4/CXCR4 transgenic mice. Although primary splenocytes and thymocytes allowed the entry and reverse transcription of HIV-1, the integration efficiency of the viral DNA was greatly reduced in these cells relative to human peripheral blood mononuclear cells (PBMCs), suggesting an additional block(s) before or at the point of host chromosome integration of the viral DNA. Pre-integration processes were further analyzed using HIV-1 pseudotyped viruses. The reverse transcription step of HIV-1 pseudotyped with the envelope of murine leukemia virus (MuLV) or vesicular stomatitis virus glycoprotein (VSV-G) was efficiently supported in both human and mouse cells, but nuclear import of the pre-integration complex (PIC) of HIV-1 was blocked in mouse cells. We found that green fluorescent protein (GFP)-labeled HIV-1 integrase, which is known to be important in the nuclear localization of the PIC, could not be imported into the nucleus of mouse cells, in contrast to human cells. On the other hand, GFP-Vpr localized exclusively to the nuclei of both mouse and human cells. These observations suggest that, due to the dysfunction of integrase, the nuclear localization of PIC is suppressed in mouse cells. We are now analyzing the host factors which interact with integrase and trying to generate transgenic mice carrying the human gene for this host barrier factor. In another attempt, we are further analyzing the host barrier steps and identifying the responsible genes.

7. Studies on the molecular mechanisms functioning in the early development of mouse embryos

Kenjiro Adachi, Seiji Takashima, Hiroaki Okae, and Yoichiro Iwakura

Blastocyst formation during mammalian preimplantation development is a unique developmental process that involves lineage segregation between the inner cell mass and the trophectoderm. To elucidate the molecular mechanisms underlying blastocyst formation, we have functionally screened a subset of preimplantation embryo-associated transcripts using siRNA and identified *Bysl (bystin-like)* as an essential gene for this process. The development of embryos injected with *Bysl* siRNA was arrested just prior to blastocyst formation, resulting in a defect in trophectoderm differentiation. Silencing of *Bysl* using an episomal shRNA expression vector inhibited proliferation of embryonic stem cells. Exogenously expressed Bysl tagged with a fluorescent protein was concentrated in the nucleolus with a diffuse nucleoplasmic distribution. Furthermore, loss of *Bysl* function using RNAi or dominant negative mutants caused defects in 40 S ribosomal subunit biogenesis. These findings provide evidence for a crucial role of Bysl as an integral factor for ribosome biogenesis, and suggest a critical dependence of blastocyst formation on active translation machinery.

As our previous studies suggest that cell-cell interaction during 8 cell compaction stage is important for the cell lineage determination during blastocyst formation, we are further trying to identify these lineage determination responsible genes which are involved in cell-cell interaction using in silico screening and RNAi techniques. We have isolated several candidate genes and are now analyzing the functions of these genes. In another approach, we are trying to identify these lineage determination responsible genes using a secretary trap method. We screened approximately 1000 trapped ES clones. From these clones, we obtained 34 clones that are specific to ES cells and encode membrane proteins. Among these clones, 12 genes were already mutated in mice, of which 10 showed recessive lethal phenotype, indicating that this screening method is extremely effective to enrich developmentally important genes. We are now trying to produce the mutant mice from the residual clones.

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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. Morphological Change Caused by Loss of the Taxon-Specific Polyalanine Tract in Hoxd-13.

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Sequence comparison of Hoxd-13 among vertebrates revealed the presence of taxon-specific polyalanine tracts in amniotes. To investigate their function at the organismal level, we replaced the wild-type Hoxd-13 gene with one lacking the 15-residue polyalanine tract by using homologous recombination. Sesamoid bone formation in knock-in mice was different from that in the wild type; this was observed not only in the homozygotes but also in heterozygotes. The present study provides the first direct evidence that taxon-specific homopolymeric amino acid repeats are involved in phenotypic diversification at the organismal level.

2. Azoospermia in mice with targeted disruption of the *Brek/Lmtk2* (brain-enriched kinase/lemur tyrosine kinase 2) gene.

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Mamiko Maekawa[3], Tohru Tezuka[2], Takahisa Nakamura[2], Takanobu Nakazawa[2], Kazumasa Yokoyama[2], Nobuaki Yoshida, Kiyotaka Toshimori[3], and Tadashi Yamamoto[2]: [2]Division of Oncology, Institute of Medical Science, University of Tokyo, [3]Department of Anatomy and Developmental Biology, Graduate School of Medicine, Chiba University.

Brek/Lmtk2 (brain-enriched kinase/lemur tyrosine kinase 2) is a member of the Aatyk family of kinases that comprises Aatyk1, Brek/Lmtk 2/Aatyk2, and Aatyk3. Although several potential roles have been proposed for Brek and other Aatyk family members, the physiological functions of these kinases remain unclear. Here, we report that *Brek*$^{-/-}$ male mice are infertile, with azoospermia. Detailed histological analysis revealed that *Brek*$^{-/-}$ germ cells differentiated normally until the round-spermatid stage, but failed to undergo the normal change in morphology to become elongated spermatids. Testicular somatic cells appeared normal in these mice. Expression of Brek in testis was restricted to the germ cells, suggesting that the maturations of germ cells in *Brek*$^{-/-}$ mice are affected in a cell-autonomous manner. On the basis of these findings, we concluded that Brek is essential for a late stage of spermatogenesis. Further clarification of the mechanism by which Brek regulates spermatogenesis may help identify new targets for reproductive contraceptives and treatments against infertility.

3. Efficient sequential gene regulation via FLP- and Cre-recombinase using adenovirus vector in mammalian cells including mouse ES cells.

Saki Kondo[4], Yuzuka Takahashi[4], Seiji Shiozawa, Hirotake Ichise, Nobuaki Yoshida, Yumi Kanegae[4], and Izumi Saito[4]: [4]Laboratory of Molecular Genetics, Institute of Medical Science, University of Tokyo.

Site-specific recombinase is widely applied for the regulation of gene expression because its regulatory action is strict and efficient. However, each system can mediate regulation of only one gene at a time. Here, we demonstrate efficient "sequential" gene regulation using Cre- and FLP-expressing recombinant adenovirus (rAd) in two different monitor cell lines, for regulation of one gene (OFF-ON-OFF) and for two genes (ON-OFF and OFF-ON, independently). Generally, serial use of Cre-and FLP-expressing rAd tends to cause significant cytotoxicity, but we here described optimum dose of the rAds for serial regulation. We also established an efficient method of rAd infection to mouse ES cell lines after removing feeder cells, showing that this system is useful for removal of FRT-flanked drug-resistance gene cassette from recombinant ES cells prior to introduction of ES cells into blastocytes for chimeric mice production. Because our sequential gene-regulation system offers efficient purpose-gene regulation and strict OFF-regulation, it is potentially valuable for elucidating not only novel gene functions using cDNA microarray analysis but also for "gene switching" in development and regeneration research.

4. The murine homolog of SALL4, a causative gene in Okihiro syndrome, is essential for embryonic stem cell proliferation, and cooperates with Sall1 in anorectal, heart, brain, and kidney development.

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Mutations in *SALL4*, the human homolog of the *Drosophila* homeotic gene *spalt (sal)*, cause the autosomal dominant disorder known as Okihiro syndrome. In this study, we show that a targeted null mutation in the mouse *Sall4* gene leads to lethality during peri-implantation. Growth of the inner cell mass from the knockout blastocysts was reduced, and *Sall4*-null embryonic stem (ES) cells proliferated poorly with no aberrant differentiation. Furthermore, we demonstrated that anorectal and heart anomalies in Okihiro syndrome are caused by *Sall4* haploinsufficiency and that *Sall4/Sall1* heterozygotes exhibited an increased incidence of anorectal and heart anomalies, exencephaly and kidney agenesis. Sall4 and Sall1 formed heterodimers, and a truncated Sall1 caused mislocalization of Sall4 in the heterochromatin; thus, some symptoms of Townes-Brocks syndrome caused by SALL1 truncations could result from SALL4 inhibition.

5. Genetic marking of hematopoietic stem and endothelial cells: Identification of the Tmtsp gene encoding a novel cell-surface protein with the thrombospondin-1 domain.

Shin-ichiro Takayanagi[9], Takashi Hiroyama[10], Satoshi Yamazaki[9], Tomoko Nakajima[11], Yohei Morita[9], Joichi Usui[9], Koji Eto[9], Tsutomu Motohashi[12], Kensuke Shiomi[13], Kazuko Keino-Masu[13], Masayuki Masu[13], Yuichi Oike[14], Shigeo Mori[15], Nobuaki Yoshida, Atsushi Iwama[16], and Hiromitsu Nakauchi[9]: [9]Laboratory of Stem Cell Therapy, the Center for Experimental Medicine, Institute of Medical Science, University of Tokyo, [10]Cell Engineering Division, RIKEN BioResource Center, [11]Division of Cancer Cell Research, Department of Cancer Biology Institute of Medical Science, University of Tokyo, [12] Department of Tissue and Organ Development, Regeneration and Advanced Medical Science, Gifu University Graduate School of Medicine, [13]Department of Molecular Neurobiology, Institute of Basic Medical Sciences, Graduate School of Comprehensive Human Sciences, University of Tsukuba, [14]Department of Cell Differentiation, the Sakaguchi Laboratory, School of Medicine, Keio University, [15]Department of Pathology, Teikyo University School of Medicine, [16]Department of Cellular and Molecular Medicine, Graduate School of Medicine, Chiba University.

Using an *in silico* database search, we identified a novel gene encoding a cell surface molecule with a thrombospondin domain, and designated the gene as transmembrane molecule with thrombospondin module (*Tmtsp*). Expression profiling of *Tmtsp* using specific monoclonal antibodies and *Venus*, a variant of yellow fluorescent protein knock-in mice in the *Tmtsp* locus, demonstrated its specific expression in hematopoietic and endothelial cells. In lymphohematopoietic cells, *Tmtsp* was predominantly expressed in hematopoietic stem and progenitor cells, and the level of expression gradually declined as the cells differentiated. Venus expression faithfully traced the expression of *Tmtsp*, and the level of Venus expression correlated well to the in vitro hematopoietic activity as well as the in vivo bone marrow repopulating capacity. Notably, Venus expression marked the development of definitive hematopoiesis in both the extraembryonic yolk sac and the intraembryonic aorta-gonadmesonephros (AGM) region and, in combination with CD41, strikingly promoted the enrichment of developing progenitors in the CD41+Venushigh fraction at embryonic day 10.5 (E10.5). In this context, *Tmtsp* is a novel marker gene for primitive hematopoietic cells and endothelial cells, and *TmtspVenus*/+ mice would serve as a valuable mouse model for the analysis of both embryonic and adult hematopoiesis, as well as for vascular biology.

6. An Establishment of Mouse Embryonic Stem Cells Derived from (C57BL/6×129) F 1 Blastocysts for High-throughput Gene Targeting.

Taeko Ichise, Reiko Sakamoto, Hirotake Ichise, Nobuaki Yoshida.

The whole genome sequence of a widely used mouse strain, C57BL/6J(B6), has been completed and almost all clones of RPCI23-BAC B6 mouse genomic DNA library are mapped on the whole genome contig of the mouse. The BAC clones harboring genes of interest and their DNA sequences can be obtained speedily and inexpensively. Moreover, recombinase-mediated modification of plasmid DNAs, the so-called "recombeneering," has been established and utilized for high-throughput DNA engineering. Hence, construction of a gene-targeting vector from a B6 BAC clone is a preferable and time-saving method. However, B6-derived BAC insert DNAs are non-isogenic to genomic DNAs of widely used ES cell lines derived from 129 substrains, though isogenic DNAs are preferred for construction of targeting vectors because mouse strain differences of DNA sequences between targeting vectors and genomic DNAs of ES cells can reduce frequencies of homologous recombination.

To utilize B6 BAC clones for gene targeting without the "non-isogenic DNA" problem, we established a new ES cell line derived from (B6 ×129) F1 blastocysts. The ES cells can contribute efficiently to both somatic and germ cells in chimeric mice as determined by coat color and germ-line transmission. We also obtained genetically modified ES cells by using the ES cell line and a B6 BAC-based targeting vector. Our new ES cell line will be helpful for BAC-assisted high -throughput genetic modification of the mouse.

7. Generation of Transgenic Mice Expressing Tamoxifen-inducible Cre Recombinase for Temporal Genetic Modification *In Vivo*.

Akiko Hori, Hirotake Ichise, Taeko Ichise, Saki Kondoh[17], Yumi Kanegae[17], Izumu Saito[17], Nobuaki Yoshida: [17]Laboratory of Molecular Genetics, IMSUT

Cre/loxP recombination system enables us to perform conditional genetic modification in the

mouse; loxP-flanked sequences are removed from modified loci in the tissue-specific manner, based on tissue-specific promoter-driven Cre expression. However, temporal genetic modification, which is often needed to circumvent unexpected lethal phenotype of the genetically engineered mice, cannot be regulated by tissue-specific promoters used for transgenesis.

To perform the temporal genetic modification, we generated transgenic mice expressing tamoxifen-inducible Cre recombinase via ES cell-mediated transgenesis. We found that administration of hydroxytamoxifen induced Cre-mediated recombination in the ES cells expressing the tamoxifen-inducible Cre recombinase gene. We are now determining whether tamoxifen-dependent Cre/loxP recombination can be induced in the transgenic mice.

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

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

Transgenically expressed temperature-sensitive SV40 large T antigen (tsA58 T Ag) has been utilized for immortalization and maintenance of terminally differentiated cells. However, its oncogenic activities *in vivo* lead to hyperproliferation of T Ag-expressing cells, resulting in unwanted lethal phenotypes. In order to circumvent this problem, we established a transgenic mouse line, termed "T26," expressing tsA 58 T Ag in Cre-loxP recombination-dependent manner. T26 mice express β-geo in a variety of tissues throughout their development, whereas T26 mice harboring Cre transgene express tsA58 T Ag instead of β-geo. tsA58 T Ag-expressing endothelial cells of T26/Tie2-Cre double transgenic mice are obtained and maintained by a simple method for at least 1-2 months without alteration of endogenous cell characteristics. These results demonstrate that our T26 mice model, in combination with Cre transgenic mice model, is a useful tool for the simple and convenient isolation and maintenance of somatic cells, providing an accessible model system for cell biology.

9. An Analysis of the Role of VEGF-C Signal in Blood Vascular Development in the Mouse

Seiji Shiozawa, Hirotake Ichise, Akiko Hori, Taeko Ichise, Nobuaki Yoshida

Vascular endothelial growth factor receptor-3 (VEGFR-3), a receptor for vascular endothelial growth factors (VEGF)-C and -D, is predominantly expressed not only on lymphatic endothelial cells but on vascular endothelial cells in early embryonic development. VEGFR-3-deficient mice fail to form large blood vessels and die before the emergence of lymphatic vessel, suggesting that VEGFR-3 plays a critical role in blood vascular development. However, knockout mice for either VEGF-C or -D exhibit no overt blood vascular phenotype. The role of VEGFR-3 signaling in blood vascular development is still unknown.

In order to analyze the function of VEGFR-3 signaling during embryonic vascular development *in vivo*, we established a transgenic ES cell line and a transgenic mouse line expressing VEGF-C in Cre-loxP recombination-dependent manner. We found that endothelial proliferation and enlargement of the blood vasculature were induced in embryoid bodies and yolk sacs of early post-implantation embryos overexpressing VEGF-C. We are performing detail analyses to understand the role of VEGFR-3 signaling in the blood vasculature.

10. A Genetic Analysis of the Generation and Separation of Blood and Lymphatic Vasculature in the Mouse

Hirotake Ichise, Taeko Ichise, Osamu Otani[18], Nobuaki Yoshida: [18]Department of Anatomy, Graduate School of Medicine and Pharmaceutical Sciences for Research, University of Toyama,

Blood vascular and lymphatic endothelial cells share a common developmental origin, as well as molecular mechanisms of proliferation, motility, and cell survival. The differentiation of lymphatic endothelial cells from a subset of venous endothelial cells is initiated by the transcription factor Prox1, followed by VEGF-C-induced centrifugal sprouting and budding of Prox-1+ endothelial cells. Recent studies have shown that bone marrow-derived circulating cells expressing Syk and SLP-76 participate in the formation and maintenance of lymphatic vasculature separately from the blood vasculature. However, the mechanism of the vascular separation is still largely unknown.

We identified a spontaneous mutant mouse strain exhibiting chylous ascites and blood-lymphatic shunt and found a candidate gene for the mutation by positional candidate cloning. We are now investigating the importance of cells for coordinating the generation and separation of blood and lymphatic vasculature.

11. Function of polypyrimidine tract binding protein in mouse embryonic stem cells

Satona Ohno, Hidehiko Tamura, Takashi Osaka, Mitsuharu Sato and Nobuaki Yoshida

Polypyrimidine tract binding protein (PTB), originally identified as an RNA binding protein in hnRNP complex, was also known to be involved in the regulation of transcription and translation. In the analysis of Rex-1 gene promoter, we found PTB bind to polypyrimidine sequence within the promoter.

To study the role of PTB for embryonic stem (ES) cell function, we established PTB deficient ES cell lines by gene targeting. The PTB deficient cells form normal colonies, but double thymidine block analysis revealed that G2/M progression was delayed in these cells. The expression levels of cyclinA or cyclinB were not affected by PTB deficiency. It is still unclear whether PTB directly regulates cell cycle machinery.

12. PTB negatively regulates brain PTB expression

Hidehiko Tamura, Mitsuharu Sato and Nobuaki Yoshida

During the course of analysis in PTB deficient ES cells, we found one of the PTB family members, brain PTB (brPTB/PTBP2) was up regulated in the absence of PTB. To test whether PTB suppresses brPTB promoter, we generated brPTB-luciferase construct and cotransfected with PTB expression vector into PTB deficient ES cells. The promoter activity was suppressed to 50% of control when PTB expression vector was cotransfected. In addition, endogenous brPTB expression was also down regulated by retrovirus infection expressing PTB in PTB deficient ES cells. These results explain why PTB and brPTB are exclusively expressed in mouse development.

13. Analysis of Fbxl10 in mouse embryonic stem cells

Tsuyoshi Fukuda, Mitsuharu Sato and Nobuaki Yoshida

Recently, one of the f-box protein family, Fbxl 10, was shown to have histone demethylase activity. To examine the role of Fbxl10 for the ES cell function, we analyzed the expression of Fbxl 10 mRNA in mouse ES cells. Two bands were detected in Northern blotting analysis when full length Fbxl10 cDNA or 3′ half of the cDNA fragment was used as a probe, but with 5′ half of the cDNA probe, only upper band was hybridized. On database, we noticed Fbxl10 has an internal promoter located between exon 12 and exon 13 of full length Fbxl10. The shorter transcript, which may corresponds to the lower band detected in ES cells, does not encode JmjC domain that is responsible for the histone demethylase activity. We postulated that the short form of Fbxl10 modulates histone demethylase activity of Fbxl10. To test this idea, we designed shRNA targeting only short form of Fbxl10 and successfully down regulated the transcript. We are analyzing the effect of the down regulation for ES cell pluripotency and the methylation state of the histone.

14. Activation of early retrotransposon (ETn) in mouse embryonic stem cells

Mio Furutani, Reiko Sakamoto, Nobuaki Yoshida and Mitsuharu Sato

We isolated early retrotransposon (ETn) as an ES or EC (embryonal carcinoma) cell specific gene by differential hybridization. ETn is an LTR type retrotransposon, and over 900 ETn LTRs are distributed throughout the mouse genome. Although ETn does not encode for any protein, it is abundantly transcribed in undifferentiated ES or EC cells. We are interested in their activation mechanism, and trying to know whether the transcription from such a repetitive sequence contributes to ES cell pluripotency. Our idea is that the activation of repetitive transcription units within the genome is essential for maintaining ES cell-specific chromatin structure and gene expressions. In order to understand the activation mechanism of ETn, we have dissected ETn LTR and identified the regulatory sequence that is essential for LTR transcriptional activity. Within the sequence, undifferentiated cell-specific DNA binding activity is observed. To identify the factor responsible for the ES cell-specific ETn transcription, we have performed protein purification and sought the DNA binding activity seen in the regulatory sequence. From the reasons listed below, we supposed BORIS (brother of regulator of imprinted sites) as the regulator of ES cell-specific ETn expression.

1) There is the CCCTC motif in the regulatory sequence.
2) BORIS is expressed in ES cells and down regulated during retinoic acid induced differentiation.
3) Proteins recovered from 75-100 kDa region on SDS-PAGE give the DNA binding activity on the regulatory sequence.

4) The shifted band observed with the regulatory sequence in EMSA is competed by the non-labeled FII sequence (which is known to bind BORIS).
5) When BORIS is over expressed in NIH 3T3, the DNA binding activity found in ES cells appears in nuclear extract.
6) Transcriptional activity from LTR is up regulated by BORIS expression in luciferase assay.

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Center for Experimental Medicine

Laboratory of Stem Cell Therapy
高次機能(幹細胞治療)研究分野

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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/cell therapy. The goal of this laboratory is to provide new insights into stem cell biology as well as approaches to therapeutic intervention for various intractable diseases.

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

a) Cytokine signals modulated via lipid rafts mimic niche signals and induce hibernation in hematopoietic stem cells

Yamazaki S, Iwama A, Takayanagi S, Morita Y, Eto K, Ema H, Nakauchi H.

Hematopoietic stem cells (HSCs) reside in the bone marrow (BM) niche in a non-cycling state and enter the cell cycle at long intervals. However, little is known about inter- and intracellular signaling mechanisms underlying this unique property of HSCs. In this study, we show that lipid raft clustering is a key event in the regulation of HSC dormancy. Freshly isolated HSCs from the BM niche lack lipid raft clustering, exhibit repression of the AKT-FOXO signaling pathway, and express abundant $p57^{Kip2}$ cyclin-dependent kinase inhibitor. Lipid raft clustering induced by cytokines is essential for HSC re-entry into the cell cycle. Conversely, inhibition of lipid raft clustering caused sustained nuclear accumulation of FOXO transcription factors and induced HSC hibernation *ex vivo*. These data establish a critical role for lipid rafts in regulating the cell cycle, the survival, and the entry into apoptosis of HSCs and uncover a striking similarity in HSC hibernation and *C. elegans* dauer formation.

b) Non-side-population hematopoietic stem cells in the bone marrow

Morita Y, Ema H, Yamazaki S, Nakauchi H.

Most hematopoietic stem cells (HSCs) are assumed to reside in the so-called side population (SP) in adult mouse bone marrow (BM). We report the coexistence of non-SP HSCs that do not significantly differ from SP HSCs in numbers, capacities, and cell-cycle states. When stained with Hoechst 33342 dye, the CD34(−/low) c-Kit(+)Sca-1(+)lineage marker(−) (CD34(−)KSL) cell population, highly enriched in mouse HSCs, was almost equally divided into the SP and the main population (MP) that represents non-SP cells. Competitive repopulation assays with single or 30 SP- or MP-CD34(−)KSL cells found similar degrees of repopulating activity and fre-

quencies of repopulating cells for these populations. Secondary transplantation detected self-renewal capacity in both populations. SP analysis of BM cells from primary recipient mice suggested that the SP and MP phenotypes are interconvertible. Cell-cycle analyses revealed that CD 34(−)KSL cells were in a quiescent state and showed uniform cell-cycle kinetics, regardless of whether they were in the SP or MP. Bcrp-1 expression was similarly detected in SP- and MP-CD34(−)KSL cells, suggesting that the SP phenotype is regulated not only by Bcrp-1, but also by other factors. The SP phenotype does not specify all HSCs; its identity with stem cell function thus is unlikely.

c) Differential impact of Ink4a and Arf on hematopoietic stem cells and their bone marrow microenvironment in Bmi1-deficient mice

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The polycomb group (PcG) protein Bmi1 plays an essential role in the self-renewal of hematopoietic and neural stem cells. Derepression of the Ink4a/Arf gene locus has been largely attributed to Bmi1-deficient phenotypes in the nervous system. However, its role in hematopoietic stem cell (HSC) self-renewal remained undetermined. In this study, we show that derepressed p16(Ink4a) and p19(Arf) in Bmi1-deficient mice were tightly associated with a loss of self-renewing HSCs. The deletion of both Ink4a and Arf genes substantially restored the self-renewal capacity of Bmi1(−/−) HSCs. Thus, Bmi1 regulates HSCs by acting as a critical failsafe against the p16(Ink4a)- and p19(Arf)-dependent premature loss of HSCs. We further identified a novel role for Bmi1 in the organization of a functional bone marrow (BM) microenvironment. The BM microenvironment in Bmi1 (−/−) mice appeared severely defective in supporting hematopoiesis. The deletion of both Ink4 a and Arf genes did not considerably restore the impaired BM microenvironment, leading to a sustained postnatal HSC depletion in Bmi1(−/−)Ink4a-Arf(−/−) mice. Our findings unveil a differential role of derepressed Ink4a and Arf on HSCs and their BM microenvironment in Bmi1-deficient mice. Collectively, Bmi1 regulates self-renewing HSCs in both cell-autonomous and nonautonomous manners.

d) Genetic marking of hematopoietic stem and endothelial cells: identification of the Tmtsp gene encoding a novel cell surface protein with the thrombospondin-1 domain.

Takayanagi S, Hiroyama T[1], Yamazaki S, Nakajima T, Morita Y, Usui J, Eto K, Motohashi T[2], Shiomi K[3], Keino-Masu K[3], Masu M[3], Oike Y[4], Mori S, Yoshida N, Iwama A[5], Nakauchi H: [1]RIKENBRC, [2]Graduate School of Medicine, Gifu University, [3] Graduate School of Comprehensive Human Sciences, University of Tsukuba, [4]School of Medicine, Keio University, [5]Graduate School of Medicine, Chiba University

Using an in silico database search, we identified a novel gene encoding a cell surface molecule with a thrombospondin domain, and designated the gene as transmembrane molecule with thrombospondin module (Tmtsp). Expression profiling of Tmtsp using specific monoclonal antibodies and Venus, a variant of yellow fluorescent protein knock-in mice in the Tmtsp locus, demonstrated its specific expression in hematopoietic and endothelial cells. In lymphohematopoietic cells, Tmtsp was predominantly expressed in hematopoietic stem and progenitor cells, and the level of expression gradually declined as the cells differentiated. Venus expression faithfully traced the expression of Tmtsp, and the level of Venus expression correlated well to the in vitro hematopoietic activity as well as the in vivo bone marrow repopulating capacity. Notably, Venus expression marked the development of definitive hematopoiesis in both the extraembryonic yolk sac and the intraembryonic aorta-gonad-mesonephros (AGM) region and, in combination with CD41, strikingly promoted the enrichment of developing progenitors in the CD41(+)Venus(high) fraction at embryonic day 10.5 (E10.5). In this context, Tmtsp is a novel marker gene for primitive hematopoietic cells and endothelial cells, and Tmtsp(Venus/)(+) mice would serve as a valuable mouse model for the analysis of both embryonic and adult hematopoiesis, as well as for vascular biology.

2. Stem/progenitor cells in hepato-biliary system

a) Side population purified from hepatocellular carcinoma cells harbors cancer stem cell-like properties.

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Recent advances in stem cell biology enable us to identify cancer stem cells in solid tumors as well as putative stem cells in normal solid organs. In this study, we applied side population (SP) cell analysis and sorting to established hepatocellular carcinoma (HCC) cell lines to detect subpopulations that function as cancer stem cells and to elucidate their roles in tumorigenesis. Among four cell lines analyzed, SP cells were detected in Huh7 (0.25%) and PLC/PRF/5 cells (0.80%), but not in HepG2 and Huh6 cells. SP cells demonstrated high proliferative potential and anti-apoptotic properties compared with those of non-SP cells. Immunocytochemistry examination showed that SP fractions contain a large number of cells presenting characteristics of both hepatocyte and cholangiocyte lineages. Non-obese diabetic/severe combined immunodeficiency (NOD/SCID) xenograft transplant experiments showed that only 1×10^3 SP cells were sufficient for tumor formation, whereas an injection of 1×10^6 non-SP cells did not initiate tumors. Re-analysis of SP cell-derived tumors showed that SP cells generated both SP and non -SP cells and tumor-initiating potential was maintained only in SP cells in serial transplantation. Microarray analysis discriminated a differential gene expression profile between SP and non-SP cells, and several so-called "stemness genes" were upregulated in SP cells in HCC cells. In conclusion, we propose that a minority population, detected as SP cells in HCC cells, possess extreme tumorigenic potential and provide heterogeneity to the cancer stem cell system characterized by distinct hierarchy.

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