Center for Experimental Medicine

Laboratory of Cell Biology
細胞機能研究分野

Professor	Yoichiro Iwakrua, D.Sc.	教　授	理学博士	岩　倉　洋一郎
Lecturer	Hisataka Yasuda, Ph.D.	講　師	理学博士	保　田　尚　孝
Research Associate	Reiko Horai*, Ph.D.	助　手	理学博士	宝　来　玲　子*
Research Associate	Dai Chida, Ph.D.	助　手	理学博士	千　田　　大
Research Associate	Shigeru Kakuta, Ph.D., D.V.M.	助　手	獣医学博士	角　田　　茂

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, Sunji Park, Keisuke Seki, and Yoichiro Iwakura

Rheumatoid arthritis (RA) is one of the most serious medical problems world-wide 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 mouse model (Horai et al., *J. Exp. Med*., 1998). 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 (Affymetrix Inc.) containing approximately 36,000 full-length mouse genes and EST clusters from UniGene database. We have identified 548 genes that showed significantly different expression levels in the inflamed joints. The gene expression profiles closely resembled between HTLV-I-Tg mice and IL-1Ra-KO mice, suggesting that common genes participate in the pathogenesis of both models. To identify the genomic regions where the expression of a gene cluster was up-regulated in arthritis, the 548-augmented genes were mapped on the whole chromosomes. Although, they were broadly assigned to all the chromosomes, some highly significant transcript density peaks were detected on several chromosomes, including the peak on chromosome 17B1, which corresponds to the major histocompatibility complex gene cluster. Other clusters such as the CC chemokine family,

schlafen family and LLR family were empathized. To examine the roles for these genes in the development of arthritis, we produced gene targeted mice of these genes. We found that one of them was resistant to collagen-induced arthritis, and the other one was more sensitive than the wild-type mice. Thus, the microarray analysis of these arthritic models seems to be useful to elucidate the pathogenesis and to establish new therapeutic targets for rheumatoid arthritis. We have also analyzed the genes responsible for the strain difference of the arthritis susceptibility between C57BL/6 and BALB/c by QTL analysis, and identified 4 genes possibly responsible for the susceptibility for arthritis on the chromosome 3 and 13.

2. TNFα is crucial for the T cell-dependent autoimmune arthritis development in IL-1 Ra-deficient mice

Reiko Horai[1,4], Akiko Nakajima[1], Katsuyoshi Habiro[2], Motoko Kotani[2], Susumu Nakae[1], Taizo Matsuki[1], Aya Nambu[1], Shinobu Saijo[1], Hayato Kotaki[1], Katsuko Sudo[1], Akihiko Okahara[3], Hidetoshi Tanioka[3], Toshimi Ikuse[3], Pamela L. Schwartzberg[4], Ryo Abe[2] and Yoichiro Iwakura[1]: [1]Center for Experimental Medicine, Institute of Medical Science, University of Tokyo; [2]Research Institute for Biological Sciences, Faculty of Science and Technology, Tokyo University of Science; [3]Santen Pharmaceutical Co. Ltd.; [4]National Human Genome Research Institute, NIH.

IL-1Ra$^{-/-}$ mice spontaneously develop autoimmune arthritis. We demonstrate here that T cells are required for the induction of arthritis; T cell-deficient IL-1Ra$^{-/-}$ mice did not develop arthritis, and transfer of IL-1Ra$^{-/-}$ T cells induced arthritis in *nu/nu* mice. Development of arthritis was also markedly suppressed by TNFα deficiency. We found that TNFα induces OX40 expression on T cells, and blocking of either CD40-CD40L or OX40-OX40L interaction suppressed development of arthritis. These findings suggest that IL-1Ra deficiency in T cells disrupts homeostasis of the immune system, and that TNFα plays an important role in activating T cells through induction of OX40 on T cells.

3. Rescue of the phenotype of RANKL-deficient mice by hepatic expression of soluble RANKL transgene

Hisataka Yasuda, Atsuko Minamida, and Yoichiro Iwakura

RANKL, a member of TNF family, is a key regulator of osteoclastogenesis, lymphocyte development and lymph node (LN) organogenesis. RANKL is a membrane-bound ligand but its soluble form is released from the different type of cells including activated T lymphocytes and stromal cells by shedding. RANKL knockout (KO) mice showed severe osteopetrosis, with no osteoclasts, marrow spaces, or tooth eruption, and exhibited profound growth retardation. These mice also showed defects in early differentiation of T and B lymphocytes, and lacked all LNs except Peyer's patches. We previously reported that Tg mice overexpressing soluble RANKL (sRANKL) only in the liver after birth showed severe osteoporosis with an increase of osteoclasts. In humans the increases of serum sRANKL level were reported in patients with rheumatoid arthritis and juvenile Paget's disease, but the physiological and pathological roles of sRANKL are unknown.

To understand the bona fide roles of sRANKL, we analyzed the phenotype of RANKL-KO mice with hepatic expression of sRANKL transgene (hereafter called RANKL-Tg/KO mice). Expression of sRANKL in RANKL-KO mice rescued osteoclast development entirely in long bones with marrow cavities but failed to restore tooth eruption and growth retardation. Although differentiation of CD4/CD8 double-negative CD44$^-$CD25$^+$ precursors to CD 44$^-$CD25$^-$ thymocytes was blocked in RANKL-KO mice, the defect was rescued in RANKL-Tg/KO mice. The total and relative numbers of splenic B220$^+$sIgM$^+$ B cells were significantly reduced in RANKL-KO mice, however, the defect was rescued in RANKL-Tg/KO mice. In RANKL-Tg mice, thymic B cells were also increased. Thus, hepatic expression of sRANKL rescued most of the phenotype of RANKL-KO mice, except for tooth eruption and growth retardation, indicating that the membrane-bound RANKL is not necessary for the restoration. We propose the potential importance of sRANKL in the pathogenesis of bone destruction in humans.

4. *IL-6*, but not *IL-1*, induction in the brain downstream of Cox-2 is essential for the induction of febrile response against peripheral IL-1α

Kyoko Kagiwada, Dai Chida, Tomoya Sakatani, Masahide Asano, Aya Nambu, Shigeru Kakuta, and Yoichiro Iwakura

IL-1 is a major mediator of host defense mechanisms against physical and psychological stresses by regulating not only the immune system but also the neuronal and endocrine sys-

tems. As IL-1Rs are expressed in the brain, particularly the hypothalamus, hippocampus and choroid plexus, a role for IL-1 in the central nervous system has been suggested. Previously, we showed that, upon injection with turpentine, IL-1 is induced in the brain in association with the development of fever (Horai et al., J. Exp. Med., 1998). The role of endogenous IL-1 in the brain and the signaling cascade to activate thermosensitive neurons, however, remains to be elucidated. In this report, febrile response was analyzed after peripheral injection of IL-1α. We found that a normal febrile response was induced even in *IL-1*α/β-deficient mice, indicating that production of IL-1 in the brain is not necessarily required for the response. In contrast, *IL-6* -deficient mice did not exhibit a febrile response. *Cyclooxygenase (Cox)-2* expression in the brain was strongly induced 1.5 h after injection of IL-1 α, while *IL-6* expression was observed 3 h after the injection. *Cox-2* expression in the brain was not influenced by *IL-6* deficiency, while indomethacin, an inhibitor of cyclooxygenases, completely inhibited induction of *IL-6*. These observations suggest a mechanism of IL-1-induced febrile response in which IL-1 in the blood activates *Cox-2*, with the resulting prostaglandin E_2 (PGE_2) inducing *IL-6* in the brain, leading to the development of fever.

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

Naomi Tsurutani, Motohiko Kadoki, Jiro Yasuda, Byung-Il Choi, 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 that 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 reactivated *in vivo* via lipopolysaccharide (LPS) administration through induction of IL-1α/β and TNF-α, although the HIV-1 genome in the lymphocytes was dormant under normal physiological conditions. After the stimulation, normal viral transcripts were detected in the splenocytes and the serum p24 Gag protein levels reached 400 pg/ml, nearly equal levels to that seen in AIDS patients. Thus, these transgenic mice represent a feature of HIV-1-infected people.

Since transgenic mice carrying the HIV genome can produce HIV-1 particles, it is suggested that the replication in murine cells is mainly blocked before the integration process. Thus, we have generated human CD4/CXCR4 transgenic mice to overcome the host-range barrier at the entry step. Although virus entry and reverse transcription were normally proceeded in the primary lymphocytes from these mice, no progeny virus production was observed. 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 trying to identify the molecules that are involved in that process.

6. Studies on the early development of mouse embryos and the developmental potential of somatic cells

Kenjiro Adachi, Deug-chan Lee, Seiji Takashima, Chie Soeda, and Yoichiro Iwakura

Successful production of cloned animals by nuclear transplantation has demonstrated that differentiated somatic cells can be reinitialized into undifferentiated embryonic cells that can form whole animal body. Moreover, it was shown recently that adult somatic stem cells (haematopoetic stem cells, neural stem cells, etc.) could differentiate into cells of all the embryonic three germ layers when they formed chimeras with normal blastocysts. Thus, it is clear that some types of somatic stem cells still maintain pluripotency and can initialize by some stimulation. We are now trying to identify molecules that are involved in the maintenance of undifferentiated state of embryonic stem cells and reprogramming of lineage-specific somatic stem cells.

Since the dedifferentiation occurred merely putting somatic cells into blastocoels, we hypothesized that cell-cell interaction may play an important role. Thus, in order to identify the molecules we are analyzing a set of genes that are specifically expressed in mouse preimplantation embryos by in silico analysis of the expressed sequence tags (ESTs) library. For this, we first clustered ESTs expressed preferentially at the preimplantation stages. Then, we annotated the encoded proteins by computer predic-

tions, and selected cell surface proteins which are uncharacterized. Finally, we confirmed pre-implantation embryo-specific expression of these molecules by RT-PCR analysis.

We analyzed the function of these molecules by using RNA-mediated interference (RNAi) technology. We injected siRNA against early embryo-specific uncharacterized genes and identified several genes of which siRNA inhibited blastocyst formation. We are now studying the roles of these genes in the early development of mouse embryos and in the reprogramming of somatic cells.

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Center for Experimental Medicine

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

Professor	Nobuaki Yoshida, M.D., D.M.Sc.	教　授	医学博士　吉　田　進　昭
Research Associate	Hirotake Ichise, D.V.M., Ph.D.	助　手	獣医学博士　市　瀬　広　武
Research Associate	Mitsuharu Sato, Ph.D.	助　手	医学博士　佐　藤　充　治
Research Associate	Taeko Ichise, Ph.D.	助　手	医学博士　市　瀬　多恵子

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 and have mapped the candidate locus to approximately 1Mb region, which contained 4 known genes and 3 unknown genes. 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. Essential role of FLK-1 (vascular endothelial growth factor receptor-2).

Yoshiko Sakurai[1], Kaori Ohgimoto[1], Yuki Kataoka, Nobuaki Yoshida, and Masabumi Shibuya[1].: [1]Division of Genetics, The Institute of Medical Science, University of Tokyo.

Flk-1 (human counterpart, KDR) tyrosine kinase, which is one of the two vascular endothelial growth factor (VEGF) receptors, is crucial for vascular development. Recently, we showed that among tyrosine residues of KDR, tyrosine residue-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/protein kinase C/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-1*^{1173F} homozygous mice died between embryonic days 8.5 (E8.5) and E9.5 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-1*^{1212F} homozygous mice were viable and fertile. These results suggest that signaling via Y 1173 of Flk-1 is essential for endothelial and he-

matopoietic development during embryogenesis.

2. Increased Numbers of B-1 Cells and Enhanced Responses against TI-2 Antigen in Mice Lacking APS, an Adaptor Molecule Containing PH and SH2 Domains.

Masanori Iseki[2], Chiyomi Kubo[2], Sang-Mo Kwon[2], Akiko Yamaguchi[2], Yuki Kataoka, Nobuaki Yoshida, Kiyoshi Takatsu[2], and Satoshi Takaki[2].: [2]Division of Immunology, Department of Microbiology and Immunology, Institute of Medical Science, University of Tokyo.

APS (adaptor molecule containing PH and SH 2 domains) is an intracellular adaptor protein that forms an adaptor family along with Lnk and SH2-B. While experiments using cultured cell lines have demonstrated that APS is phosphorylated in response to various stimuli, its in vivo functions remain unclear. We attempted to determine the physiological roles of APS by generating *APS*-deficient (*APS*-/-) mice. *APS*-/- mice were viable and fertile and showed no abnormalities or growth retardation. Immunologically, *APS*-/- mice showed normal development and distribution of lymphocytes and myeloid cells, except for increased numbers of B-1 cells in the peritoneal cavity. *APS*-/- mice exhibited an enhanced humoral immune response against trinitrophenol-Ficoll, a thymus-independent type 2 antigen, while *APS*-/- B-2 cells exhibited normal proliferative responses and tyrosine phosphorylation of intracellular proteins upon B-cell receptor (BCR) cross-linking. APS colocalized with filamentous actin (F-actin) accumulated during the capping of BCRs in *APS*-transgenic B cells. After BCR stimulation, F-actin contents were lower in *APS*-/- B-1 cells than in wild-type B-1 cells. Our results indicate that APS might have a novel regulatory role in actin reorganization and control of B-1 cell compartment size.

3. Absence of mechanical allodynia and Aβ-fiber sprouting after sciatic nerve injury in mice lacking membrane-type 5 matrix metalloproteinase.

Kiyoshi Komori[3], Takahiro Nonaka[3], Akiko Okada[3], Hiroaki Kinoh[3], Hiromi Hayashita-Kinoh[3], Nobuaki Yoshida, Ikuo Yana[3] and Motoharu Seiki[3].: [3]Department of Cancer Cell Research, Institute of Medical Science, University of Tokyo.

Matrix metalloproteinases (MMPs) are a family of endopeptidases that degrade extracellular matrix components. Membrane-type 5 MMP (MT5-MMP/MMP-24) was identified as neuron-specific, and is believed to contribute to neuronal circuit formation and plasticity. To elucidate its function in vivo, we have generated mice lacking MT5-MMP by gene targeting. MT5-MMP-deficient mice were born without obvious morphological abnormalities. No apparent histological defects were observed in the nervous system either. However, MT5-MMP-deficient mice did not develop neuropathic pain with mechanical allodynia after sciatic nerve injury, though responses to acute noxious stimuli were normal. Neuropathic pain induced by peripheral nerve lesions is known to accompany structural reorganization of the nervous system. Intraneural injection of cholera toxin B subunit, a transganglionic tracer, into the injured sciatic nerve of wild-type mice revealed that the myelinated Aβ-fiber primary afferents sprouted from laminae III-VI of the dorsal horn of the spinal cord and invaded lamina II. However, no such sprouting and invasion of Aβ-fibers were observed in MT5-MMP-deficient mice. These findings suggest that MT5-MMP is essential for the development of mechanical allodynia and plays an important role in neuronal plasticity in this mouse model.

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

Taeko Ichise, Hirotake Ichise, Seiji Shiozawa, Takashi Yamaguchi, Yuhko Ayabe, Akiko Hori, Jun-ichi Matsuo, 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 lymphatic endothelial cell-specific expression of Cre recom-

binase gene was observed in some VEGFR3-Cre transgenic mouse lines, which was revealed by the use of the site-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.

5. Analysis of regulation of blood and lymphatic vascular separation in mice

Hirotake Ichise, Taeko Ichise, 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. 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 these mice. 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.

6. Studies on the maintenance of mouse embryonic stem cells.

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

Plasticity and self-renewing activity of stem cells are the most important characteristics of stem cells on which cells of every kind are standing. We can see typical examples such as the development of organs during embryogenesis and the emergence of multiple lineages of blood cells in the bone marrow. In both cases, the stem cell activity for tissues or organs is crucial.

Mouse embryonic stem (ES) cells are the cell line which gives rise to the complete range of cells in the organism and can be maintained infinitely in culture. This enables us to obtain mutant mice by manipulating genes in ES cells. Although ES cells could be a powerful tool for the analysis of gene functions in vivo, little is known about how the plasticity and self-renewal activity are maintained.

Our main purpose is to know what makes ES cells remain in the pluripotent state.

6.1 *Analysis of Rox-1 function in ES cell and mouse development*

We started our research to identify a factor which binds to Rex-1 gene promoter by protein purification. Rex-1 is one of the marker genes of undifferentiated ES cells. The purified factor, termed Rox-1, specifically binds to C/T-rich sequence which is critical for the Rex-1 promoter activity. To test whether Rox-1 contributes to the Rex-1 expression in ES cells, we introduced siRNA expression vector to down-regulate Rox-1 expression. When mRNA level of Rox-1 was down-regulated to 60 % of the control, the expression of Rex-1 was reduced. In addition, the mRNA level of Nanog was also affected by the expression of Rox-1 siRNA. This prompted us to examine the promoter of Nanog gene and we found a region which contains undifferentiated-state specific enhancer activity. Rox-1 binding to this region was confirmed by EMSA. These results indicate that Rox-1 plays an important role for the expression of ES cell specific genes. However, some questions arise at this point.

a) Is Rox-1 DNA binding activity specific in undifferentiated ES cells ?

b) How Rox-1 DNA binding activity is regulated ?

These questions arise because the protein level of Rox-1 is not altered in the course of ES cell differentiation. The answer to the question a) is yes. We examined Rox-1 DNA binding activity to the sequence from Rex-1 or Nanog promoter by EMSA and found that the binding was specific in undifferentiated ES cells. Then how Rox-1 DNA binding activity is regulated ? To answer this question, we performed 2D analysis of Rox-1 protein. Some spots appear specifically in undifferentiated ES cell extracts. This result implies that Rox-1 activity is regulated by modification. According to this idea, we treated ES cell extracts with phosphatase and subjected to EMSA to see the Rox-1 DNA binding activity.

The binding activity was reduced by the treatment. This result shows that the DNA binding activity of Rox-1 is regulated by protein phosphorylation. We are now trying to identify Rox-1 kinase and associated protein(s) by protein purification. The kinase or associated protein(s) will give us a clue to further understand the ES cell pluripotency.

To study the role of Rox-1 in vivo, we established ES cell lines whose Rox-1 alleles were mutated. One allele was disrupted by neomycin resistant cassette (Neo) and the other allele was modified by the insertion of two loxP sites (flox). In this cell line (Neo/flox), the promoter and first exon are excised out by the expression of Cre recombinase to obtain Neo/-(Rox-1 null) cell. We introduced Cre recombinase by retrovirus vector. Although Rox-1 negative colonies were observed in the early period of time, they disappeared within a few weeks of cell culture. This indicates Rox-1 is a critical factor for the maintenance of ES cells. To examine the growth rate and cell cycle distribution, we are setting the condition to enrich Rox-1 null ES cells.

During the course of establishment of Neo/flox ES cells, we obtained Neo/+ ES cells. Using this cell, we made chimeric mice to produce Rox-1 mutant mice. Heterozygous mutants grow normally and showed no behavioral abnormality. By intercrossing Rox-1 heterozygous mice, we found that Rox-1 homozygous mice are lethal at the stage of implantation. This result also indicates the importance of Rox-1 in early mouse development.

6.2 *Early transposon (ETn) and pluripotency of ES cells*

Lately, many researchers came to think that trying to find a single factor that defines stemness by itself may not be realistic. Even though several transcription factors that are ES cell-specific were reported, how ES cells acquire pluripotency, the major characteristic of stem cells, has not been understood yet. Therefore seeing stemness from different aspects is crucial for the understanding of pluripotency. Stem cells, including ES cells are regarded as expressing far more kinds of transcripts when compared to mature, differentiated cells. Carrying out proteomic analysis using ES cell lysates is especially difficult because in ES cells many kinds of proteins are expressed, at least at low level, which causes the increase in background. It is also shown that stem cells show miscellaneous gene expression pattern: hematopoietic stem cells express neuronal genes, and mesenchymal stem cells express genes which are markers for different lineages. Furthermore, when several groups identified genes overexpressed in stem cells using microarray, they have come up with totally different results. From these facts, we are convinced that expressing miscellaneous genes is the most important property of stem cells, and that type of expression is necessary for cells to acquire pluripotency. During early development, individual cell would not have a clue to which lineage it will differentiate, and commitment of a cell to differentiate into specific cell lineage must be controlled by its niche or spatial distribution within the environment. When cells differentiate, some signals are transmitted from their niche, and that signals push the cells to commit to specific lineages. Therefore, cells which can rapidly respond to signals from their niche can undergo differentiation process, while cells which are not prepared to receive the signals cannot respond and differentiate. Expressing miscellaneous genes may be the outcome of stem cells' preparation for responding to all kinds of differentiation-inducing signals.

From this aspect, we intend to elucidate the nature of pluripotency. In differential hybridization screening, we have identified ETn, a retrotransposon whose expression is limited to early stages of development and in undifferentiated cells. ETn is located in every chromosome and there are about 400 copies per 2n. DNase I hypersensivity assay showed that genomes near ETn sequence is highly sensitive to DNase I in undifferentiated ES cells. This means that chromatin structure in ETn surroundings is loose and euchromatic. We are trying to find out why this retrotransposon is actively transcribed in ES cells. When this kind of sequence is transcribed, genes near the sequence can also be recruited to transcription factory in which transcription occurs. That may increase the chance of expressing miscellaneous genes, which are needed for responding differentiation inducing signals, or after completing differentiation. In order to clarify this idea, we are now trying to find a transcription factor which enables the expression of ETn.

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Center for Experimental Medicine

Laboratory of Stem Cell Therapy
幹細胞治療動物モデル分野

Professor	Hiromitsu Nakauchi, M.D., Ph.D.	教　授	中　内　啓　光
Lecturer	Atsushi Iwama, M.D., Ph.D.	講　師	岩　間　厚　志
Research Associate	Koji Eto, M.D., Ph.D.	助　手	江　藤　浩　之
Research Associate	Akihide Kamiya, Ph.D.	特任助手	紙　谷　聡　英

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) Asymmetric division and lineage Commitment at the Level of Hematopoietic Stem Cells

How hematopoietic stem cells (HSCs) commit to a particular lineage is unclear. A high degree of HSC purification enabled us to address this issue at the clonal level. Single-cell transplantation studies revealed that 40% of the CD34 / low, c-Kit, Sca-1, and lineage marker (CD34 KSL) cells in adult mouse bone marrow were able, as individual cells, to reconstitute myeloid and Band T-lymphoid lineages over the long-term. Single-cell culture showed that 40% of CD 34 KSL cells could form neutrophil (n)/macrophage (m)/erythroblast (E)/megakaryocyte (M) (nmEM) colonies. Assuming that a substantial portion of long-term repopulating cells can be detected as nmEM cells within this population, we compared differentiation potentials between individual pairs of daughter and granddaughter cells derived in vitro from single nmEM cells. One of the two daughter or granddaughter cells remained an nmEM cell. The other showed a variety of combinations of differentiation potential. In particular, an nmEM cell directly gave rise, after one cell division, to progenitor cells committed to nm, EM, or M lineages. The probability of asymmetric division of nmEM cells depended on the cytokines used. These data strongly suggest that lineage commitment takes place asymmetrically at the level of HSCs under the influence of external factors.

b) Epigenetic regulation of stem cell self-renewal by Polycomb Group protein Bmi-1

As described above, in physiological condition, HSCs divide asymmetrically producing one HSC and one committed progenitor cell. Self-renewal of HSC indeed means maintenance of the same gene expression pattern through cell division at least in one of the daughter cells. Polycomb Group (PcG) proteins have been known to play a role in the cellular memory. PcG proteins form multiprotein complexes that play an important role in the maintenance of transcriptional repression of target genes. Two distinct PcG complexes have been identified and

characterized utilizing drosophila or mammalian epithelial cells. One complex includes Eed, EzH 1, and EzH2, and the other includes Bmi-1, Mel-18, Mph1/Rae28, M33, Scmh1, and Ring1A/B. These two types of complexes coordinately maintain positional memory along the anterior-posterior axis by regulating *Hox* gene expression patterns during development. Recent studies have revealed a role of the Bmi-1-containing complex in the maintenance of hematopoietic and leukemic stem cells. *Mph1/Rae28*$^{-/-}$ fetal liver contains 20-fold fewer long-term lympho-hematopoietic repopulating HSCs than wild type. More importantly, although *Bmi-1*$^{-/-}$ mice show normal development of embryonic hematopoiesis, *Bmi-1*$^{-/-}$ HSC have a profound defect in self-renewal capacity. They cannot repopulate hematopoiesis long-term and these lead to progressive postnatal pancytopenia. Notably, the self-renewal defect is not confined to HSC, but also applicable to leukemic stem cells and neuronal stem cells. However, how those complexes are formed in neural, leukemic or hematopoietic stem cells are not known. In addition, the impact of forced expression of PcG genes on HSC self-renewal remains to be elucidated.

To address these issues, we performed both loss-of-function and gain-of-function analysis on various PcG proteins. Expression analysis revealed that not only *Bmi-1* but also other PcG genes are predominantly expressed in HSCs. Loss-of-function analyses, however, demonstrated that absence of *Bmi-1* is preferentially linked with a profound defect in HSC self-renewal. On the other hand, forced expression of Bmi-1 but not other PcG genes led to a striking ex vivo expansion of multipotential progenitors and marked enhancement of HSC repopulating capacity in vivo, indicating a central role for Bmi-1, but not the other components, in the maintenance of HSC self-renewal both *in vitro* and *in vivo,* and in augmentation of HSC activity *ex vivo.* Our findings indicate that the expression level of Bmi-1 is the critical determinant for the self-renewal capacity of HSC. These findings uncover novel aspects of stem cell regulation exerted through epigenetic modifications by the PcG proteins.

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 that can differentiate into hepatocytes and cholangiocytes. Using the same approach, in collaboration with Dr. Taniguchi of Yokohama City University and Dr. Suzuki of Salk Institute, we attempted a prospective isolation of pancreatic progenitors.

During pancreatic development, neogenesis, and regeneration, stem cells might act as a central player to generate endocrine, acinar, and duct cells. Although these cells are well known as pancreatic stem cells (PSCs), indisputable proof of their existence has not been reported. Identification of phenotypic markers for PSCs leads to their prospective isolation and precise characterization to clear whether stem cells exist in the pancreas. By combining flow cytometry and clonal analysis, we show here that a possible pancreatic stem or progenitor cell candidate that resides in the developing and adult mouse pancreas expresses the receptor for the hepatocyte growth factor (HGF) c-Met, but does not express hematopoietic and vascular endothelial antigens such as CD45, TER119, c-Kit, and Flk-1. These cells formed clonal colonies in vitro and differentiated into multiple pancreatic lineage cells from single cells. Some of them could largely expand with self-renewing cell divisions in culture, and, following cell transplantation, they differentiated into pancreatic endocrine and acinar cells in vivo. Furthermore, they produced cells expressing multiple markers of nonpancreatic organs including liver, stomach, and intestine in vitro. Our data strongly suggest that c-Met/HGF signaling plays an important role in stem/progenitor cell function in both developing and adult pancreas. By using this antigen, PSCs could be isolated prospectively, enabling a detailed investigation of stem cell markers and application toward regenerative therapies for diabetes.

b) Cell fate determination of primitive endodermal progenitor cells

As described above, we have identified hepatic and pancreatic stem/progenitor cells from the fetus. With lower frequency, those cells also differentiated into hepatocytes, pancreatic acinar as well as endocrine cells. However, the mechanism of cell fate determination was never understood in hepato-biliary system. To address this issue, in collaboration with Dr. Sumazaki of University of Tsukuba and Dr. Kageyama of Kyoto University, we investigated a role of Hes-1 in hepato-biliary cell differentiation using Hes-1 knockout mice.

The biliary system, pancreas and liver all develop from the nearby foregut at almost the same time in mammals. The molecular mechanisms that determine the identity of each organ in this complex area are unknown. *Hes1* encodes

the basic helix-loop-helix protein Hes1, which represses positive basic helix-loop-helix genes2 such as *Neurog3.* Expression of Hes1 is controlled by the evolutionarily conserved Notch pathway. Hes1 operates as a general negative regulator of endodermal endocrine differentiation, and defects in Notch signaling lead to accelerated pancreatic endocrine differentiation. Mutations in *JAG1,* encoding a Notch ligand, cause the Alagille syndrome in humans, characterized by poor development of the biliary system, suggesting that the Notch pathway is also involved in normal biliary development. We demanstrated that *Hes1* is expressed in the extrahepatic biliary epithelium throughout development and that *Hes1*-deficient mice have gallbladder agenesis and severe hypoplasia of extrahepatic bile ducts. Biliary epithelium in *Hes1*-/- mice ectopically expresses the proendocrine gene *Neurog3,* differentiates into endocrine and exocrine cells and forms acini and islet-like structures in the mutant bile ducts. Thus, biliary epithelium has the potential for pancreatic differentiation and Hes1 determines biliary organogenesis by preventing the pancreatic differentiation program, probably by directly repressing transcription of *Neurog3.*

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