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: Identification of C-type lectins as novel causative genes for autoimmunity

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Rheumatoid arthritis (RA) is one of the most serious medical problems worldwide with approximately 1% of the people in the world affected. The disease is autoimmune in nature and characterized by chronic inflammation of the synovial tissues in multiple joints that leads to joint destruction. High levels of 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 transgenic mice (Iwakura et al., *Science*, 1991) and the other is IL-1 receptor antagonist-deficient mice (Horai et al., *J. Exp. Med*., 2000). Both of these models develop autoimmunity and chronic inflammatory arthropathy closely resembling RA in humans.

The dendritic cell immunoreceptor (official gene symbol *Clec4a2*, called *Dcir* here) is a C-type lectin receptor expressed mainly in dendritic cells (DCs) that has a carbohydrate recognition domain in its extracellular portion and an immunoreceptor tyrosine-based inhibitory motif, which transduces negative signals into cells, in its cytoplasmic portion. We found high *Dcir* expression in the joints of these two mouse rheumatoid arthritis models. Because the structural characteristics of Dcir suggest that it may have an immune regulatory role, and because autoimmune-related genes are mapped to the *DCIR* locus in humans, we generated $Dcir^{-/-}$ mice to learn more about the pathological roles of this molecule. We found that aged $Dcir^{-/-}$ mice spontaneously develop sialadenitis and enthesitis associated with elevated serum autoantibodies. $Dcir^{-/-}$ mice showed a markedly exacerbated response to collagen-induced arthritis. The DC population was expanded excessively in aged and type II collagen-immunized $Dcir^{-/-}$

mice. Upon treatment with granulocyte-macrophage colony-stimulating factor, *Dcir*$^{-/-}$ mouse-derived bone marrow cells (BMCs) differentiated into DCs more efficiently than did wild-type BMCs, owing to enhanced signal transducer and activator of transcription-5 phosphorylation. These observations indicate that Dcir is a negative regulator of DC expansion and has a crucial role in maintaining the homeostasis of the immune system. We are now further analyzing the molecular mechanisms of the Dcir signal transduction and trying to develop Dcir inhibitors in order to examine possible application for clinical use.

2. Studies on rheumatoid arthritis models: Analysis of the roles of arthritis-related genes

Reiko Ichikawa, Toshimasa Kusaka, Soo-Hyun Chung, Keiko Yamabe, Yuko Tanahashi, Masanori Murayama, Sun-Ji Park, Shinobu Saijo, Noriyuki Fujikado, and Yoichiro Iwakura

As described above, we have developed 2 arthritis models; one is HTLV-I transgenic mice and the other is IL-1 receptor antagonist-deficient mice. To identify genes involved in the pathogenesis of arthritis, we analyzed the gene expression profiles of these animal models by using high-density oligonucleotide arrays. We extracted 554 genes whose expression significantly changed in both models. Then, each of these commonly changed genes was mapped into the whole genome in a scale of the 1-megabase pairs. We found that the transcriptome map of these genes formed clusters including the major histocompatibility complex class I and class II genes, complement genes, and chemokine genes, which are well known to be involved in the pathogenesis of RA at the effector phase. By searching for such clusters, we could detect genes with marginal expression changes, including several genes whose involvement in the arthritis pathogenesis has previously not known. We have also searched for pathogenesis-related genes by analyzing the genetic background dependency of the arthritis development. We identified several genes which were involved in the BALB/c strain-specific development of arthritis in these model mice. We are now analyzing the roles of these genes in the pathogenesis of arthritis and autoimmunity by generating the knockout mice of these genes.

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

Harumichi Ishigame, Yuko Tanahashi, Aoi Akitsu, Satoshi Ikeda, Shigeru Kakuta, Aya Nambu, Yutaka Komiyama, Susumu Nakae, Shinobu Saijo, and Yoichiro Iwakura

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

IL-17F is an IL-17 family member and has the highest amino acid sequence homology to IL-17 A. Although IL-17F is also produced by Th17 cells and binds the same IL-17 receptor, its role remains largely unknown. To distinguish the roles of IL-17A and IL-17F in allergy, autoimmunity, and infection, we generated *Il17a*$^{-/-}$, *Il 17f*$^{-/-}$ and *Il17a/f*$^{-/-}$ mice and demonstrated that IL-17A, but not IL-17F, is involved in the development of delayed-type hypersensitivity and encephalomyelitis. Similarly, IL-17A played a greater role than IL-17F in a spontaneous autoimmune arthritis model. These results indicate that IL-17A and IL-17F have distinct roles in immune responses.

4. Studies on the roles of cytokines in the neuro-immuno-endocrine system: Peripheral TNFα, but not peripheral IL-1, requires endogenous IL-1 or TNFα induction in the brain for the febrile response

Dai Chida, and Yoichiro Iwakura

It is known that peripherally administered IL-1 and TNFα induce fever through mechanisms involving Prostaglandin (PG) E_2. In this report, we compared the signaling cascade induced in the brain by TNFα and IL-1. Peripheral administration of TNFα induced enhanced fever in IL-1 receptor antagonist KO mice, suggesting that IL-1 is involved in the TNFα mediated fever. IL-1α, but not TNFα, induced fever in IL-1α/β/TNFα KO mice, although central administration of TNFα induced fever. Only IL-1α, but not TNFα, induced IL-6 in the IL-1α/β/TNFα KO mouse brain, while both cytokines induced Cyclooxyge-

nase (Cox)-2. Icv administration of PGE_2 induced only transient fever in contrast to the TNFα- or IL-1α-induced fever that lasted longer. Taken together, either IL-1 or TNFα induction in the brain is required for the response induced by TNFα but not by IL-1α, and that both Cox-2 and IL-6 induction are required for prolonged febrile response against these cytokines.

5. Melanocortin receptor 2 is required for adrenal gland development, steroidogenesis and neonatal gluconeogenesis.

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ACTH is the principal regulator of the hypothalamus-pituitary-adrenal axis and stimulates steroidogenesis in the adrenal gland via the specific cell surface melanocortin 2 receptor (MC2R). In this study, we generated mice with an inactivation mutation of the MC2R gene to elucidate the roles of MC2R in adrenal development, steroidogenesis and carbohydrate metabolism. These mice, the last of the KO mice to be generated for the melanocortin family receptors, provide the opportunity to compare the phenotype of proopiomelanocortin KO mice with the MC1-5R KO mice. We found the MC2R KO mutation led to neonatal lethality in three-quarters of the mice possibly due to hypoglycemia. Those surviving to adulthood exhibited macroscopically detectable adrenal glands with markedly atrophied zona fasciculata, while the zona glomerulosa and the medulla remained fairly intact. Mutations of MC2R were reported to be responsible for 25% of cases of Familial Glucocorticoid Deficiency (FGD). Adult MC2R KO mice resembled FGD patients in several aspects such as undetectable levels of corticosterone despite high levels of ACTH, unresponsiveness to ACTH and hypoglycemia after a prolonged 36 h of fasting. However, MC2R KO mice are different from patients with MC2R-null mutations in several aspects, such as low aldosterone levels and unaltered body length. These results indicate that MC2R is required for postnatal adrenal development and adrenal steroidogenesis and MC2R KO mice provide a useful animal model to study FGD.

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

Shigeru Kakuta, Reiko Ichikawa, and Yoichiro Iwakura

2′, 5′-oligoadenylate synthetase (OAS) is one of IFN-inducible proteins and is known to be involved in the major antiviral mechanism through activation of RNaseL. In mice, the OAS family consists of 12 genes with a well-conserved motif. However, the physiological role and functional difference among these genes are not elucidated completely. In this study, to elucidate the roles of OAS family genes, we generated each of *Oas1a*-, *Oas1c*-, *Oas 2*-, *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 *loxP* sites was created by a Cre-mediated recombination. *OASC*-deficient mice were generated by injecting a Cre expression plasmid, pCAG-Cre, into 1-cell embryos derived from *Oas1a/Oas2*-deficient (flox) mice. *OASC* mice were born in a Mendelian ratio, fertile, and appeared healthy under SPF conditions. The thymus appeared normal, in contrast to *RNaseL*-deficient mice in which the thymus becomes hyperplastic because of the deficiency of thymocyte apoptosis. As *OASC*-deficient mice lack 10 conventional OAS genes, but still express 2 OASL genes, which encode enzymatically inactive and functionally unknown, these observations suggest that OASL may compensate the OAS 1-3 functions in the 2-5A system, or RNaseL could be activated in an OAS-independent manner.

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

Motohiko Kadoki, Takuya Tada, and Yoichiro

Iwakura

Studies of AIDS pathogenesis and development of therapeutic drugs and vaccines have been hampered by the lack of appropriate small animal models such as mouse models for AIDS, because HIV-1 cannot infect and replicate in mice due to the difference of the structure of some host factors necessary for HIV-1 replication. As primate models are difficult to handle, expensive and require expensive facilities, genetically not well characterized, often infected with pathogens, and ethically less acceptable, we are trying to generate mouse models for AIDS. We are taking two approaches to generate 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.

We previously showed that HIV-1 expression is efficiently activated in the spleen of HIV-1 transgenic mice by bacterial lipopolysaccharide (LPS) (Tanaka et al., *AIDS*, 2000; 2003). This year, we analyzed the molecular mechanism of the HIV-1 activation in macrophages of HIV-1 transgenic mice. We found that, in contrast to spleen cells in which HIV-1 expression was induced by cytokines such as TNF-α or IL-1, HIV-1 was directly activated by the TLR-mediated signaling. We are now analyzing the mechanisms. We are also trying to identify host factors that are involved in the nuclear localization of pre-integration complex, that consists one of species-specific host range barriers.

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

Hiroaki Okae and Yoichiro Iwakura

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

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

By using these methods, 71 genes were disrupted in ES cells. Approximately the half of the genes encoded membrane bound proteins and this ratio was significantly higher than that in ordinary gene trap. Among these trapped genes, gene knockout mice of 13 genes were already generated, of which 11 caused recessive lethal phenotypes. The extremely high lethality ratio indicates that this screening efficiently enriched developmentally important genes.

We have selected three genes which are evolutionally conserved and have signal transduction motifs, and generated gene knockout mice. Two knockout mice showed recessive lethal phenotypes. One showed neural tube defects and the other showed cleft palates. We are now analyzing these knockout mice in detail and trying to understand the molecular mechanisms underlying these defects.

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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. Establishment of an MT4-MMP-deficient mouse strain representing an efficient tracking system for MT4-MMP/MMP-17 expression in vivo using β-galactosidase.

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The biological functions of membrane-type 4 matrix metalloproteinase (MT4-MMP/MMP-17) are poorly understood because of the lack of a sensitive system for tracking its expression *in vivo*.

We established a mutant mouse strain (*Mt4-mmp*$^{-/-}$) in which *Mt4-mmp* was replaced with a reporter gene encoding β-galactosidase(LacZ). *Mt4-mmp*$^{-/-}$ mice had normal gestations, and no apparent defects in growth, life span and fertility. Using LacZ as a maker, we were able to as Monitor the expression and promoter activity of *Mt4-mmp* for the first time *in vivo*. The tissue distribution of *Mt4-mmp* mRNA correlated with *LacZ* expression, and we showed that *Mt4-mmp* is expressed primarily in cerebrum, lung, spleen, intestine and uterus. We identified LacZ-positive neurons in the cerebrum, smooth, muscle cells in the intestine and uterus, and macrophages located in the lung alveolar or intraperitoneal space. Contrary to the reported role of MT4-MMP as a tumor necrosis factor-α (TNF-α) sheddase, the lipopolysaccharide (LPS)-induced release of TNF-α from *Mt4-mmp*$^{-/-}$ macrophages was similar to that in wild-type cells,

and expression of *Mt4-mmp* mRNA was repressed following LPS stimulation. Thus, we have established a mutant mouse strain for analyzing the physiological functions of MT4-MMP, which also serves as a sensitive system for monitoring and tracking the expression of MT4-MMP *in vivo*.

2. Mastermind-1 is required for Notch signal-dependent steps in lymphocyte development *in vivo*.

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Mastermind (Mam) is one of the elements of Notch signaling, an ancient system that plays a pivotal role in metazoan development. Genetic analyses in *Drosophila* and *Caenorhabdit is elegans* have shown Mam to be an essential positive regulator of this signaling pathway in these species. Mam proteins bind to and stabilize the DNA-binding complex of the intracellular domains of Notch and CBF-1, Su(H), Lag-1 (CSL) DNA-binding proteins in the nucleus. Mammals have three Mam proteins, which show remarkable similarities in their functions while having an unusual structural diversity. There have also been recent indications that Mam-1 functionally interacts with other transcription factors including p53 tumor suppressor. We here in describe that Mam-1 deficiency in mice abolishes the development of splenic marginal zone B cells, a subset strictly dependent on Notch 2, a CSL protein and Delta 1 ligand. Mam-1 deficiency also causes a partially impaired development of early thymocytes, while not affecting the generation of definitive hematopoiesis, processes that are dependent on Notch 1. We also demonstrate the transcriptional activation of a target promoter by constitutively active forms of Notch to decrease severalfold in cultured Mam-1-deficient cells. These results indicate that Mam-1 is thus required to some extent for Notch-dependent stages in lymphopoiesis, thus supporting the notion that Mam is an essential component of the canonical Notch pathway in mammals.

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

Keiichi Anan[6], Nobuaki Yoshida, Yuki Kataoka, Mitsuharu Sato, Hirotake Ichise, Makoto Nasu[6] and Shintaro Ueda[6]: [6]Laboratory for Molecular Anthropology and Molecular Evolution, University of Tokyo.

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.

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

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

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 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.

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

Satona Ohno, Mitsuharu Sato and Nobuaki Yoshida

Polypyrimidine tract-binding protein (PTB) is a widely expressed RNA-binding protein with multiple functions in RNA processing and IRES-mediated translation. In the course of promoter analysis of Rex-1 and Nanog genes, they are the markers for undifferentiated state of mouse embryonic stem (ES) cells, we found that PTB binds to polypyrimidine sequences within the promoters. To study the role of PTB for ES cell function, we established PTB deficient ES cell lines by homologous recombination.

The mutant cells form normal compact colonies, but they showed defect in cell proliferation. Cell cycle analysis using thymidine and nocodazole revealed that the mutant cells have prolonged G2/M phase and this may explain the defect in cell proliferation. Recent studies reported that cell cycle specific translation mechanism during mitosis. For example, ornithine decarboxylase and c-myc mRNAs contain an internal ribosome entry site (IRES) that exclusively functions during mitosis, and the protein level of these gene product peaks at G2/M phase. One of those genes, p58 is translated from IRES located at coding region of cdk11 mRNA. The expression of p58 is tightly regulated at mitosis and shown to facilitate proper mitotic progression and termination. Then we examined the expression level of cdk11/p58 in PTB deficient ES cells. The mutant cells showed higher p58 levels than that of control cells during mitotic phase. Consistent with this result, reporter gene assay using p58 IRES has higher IRES activity in PTB deficient cells. Furthermore, we found that the level of PTB was transiently down regulated in mitotic phase. These results suggest the importance of PTB in cell cycle regulation via IRES mediated translation mechanism.

6. Analysis of Fbxl10 in mouse embryonic stem cells

Tsuyoshi Fukuda, Mitsuharu sato and Nobuaki Yoshida

One of the f-box protein family, Fbxl10, was shown to have histone demethylase activity through jmj domain. Interestingly, Fbxl10 has two kinds of transcripts with different sizes and one of those does not contain exons coding for jmj domain. This raises two questions: 1) Does Fbxl10 without jmj domain (hereafter short form) modulate histone demethylase activity of full length Fbxl10? 2) What function does Fbxl10 have which does not require histone demethylase activity? For the question 2, other group recently showed that Fbxl10 represses c-jun transcription and histone demethylase activity is not required for this function. We postulated that methylation status of histone is balanced by two forms of Fbxl10. Namely, the methylation level of histone H3 is positively regulated by short form of Fbxl10 and negatively by full length Fbxl10. However, when short form or full length version of Fbxl10 is over expressed in 293T cells, the methylation level of histone H3 (K36me3) was down regulated by both forms of Fbxl10. To elucidate the function of Fbxl10 further, we are generating mutant ES cell lines for both forms of Fbxl10.

7. 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 for the candidates. Among the candidates, we focused on two factors, CTCF and its paralogue, BORIS. CTCF and BORIS shares 11 zinc fingers and the expression of CTCF is ubiquitous, while the expression of BORIS is restricted in cancer and CTCF-negative cells in testis. CTCF is well known for its function as an insulator protein and a candidate tumor suppressor. Meanwhile, BORIS is not well characterized owing to its highly restricted expression pattern. In contrary to the previous reports, we found that CTCF and BORIS are ex-

pressed simultaneously in mouse ES cells. When coexpressed, CTCF and BORIS interact with each other, and activates ETn promoter. Down-regulation of CTCF and BORIS using shRNA resulted in the reduction of ETn mRNA. Chromatin immunoprecipitation assay has indicated the binding of CTCF to ETn promoter in ES cells. From these results, we concluded that CTCF and BORIS are the transcriptional activators of ETn in mouse ES cells.

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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) Lnk negatively regulates self-renewal of hematopoietic stem cells by modifying thrombopoietin-mediated signal transduction

Seita J, Ema H, Ooehara J, Yamazaki S, Tadokoro Y, Yamasaki A, Eto K, Takaki S, Takatsu K, Nakauchi H.

One of the central tasks of stem cell biology is to understand the molecular mechanisms that control self-renewal in stem cells. Several cytokines are implicated as crucial regulators of hematopoietic stem cells (HSCs), but little is known about intracellular signaling for HSC self-renewal. To address this issue, we attempted to clarify how self-renewal potential is enhanced in HSCs without the adaptor molecule Lnk, as in Lnk-deficient mice HSCs are expanded in number >10-fold because of their increased self-renewal potential. We show that Lnk negatively regulates self-renewal of HSCs by modifying thrombopoietin (TPO)-mediated signal transduction. Single-cell cultures showed that Lnk-deficient HSCs are hypersensitive to TPO. Competitive repopulation revealed that long-term repopulating activity increases in Lnk-deficient HSCs, but not in WT HSCs, when these cells are cultured in the presence of TPO with or without stem cell factor. Single-cell transplantation of each of the paired daughter cells indicated that a combination of stem cell factor and TPO efficiently induces symmetrical self-renewal division in Lnk-deficient HSCs but not in WT HSCs. Newly developed single-cell immunostaining demonstrated significant enhancement of both p 38 MAPK inactivation and STAT5 and Akt activation in Lnk-deficient HSCs after stimulation with TPO. Our results suggest that a balance in positive and negative signals downstream from the TPO signal plays a role in the regulation of the probability of self-renewal in HSCs. In general, likewise, the fate of stem cells may be determined by combinational changes in multiple signal transduction pathways.

b) De novo DNA methyltransferase is essential for self-renewal, but not for differentiation, in hematopoietic stem cells

Tadokoro Y, Ema H, Okano M, Li E, Nakauchi H

DNA methylation is an epigenetic modification essential for development. The DNA methyltransferases Dnmt3a and Dnmt3b execute de novo DNA methylation in gastrulating embryos and differentiating germline cells. It has been assumed that these enzymes generally play a role in regulating cell differentiation. To test this hypothesis, we examined the role of Dnmt3a and Dnmt3b in adult stem cells. CD34(-/low), c-Kit (+), Sca-1(+), lineage marker(−) (CD34(−) KSL) cells, a fraction of mouse bone marrow cells highly enriched in hematopoietic stem cells (HSCs), expressed both Dnmt3a and Dnmt3b. Using retroviral Cre gene transduction, we conditionally disrupted Dnmt3a, Dnmt3b, or both Dnmt3a and Dnmt3b (Dnmt3a/Dnmt3b) in CD 34(−) KSL cells purified from mice in which the functional domains of these genes are flanked by two loxP sites. We found that Dnmt3a and Dnmt3b function as de novo DNA methyltransferases during differentiation of hematopoietic cells. Unexpectedly, in vitro colony assays and in vivo transplantation assays showed that both myeloid and lymphoid lineage differentiation potentials were maintained in Dnmt3a-, Dnmt 3b-, and Dnmt3a/Dnmt3b-deficient HSCs. However, Dnmt3a/Dnmt3b-deficient HSCs, but not Dnmt3a- or Dnmt3b-deficient HSCs, were incapable of long-term reconstitution in transplantation assays. These findings establish a critical role for DNA methylation by Dnmt3a and Dnmt 3b in HSC self-renewal.

2. Stem/progenitor cells in hepato-biliary system

a) Prospero-related homeobox 1 and liver receptor homolog 1 coordinately regulate long-term proliferation of murine fetal hepatoblasts.

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We investigated the contribution of Prospero-related homeobox 1 gene (Prox1) and liver receptor homolog 1 (Lrh1) in early liver development. Embryonic day 13 liver-derived $CD45^{-}Ter\ 119^{-}Dlk^{+}$ cells were purified as fetal hepatic stem/progenitor cells and formation of colonies derived from single-cells was detected under low-density culture conditions. We found that overexpression of Prox1 using retrovirus infection induced migration and proliferation of fetal hepatic stem/progenitor cells. In contrast, overexpression of Lrh1 suppressed colony formation. Prox1 induced the long-term proliferation of fetal hepatic stem/progenitor cells, which exhibited both high proliferative activity and bipotency for differentiation. Prox1 upregulated expression of cyclins D2, E1 and E2, whereas it suppressed expression of $p16^{ink4a}$, the cdk inhibitor. In addition, overexpression of Prox1 significantly inhibited the proximal promoter activity of $p16^{ink4a}$.

b) Limited Role of TCF/LEF Signaling in Liver Development

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The Wnt-TCF/LEF signaling pathway controls development and maintenance of stem cells in various tissues. Its role in hepatic stem/progenitor cells is debatable, however, in terms of liver development. We examined in detail the role of the Wnt-TCF/LEF pathway in the development of fetal hepatic stem/progenitor cells prospectively purified using cell surface markers. Expression analyses detected many Wnt ligands in whole fetal liver. Several Wnt ligands, Frizzled receptors, members of the catenin family, and all known TCF/LEF transcription factors were expressed in an overlapping pattern in E14.5 fetal liver by both primary hepatic stem/progenitor cells and hematopoietic cells. We also assessed the developmental kinetics of TCF/LEF activation using TCF/LEF-reporter mice. *In vivo* analysis using TCF/LEF-reporter mice demonstrated that TCF/LEF activation was transient; it was detected in a minority of E14.5 hepatic stem /progenitor cells, suggesting that the Wnt-TCF/LEF pathway is inactivated in most hepatic stem /progenitor cells in normal liver ontogeny. These results indicate that TCF/LEF activation plays only a limited role in the proliferation of hepatic stem/progenitor cells during normal development, and that the expansion of liver stem cells is predominantly regulated by Wnt-TCF/LEF-independent pathways.

3. ES cell differentiation

a) Generation of functional platelets from human embryonic stem cells in vitro via a VEGF-promoted structure, the ES-sac, that concentrates hematopoietic progenitors

Takayama N, Nishikii H, Usui J, Tsukui H, Eto K, Nakauchi H

Human embryonic stem cells (hESCs) are proposed as an alternative source for blood transfusion therapies or as a promising tool to study the ontogeny of hematopoiesis. When hESCs were cultured on stroma cells for facilitating hematopoiesis, we found that exogenous administration of VEGF promoted the emergence of 'sac'-like structures, which we named "embryonic stem cell-derived sacs" (ES-sacs). ES-sacs consisted of multiple cysts with cellular monolayers that retained properties of endothelial cells. The round cells inside ES-sacs could form hematopoietic colonies in semisolid culture and could differentiate into mature megakaryocytes in the presence of thrombopoietin, suggesting that ES-sacs provide a suitable environment for hematopoietic progenitors. Relatively large numbers of mature megakaryocytes could be induced from hematopoietic progenitors within ES-sacs, which were also able to release platelets. Platelets from hESCs displayed integrin αIIbβ3 activation and spreading in response to platelet agonists such as ADP and thrombin. This novel protocol provides a means of generating platelets from hESC that may contribute to efficient production of platelets for clinical transfusion or studies of thrombopoiesis.

b) The WAVE2/Abi1 complex differentially regulates megakaryocyte development and spreading: implications for platelet biogenesis and spreading machinery

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Actin polymerization is crucial in thrombopoiesis, platelet adhesion, and megakaryocyte (MK) and platelet spreading. The WASp-homologue WAVE functions downstream of Rac and plays a pivotal role in lamellipodia formation. While MKs and platelets express principally WAVE1 and -2, which are associated with Abi1, the physiological significance of WAVE isoforms remains undefined. We generated WAVE2$^{-/-}$ ES cells because WAVE2-null mice die by E12.5. We found that while WAVE2$^{-/-}$ ES cells differentiated into immature MKs on OP9 stroma, they were severely impaired in terminal differentiation and in platelet production. WAVE2$^{-/-}$ MKs exhibited a defect in peripheral lamellipodia on fibrinogen even with PMA costimulation, indicating a requirement of WAVE2 for integrin αIIbβ3-mediated full spreading. MKs in which expression of Abi1 was reduced by siRNA exhibited striking similarity to WAVE 2$^{-/-}$ MKs in maturation and spreading. Interestingly, the knockdown of IRSp53, a Rac effector that preferentially binds to WAVE2, impaired the development of lamellipodia without affecting proplatelet production. In contrast, thrombopoiesis *in vivo* and platelet spreading on fibrinogen *in vitro* were intact in WAVE1-null mice. These observations clarify indispensable roles for the WAVE2/Abi1 complex in αIIbβ3-mediated lamellipodia by MKs and platelets through Rac and IRSp53, and additionally in thrombopoiesis independent of Rac and IRSp53.

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