Center for Experimental Medicine

Laboratory of Cell Biology
細胞機能研究分野

Professor	Yoichiro Iwakura, 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	Katsuko Sudo, Ph.D.	助　手	獣医学博士	須　藤　カツ子
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. IL-17 production from activated T cells is required for the spontaneous development of destructive arthritis in mice deficient in IL-1 receptor antagonist

Susumu Nakae, Shinobu Saijo, Reiko Horai, Katsuko Sudo, Shigeo Mori[1], and Yoichiro Iwakura: [1]Division of Pathology, Department of Oncology

IL-17 is a T cell-derived, proinflammatory cytokine, which is suspected to be involved in the development of various inflammatory diseases. Although there are elevated levels of IL-17 in synovial fluid of rheumatoid arthritis (RA) patients, the pathogenic role of IL-17 in the development of RA, however, remains to be elucidated. In this report, the effects of IL-17-deficiency were examined in IL-1 receptor antagonist-deficient (IL-1Ra$^{-/-}$) mice, which spontaneously develop an inflammatory and destructive arthritis due to unopposed excess IL-1 signaling. IL-17 expression is greatly enhanced in IL-1Ra$^{-/-}$ mice, suggesting that IL-17 activity is involved in the pathogenesis of arthritis in these mice. Indeed, the spontaneous development of arthritis did not occur in IL-1Ra$^{-/-}$ mice also deficient in IL-17. The proliferative response of ovalbumin (OVA)-specific T cells from DO 11.10 mice against OVA co-cultured with antigen-presenting cells (APCs) from either IL-1 Ra$^{-/-}$ mice or wild-type mice was reduced by IL-17-deficiency, indicating insufficient T cell activation. Cross-linking OX40, a co-signaling molecule on CD4+ T cells that plays an important role in T cell-antigen presenting cell-interaction, with anti-OX40 antibody accelerated the production of IL-17 induced by CD3 stimulation. Since OX40 is induced by IL-1 signaling, IL-17 induction is likely to be downstream of IL-1 through activation of OX40. These observations suggest that IL-17 plays a crucial role in T cell activation, downstream of IL-1, causing the development of autoimmune arthritis.

2. Suppression of immune induction of collagen-induced arthritis in IL-17-deficient mice

Susumu Nakae, Aya Nambu, Katsuko Sudo, and Yoichiro Iwakura

IL-17 is a T cell-derived proinflammatory cytokine. This cytokine is suspected to be involved in the development of rheumatoid arthritis (RA) since this cytokine expression is augmented in synovial tissues of RA patients. The pathogenic roles of IL-17 in the development of RA, however, still remain to be elucidated. In this report, effects of IL-17-deficiency on collagen-induced arthritis (CIA) model were examined using IL-17 -deficient mice (IL-17$^{-/-}$ mice). We found that CIA was markedly suppressed in IL-17$^{-/-}$ mice. IL-17 was responsible for the priming of collagen-specific T cells and collagen-specific IgG2a production. Thus, these observations suggest that IL-17 plays a crucial role in the development of CIA by activating autoantigen-specific cellular and humoral immune responses.

3. IL-1 plays an important role in lipid metabolism by regulating insulin levels under physiological conditions

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

Interleukin-1 (IL-1) is a proinflammatory cytokine which plays important roles in inflammation. The role of this cytokine under physiological conditions, however, is not known completely. In this study, we analyzed the role of IL -1 in maintaining body weight, because IL-1 receptor antagonist-deficient (IL-1Ra$^{-/-}$) mice, in which excess IL-1 signaling may be induced, show a lean phenotype. Body fat accumulation was impaired in IL-1Ra$^{-/-}$ mice, but feeding behavior, expression of hypothalamic factors involved in feeding control, energy expenditure, and heat production were normal. When IL-1 Ra$^{-/-}$ mice were treated with monosodium glutamate (MSG), which causes obesity in wild-type mice by ablating cells in the hypothalamic arcuate nucleus, they were resistant to obesity, indicating that excess IL-1 signaling antagonizes the effect of MSG-sensitive-neuron-deficiency. IL-1 Ra$^{-/-}$ mice showed decreased weight gain when they were fed the same amount of food as wild-type mice, and lipid accumulation remained impaired even when they were fed a high-fat diet. Interestingly, serum insulin levels and lipase activity were low in IL-1Ra$^{-/-}$ mice, and the insulin levels were low in contrast to wild-type mice after MSG treatment. These observations suggest that IL-1 plays an important role in lipid metabolism by regulating insulin levels and lipase activity under physiological conditions.

4. Deficiency of interleukin-1 receptor antagonist promotes neointimal formation after injury

Kikuo Isoda[2], Masaru Shiigai[2], Norio Ishigami[2], Taizo Matsuki, Reiko Horai, Kenichirou Nishikawa[2], Masatoshi Kusuhara[2], Yasuhiro Nishida[3], Yoichiro Iwakura, and Fumitaka Ohsuzu[2]: [2]Internal Medicine I, [3]Physiology II, National Defense Medical College

Interleukin (IL)-1 plays an important role in inflammation, cell damage, and cell-proliferating reactions. IL-1 receptor antagonist (Ra) is an endogenous inhibitor of IL-1. However, the role of IL-1Ra in the formation of neointima after injury is poorly understood. To elucidate the role of IL -1Ra, IL-1Ra-deficient (IL-1Ra$^{-/-}$) mice were produced and backcrossed to C57BL/6J mice for 8 generations. We investigated neointimal formation in both wild type (IL-1Ra$^{+/+}$) and IL-1Ra$^{-/-}$ mice after injury. Vascular intima formation was induced by an external vascular cuff model. Three weeks after injury, the thickness of the intima and media were measured and the ratio of the intima to media (I/M) was calculated. The mean intimal thickness and the I/M ratio of IL-1 Ra$^{-/-}$ mice were increased by 262% ($P<0.0001$) and 284% ($P<0.0001$) in comparison to the IL-1 Ra$^{+/+}$ mice, but there was no significant difference in the medial thickness. Immunostaining for IL-1Ra showed IL-1Ra protein to be present in the endothelium and some inflammatory cells of the adventitia in IL-1Ra$^{+/+}$ mice, but it was not detected in the IL-1Ra$^{-/-}$ mice. These observations suggest that IL-1Ra plays an important role in the suppression of neointimal formation after angioplasty and atherosclerosis in vivo.

5. Interleukin 1 is required for tumor invasiveness and angiogenesis

Elena Voronov[4], Dror S. Shouval[4], Yakov Krelin[4], Emanuela Cagnano[4], Daniel Benharroch[4], Yoichiro Iwakura, Charles A. Dinarello[5], and Ron N. Apte[4]: [4]Departments of Microbiology and Immunology and of Pathology, Faculty of Health Sciences and The Cancer Research Center, Ben-Gurion University of the Negev, Beer-Sheva, Israel; [5]University of Colorado Health Sciences Center, Denver, USA

Here we describe that microenvironmental interleukin-1β (IL-1β), and to a lesser extent IL-1 α, are required for in vivo angiogenesis and invasiveness of different tumor cells. In IL-1β knockout (KO) mice, local tumor or lung metastases of B16 melanoma cells were not observed compared to wild-type (WT) mice. Angiogenesis

was assessed by the recruitment of blood vessel networks into Matrigel plugs containing B16 melanoma cells; vascularization of the plugs was present in WT mice, but was absent in IL-1 β KO mice. The addition of exogenous IL-1 into B16-containing Matrigel plugs in IL-1β KO mice partially restored the angiogenic response. Moreover, the incorporation of IL-1 receptor antagonist (IL-1Ra) to B16-containing plugs in WT mice inhibited the ingrowth of blood vessel networks into Matrigel plugs. In IL-1α KO mice, local tumor development and induction of an angiogenic response in Matrigel plugs was less pronounced than in WT mice, but significantly higher than in IL-1β KO mice. These effects of host-derived IL-1α and IL-1β were not restricted to the melanoma model, but were also observed in DA/3 mammary and prostate cancer cell models. In addition to the in vivo findings, IL-1 contributed to the production of vascular endothelial cell growth factor and tumor necrosis factor in co-cultures of peritoneal macrophages and tumor cells. Host-derived IL-1 appears to control tumor angiogenesis and invasiveness. Furthermore, the anti-angiogenic effects of IL-1Ra, shown here, suggest a possible therapeutic role in cancer, in addition to its current use in rheumatoid arthritis.

6. Studies on the roles of cytokines in physiological and pathological bone remodeling

Hisataka Yasuda, Young-Mi Lee, Atsuko Minamida, and Yoichiro Iwakura

We previously molecular cloned osteoclastoge nesis-inhibitory factor (OCIF) (also called osteoprotegerin [OPG]), osteoclast differentiation factor (ODF; also called OPG ligand [OPGL], TNF-related activation-induced cytokine [TRANCE], and receptor activator of NF-κB ligand [RANKL]), and receptor activator of NF-κB (RANK), all of which are important for regulating osteoclast differentiation and activation. ODF/RANKL is a member of the membrane-associated tumor necrosis factor (TNF) ligand family and it induces osteoclast differentiation from progenitor cells co-treated with macrophage colony-stimulating factor (M-CSF) in the absence of osteoblasts/stromal cells and osteotropic factors. ODF/RANKL is a long-sought ligand expressed on osteoblasts/stromal cells in response to osteotropic factors, and it mediates an essential signal to osteoclast progenitors for their differentiation into active osteoclasts. OCIF /OPG is a secreted member of the tumor necrosis factor receptor (TNFR) family, and it inhibits osteoclastogenesis in vitro and in vivo. RANK is the signaling receptor essential for ODF/RANKL-mediated osteoclastogenesis, and that OCIF/OPG acts as a decoy receptor for ODF/RANKL to compete against RANK. The discovery of ODF/RANKL, OCIF/OPG, and RANK opens a new era in the investigation of the regulation of osteoclast differentiation/function. Even though molecular mechanism of osteoclast differentiation and activation is almost clarified, factors (e.g. cytokines and hormones) regulating the expression of ODF/RANKL, OCIF/OPG, and RANK in vivo are not well studied.

Inflammatory cytokines (e.g. IL-1) play a major role in bone resorption in pathological conditions (e.g. rheumatoid arthritis and periodontal diseases). IL-1 also regulates the expression of ODF/RANKL and OCIF/OPG in vitro. However, the roles of these cytokines in bone development in physiological conditions are unknown. In addition, the relationship between inflammatory cytokines and ODF/RANKL or OCIF/OPG is not known in physiological conditions. Previous studies demonstrated that no obvious abnormality in bone in IL-1 receptor type I (IL-1R1) KO mice whose genetic background were C57BL/6 x 129/SV. We addressed the role of IL-1 in physiological bone remodeling using IL-1α KO mice, IL-1β KO mice, and IL-1α, β double KO mice, all of which were backcrossed to BALB/cA strain mice for 8 generations. Measurement of Bone Mineral Density (BMD) of femur with dual energy X-ray absorptiometry and peripheral quantitative computed tomography (pQCT) revealed significant increases in 8-week old mice with each genotype. Radiographs showed massive increase in bone density especially in the epiphysis and metaphysis of femur of these KO mice. Histological analysis also showed that marked increase of bone volume in trabecular bone of these KO mice. The thickness of cortical bone also was increased in these KO mice. The morphology of the growth plate and the columnar organization of chondrocytes are normal, but cartilaginous remnants were markedly observed in the cortical bone of these KO mice, which suggests a decrease in osteoclastic activity in resorption of bone and cartridge. Taken together these results indicate that IL-1 may have an important role in physiological bone development. The mechanism by which IL -1 KO mice have increased bone mass is under investigation.

To treat rheumatoid arthritis (RA), it is important to inhibit inflammation and bone resorption. It is known that activated T cells expressing ODF/RANKL are involved in RA. We proposed a hypothesis that ODF/RANKL on the activated T cells was important in inflammation and bone resorption in RA. We generated transgenic mice (TG) overexpressing OCIF/OPG in T

cells to inhibit ODF/RANKL on the activated T cells. OCIF/OPG-TG mice were crossed with IL-1 receptor antagonist KO mice that develop autoimmunity and chronic inflammatory artropathy closely resembling RA in humans. The effects of OCIF/OPG on the development of RA are under investigation. We are also investigating the roles of soluble ODF/RANKL in bone remodeling using TG-mice overexpressing soluble ODF/RANKL.

7. Impaired selectin ligand biosynthesis and reduced inflammatory responses in β-1,4-galactosyltransferase-I-deficient mice

Masahide Asano[6], Susumu Nakae, Norihiro Kotani[7], Naoki Shirafuji[8], Aya Nambu, Noriyoshi Hashimoto[6], Hiroto Kawashima[9], Mayumi Hirose[9], Masayuki Miyasaka[9], Seiichi Takasaki[9] and Yoichiro Iwakura: [6]Center for Experimental Medicine, Institute of Medical Science, University of Tokyo, and Department of Transgenic Animal Science, Graduate School of Medical Science, Kanazawa University; [7]Department of Biochemistry and [8]Department of Advanced Medical Science, Institute of Medical Science, University of Tokyo; [9]Department of Post-Genomics and Diseases, Osaka University Graduate School of Medicine.

Selectin ligands are known to be carbohydrate chains such as sialyl Lewis x (sLe^x), which are mainly expressed at the terminal of N-acetyllactosamine repeats on core 2 O-linked glycans. Several glycosyltransferases are successively acting to extend N-acetyllactosamine repeats and to synthesize sLex, and β-1,4-galactosyltransferase (GalT) plays a key role in these processes. However, seven members of GalT genes have been recently isolated so far and individual roles of them including selectin ligand biosynthesis remain to be elucidated. Using β-1,4-galactosyltransferase-I (GalT-I)-deficient mice, we evaluate the contribution of GalT-I in selectin ligand biosynthesis. More than 80% of core 2 O-glycans in leukocyte membrane glycoproteins of GalT-I-deficient mice were not galactosylated by β-1,4 linkage and binding of soluble P-selectin to their leukocytes was significantly reduced, indicating that biosynthesis of selectin ligands was impaired. GalT-I-deficient mice exhibited blood leukocytosis, but normal lymphocyte homing to peripheral lymph nodes. Acute and chronic inflammatory responses were suppressed and infiltration of neutrophils into inflammatory sites was largely reduced during inflammatory responses in GalT-I-deficient mice. Our results clearly demonstrate that GalT-I is a major galactosyltransferase responsible for selectin ligand biosynthesis and that inflammatory responses of GalT-I-deficient mice are impaired due to the defect in selectin ligand biosynthesis.

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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

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. Role of Phospholipase C-L2, a negative regulator of calcium signaling, is required for B cell development and immune response.

Kei Takenaka[1], Kiyoko Fukami[1,2], Makiko Otsuki[1], Yoshikazu Nakamura[1], Yuki Kataoka, Mika Wada[3], Kohichiro Tsuji[3], Shin-Ichi Nishikawa[4], Nobuaki Yoshida, and Tadaomi Takenawa.[1,2]: Department of Biochemistry[1] Division of Cellular Therapy[3], The Institute of Medical Science, University of Tokyo, [2]Core Research for Evolutional Science and Technology of Japan Science and Technology Corporation, [4]Department of Molecular Genetics, Kyoto University Graduate School of Medicine.

Phospholipase C (PLC) plays important roles in phosphoinositide turnover by regulating the calcium-protein kinase C signaling pathway. PLC-L2 is a novel PLC-like protein which lacks PLC activity, although it is very homologous with PLCδ. PLC-L2 is expressed in hematopoietic cells, but its physiological roles and intracellular functions in the immune system have not yet been clarified. To elucidate the physiological function of PLC-L2, we generated mice which had a genetic *PLC-L2* deficiency. PLC-L2-deficient mice grew with no apparent abnormalities. However, mature B cells from PLC-L2-deficient mice were hyperproliferative in response to B-cell receptor (BCR) cross-linking, although B2 cell development appeared to be normal. Molecular biological analysis revealed that calcium influx and NFATc accumulation in nuclei were increased in PLC-L2-deficient B cells. Extracellular signal-regulated kinase activity was also enhanced in PLC-L2-deficient B cells. These

mice had a stronger T-cell-independent antigen response. These results indicate that PLC-L2 is a novel negative regulator of BCR signaling and immune responses.

2. WAVE 2 is required for directed cell migration and cardiovascular development.

Daisuke Yamazaki[1,6], Shiro Suetsugu[1,6], Hiroaki Miki[5,7], Yuki Kataoka, Shin-Ichi Nishikawa[9], Takashi Fujiwara[8], Nobuaki Yoshida and Tadaomi Takenawa.[1,6]: [5]Division of Cancer Genomics, Institute of Medical Science, University of Tokyo, [6]CREST, Japan Science and Technology Corporation, [7]PRESTO, Japan Science and Technology Corporation; Department of Molecular Genetics, Graduate School of Medicine, Kyoto University, [8]Laboratory Animal Center, Ehime University School of Medicine, [9]RIKEN, Center for Developmental Biology.

WAVE2, a protein related to Wiskott-Aldrich syndrome protein, is crucial for Rac-induced membrane ruffling, which is important in cell motility. Cell movement is essential for morphogenesis, but it is unclear how cell movement is regulated or related to morphogenesis. Here we show the physiological functions of WAVE2 by disruption of the WAVE2 gene in mice. WAVE2 was expressed predominantly in vascular endothelial cells during embryogenesis. WAVE2-/- embryos showed haemorrhages and died at about embryonic day 10. Deficiency in WAVE2 had no significant effect on vasculogenesis, but it decreased sprouting and branching of endothelial cells from existing vessels during angiogenesis. In WAVE2-/- endothelial cells, cell polarity formed in response to vascular endothelial growth factor, but the formation of lamellipodia at leading edges and capillaries was severely impaired. These findings indicate that WAVE2-regulated actin reorganization might be required for proper cell movement and that a lack of functional WAVE2 impairs angiogenesis in vivo.

3. Forkhead transcription factor Foxf2 (LUN)-deicient mice exhibit abnormal development of secondary palate.

Tao Wang[10], Tomoki Tamakoshi[10], Tadayoshi Uezato[10], Fang Shu[11], Naoko Kanzaki-Kato[12], Yan Fu[12], Haruhiko Koseki[13], Nobuaki Yoshida, Toshihiro Sugiyama[12] and Naoyuki Miura.[10]: [10]Department of Biochemistry, Hamamatsu University School of Medicine, [11]Department of Obstetrics and Gynecology, Hamamatsu University School of Medicine, [12]Department of Biochemistry, Akita University School of Medicine, [13]Graduate School of Medicine, Chiba University.

The forkhead genes encode a transcription factor involved in embryogenesis and pattern formation in multicellular organisms. They are mammalian transcriptional regulators that bind DNA as a monomer through their forkhead domain. The Foxf2 (LUN) mRNA is expressed in the mesenchyme directly adjacent to the ectoderm-derived epithelium in the developing tongue and in the mesenchyme adjacent to the endoderm-derived epithelium in the gastrointestinal (GI) tract, lungs, and genitalia. To investigate the developmental role of the Foxf2 gene during embryogenesis, we disrupted the Foxf2 gene and showed that these mutant mice died shortly after birth. Mice lacking the Foxf2 gene were found to develop cleft palate and an abnormal tongue. In addition, we found that the GI tract and the lungs of Foxf2-deficient newborn mice were normal in both morphology and function. These results suggest that the Foxf2 gene plays key roles in palatogenesis by reshaping the growing tongue.

4. Thromboxane A2 modulates interaction of dendritic cells and T cells and regulates acquired immunity.

Kenji Kabashima[14,15,20], Takahiko Murata[14,20], Hiroyuki Tanaka[16], Toshiyuki Matsuoka[14], Daiji Sakata[14], Nobuaki Yoshida, Koko Katagiri[17], Tatsuo Kinashi[17], Toshiyuki Tanaka[18], Masayuki Miyasaka[18], Hiroichi Nagai[16], Fumitaka Ushikubi[19] and Shuh Narumiya.[14]: [14]Department of Pharmacology, Kyoto University Faculty of Medicine, [15]Department of Dermatology, Kyoto University Faculty of Medicine, [16] Department of Pharmacology, Gifu Pharmaceutical University, [17]Department of Molecular Immunology and Allergy, Kyoto University Faculty of Medicine, [18]Molecular and Cellular Recognition, Osaka University Graduate School of Medicine, [19] Department of Pharmacology, Asahikawa Medical College.[20]

Physical interaction of T cells and dendritic cells (DCs) is essential for T cell proliferation and differentiation, but it has been unclear how this interaction is regulated physiologically. Here we show that DCs produce thromboxane A2 (TXA2), whereas naive T cells express the thromboxane receptor (TP). In vitro, a TP agonist enhances random cell movement (chemokinesis) of naive but not memory T cells, impairs DC-T cell adhesion, and inhibits DC-dependent proliferation of T cells. In vivo, immune responses to foreign antigens are enhanced in TP-

deficient mice, which also develop marked lymphadenopathy with age. Similar immune responses were seen in wild-type mice treated with a TP antagonist during the sensitization period. Thus, TXA2-TP signaling modulates acquired immunity by negatively regulating DC-T cell interactions.

5. Phospholipase Cδ1 is required for skin stem cell lineage commitment.

Yoshikazu Nakamura[1,6], Kiyoko Fukami[1,6], Haiyan Yu[1,22], Kei Takenaka[1,6], Yuki Kataoka, Yuji Shirakata[21], Shin-Ichi Nishikawa[3], Koji Hashimoto[21], Nobuaki Yoshida and Tadaomi Takenawa[1,6]: [21]Department of Dermatology, Ehime University School of Medicine, [22]Joslin Diabetes Center, Harvard Medical School.

Phosphoinositide-specific phospholipase C (PLC) is a key enzyme in phosphoinositide turnover and is involved in a variety of physiological functions. Here we report that PLCdelta(1)-deficient mice undergo progressive hair loss in the first postnatal hair cycle. Epidermal hyperplasia was observed, and many hairs in the skin of PLCdelta(1)-deficient mice failed to penetrate the epidermis and became zigzagged owing to occlusion of the hair canal. Two major downstream signals of PLC, calcium elevation and protein kinase C activation, were impaired in the keratinocytes and skin of PLCdelta(1)-deficient mice. In addition, many cysts that had remarkable similarities to interfollicular epidermis, as well as hyperplasia of sebaceous glands, were observed. Furthermore, PLCdelta(1)-deficient mice developed spontaneous skin tumors that had characteristics of both interfollicular epidermis and sebaceous glands. From these results, we conclude that PLCdelta(1) is required for skin stem cell lineage commitment.

6. Phospholipase Cδ4 is required for Ca^{2+} mobilization essential for acrosome reaction in sperm.

Kiyoko Fukami[1,26], Manabu Yoshida[24,27], Takafumi Inoue[24,27], Manabu Kurokawa[25], Rafael A. Fissore[25], Nobuaki Yoshida, Katsuhiko Mikoshiba[24,27,28] and Tadaomi Takenawa[1,26]: [24]Division of Molecular Neurobiology, The Institute of Medical Science, The University of Tokyo, [25]Department of Veterinary and Animal Sciences, Paige Laboratory, University of Massachusetts, [26]Center for Renewable Energy and Sustainable Technology, International Cooperative Research Project, Japan Science and Technology Corporation, [27] Calcium Oscillation Project, International Cooperative Research Project, Japan Science and Technology Corporation, [28] RIKEN Brain Science Institute.

Zona pellucida (ZP)-induced acrosome reaction in sperm is a required step for mammalian fertilization. However, the precise mechanism of the acrosome reaction remains unclear. We previously reported that PLCdelta4 is involved in the ZP-induced acrosome reaction in mouse sperm. Here we have monitored Ca^{2+} responses in single sperm, and we report that the $[Ca^{2+}]i$ increase in response to ZP, which is essential for driving the acrosome reaction in vivo, is absent in PLCdelta4-/- sperm. Progesterone, another physiological inducer of the acrosome reaction, failed to induce sustained $[Ca^{2+}]i$ increases in PLCdelta4-/- sperm, and consequently the acrosome reaction was partially inhibited. In addition, we observed oscillatory $[Ca^{2+}]i$ increases in wild-type sperm in response to these acrosome inducers. Calcium imaging studies revealed that the $[Ca^{2+}]i$ increases induced by exposure to ZP and progesterone started at different sites within the sperm head, indicating that these agonists induce the acrosome reaction via different Ca^{2+} mechanisms. Furthermore, store-operated channel (SOC) activity was severely impaired in PLCdelta4-/- sperm. These results indicate that PLCdelta4 is an important enzyme for intracellular $[Ca^{2+}]i$ mobilization in the ZP-induced acrosome reaction and for sustained $[Ca^{2+}]i$ increases through SOC induced by ZP and progesterone in sperm.

7. Mammalian Twisted gastrulation is essential for skeleto-lymphogenesis.

Tetsuya Nosaka[29], Sumiyo Morita[29], Hidetomo Kitamura[31], Hideaki Nakajima[30], Fumi Shibata[29], Yoshihiro Morikawa[32], Yuki Kataoka, Yasuhiro Ebihara[30], Toshiyuki Kawashima[29], Tsuneo Itoh[31], Katsutoshi Ozaki[29], Emiko Senba[32], Kohichiro Tsuji[30], Fusao Makishima[31], Nobuaki Yoshida and Toshio Kitamura[30]: Division of Hematopoietic Factors[29], Division of Cellular Therapy, Advanced Clinical Research Center[30], Institute of Medical Science, The University of Tokyo, [31]Fuji Gotemba Research Labs, Chugai Pharmaceutical Co., Ltd., [32]Department of Anatomy and Neurobiology, Wakayama Medical School.

Dorsoventral patterning depends on the local concentrations of the morphogens. Twisted gastrulation (TSG) regulates the extracellular availability of a mesoderm inducer, bone morphogenetic protein 4 (BMP-4). However, TSG function in vivo is still unclear. We isolated a TSG cDNA as a secreted molecule from the mouse aorta-

gonad-mesonephros region. Here we show that TSG-deficient mice were born healthy, but more than half of the neonatal pups showed severe growth retardation shortly after birth and displayed dwarfism with delayed endochondral ossification and lymphopenia, followed by death within a month. TSG-deficient thymus was atrophic, and phosphorylation of SMAD1 was augmented in the thymocytes, suggesting enhanced BMP-4 signaling in the thymus. Since BMP-4 promotes skeletogenesis and inhibits thymus development, our findings suggest that TSG acts as both a BMP-4 agonist in skeletogenesis and a BMP-4 antagonist in T-cell development. Although lymphopenia in TSG-deficient mice would partly be ascribed to systemic effects of runtiness and wasting, our findings may also provide a clue for understanding the pathogenesis of human dwarfism with combined immunodeficiency.

8. Zinc finger protein sall2 is not essential for embryonic and kidney development.

Akira Sato[33,34], Yuko Matsumoto[33], Urara Koide[33,34], Yuki Kataoka, Nobuaki Yoshida, Takashi Yokota[33], Makoto Asashima[34] and Ryuichi Nishinakamura[33]: [33]Division of Stem Cell Regulation, The Institute of Medical Science, The University of Tokyo, [34]Department of Life Sciences, The University of Tokyo.

SALL/Sall is a mammalian homolog of the Drosophila region-specific homeotic gene spalt (sal), and heterozygous mutations in SALL1 in humans lead to Townes-Brocks syndrome. We earlier reported that mice deficient in Sall1 die in the perinatal period and that kidney agenesis or severe dysgenesis are present. We have now generated mice lacking Sall2, another Sall family gene. Although Sall2 is expressed mostly in an overlapping fashion versus that of Sall1, Sall2-deficient mice show no apparent abnormal phenotypes. Morphology and gene expression patterns of the mutant kidney were not affected. Mice lacking both Sall1 and Sall2 show kidney phenotypes comparable to those of Sall1 knockout, thereby demonstrating the dispensable roles of Sall2 in embryonic and kidney development.

9. Genetic analysis of lymphatic development and functions in mice.

Hirotake Ichise, Taeko Ichise, Seiji Shiozawa, Takashi Yamaguchi, Akiko Hori, Kaori Yamanaka and Nobuaki Yoshida.

The lymphatics are 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 some roles on tumor metastasis. However, the lymphatic development in mammals has been unknown from lack of useful mutant animal that has obvious lymphatic abnormality.

In order to understand the mechanism of lymphatic development and functions, we are generating genetically-engineered mice for some genes that are thought to regulate lymphangiogenesis, such as *VEGF-C, Angiopoietin-2* and *Prox-1*. Using Cre/*loxP* recombination system, the overexpression of transgene can be induced temporally or tissue-specifically in them. For creating cre transgenic mice, the expression of *cre* recombinase gene is drived by either endothelial cell-specific promoter or ubiquitous promoter. We have already established some tamoxifen-inducible cre transgenic mouse lines, so we are investigating the recombination efficiencies of floxed ROSA26 (ROSA26R) loci in tamoxifen-inducible *cre* Tg/ROSA26R double transgenic mice and seeking for efficient methods of tamoxifen administration. However, cre-mediated recombination has not been observed in these lines, although *cre* expression can be detected by Western blot analysis. We have also established some cre expressing transgenic mouse lines. In several of them, cre-mediated recombination is confirmed to be endothelial cell-specific by the observation of *lacZ* expression in *cre* Tg/ROSA26R double transgenic mice.

We are also under investigation of an original spontaneous mutant mouse line developing chylous ascites and lymphedema that are thought to be due to lymphatic abnormality. In the homozygous mutant mice, the blood flow is found not only in blood vessels but also in lymphatic vessels of intestine and a part of skin. The peripheral capillary-lacteal shunt at the intestinal villi is observed in homozygous mutant mice. It is thought to be one of the cause for blood flow observed in lymphatics of the homozygous mutant mice. In our immunohistochemical study, VEGFR-3, one of the receptor tyrosine kinase regulating lymphangiogenesis, is expressed intensely in the intestinal lymphatic endothelial cells of the wild-type mice, but not in those of the homozygous mutant mice. The intestinal lymphatic vascular structure of the homozygous mutant mice is immature and dilated. In addition to the lymphatic defect, the intestinal vascular structure of blood vessels is also impaired in them. The candidate 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. We are trying forward genetic approaches

to find the candidate for this mutation. As they have an autosomal recessive mutation, we have performed F2 intercrosses between our mutant strain in 129/SvEv genetic background and wild -derived inbred strain, CAST/Ei, in order to map the mutant locus.

10. Functional analysis of ROX-1 in ES cells.

Mitsuharu Sato, Yuhki Nakatake, Masaki Shibayama, Toshiaki Isobe[35], Tomonori Izumi[35], and Nobuaki Yoshida.: [35]Division of Proteomics research, The Institute of Medical Science, The University of Tokyo.

Mouse embryonic stem (ES) cells are one of pluripotent cells which can be expanded *in vitro* and extensively utilized to generate gene knockout mouse. However, how ES cells attain pluripotency and keep themselves in undifferentiated state is not fully understood. To elucidate how ES cells maintain undifferentiated state, we started our study to clone the factor named ROX-1, which binds to *Rex-1* promoter.

We have determined amino acid sequence of the candidates and obtained cDNAs. One of the candidates translated *in vitro* showed the same property as ROX-1 for DNA binding observed in ES cells. In addition, *in situ* hybridization revealed the mRNA expression of this candidate was confined in inner cell mass of 3.5 d mouse embryo. We concluded that this is the ROX-1.

To determine the role of ROX-1 in maintaining the undifferentiated state of ES cells, ROX-1 mRNA expression was down regulated using RNA interference. For this purpose, retrovirus vector carrying siRNA expression unit driven by RNA polymerase III was constructed. When siRNA for ROX-1 was expressed, mRNA level of *Rex-1* was reduced. Furthermore, we found that the mRNA level of *Nanog* has also decreased. This finding prompted us to test whether ROX-1 was involved in the regulation of gene expression of *Nanog*. Using several promoter construct, we nailed down the region containing enhancer activity that is specific to undifferentiated ES cell. Then we tested whether ROX-1 could bind to this region in electromobility shift assay and showed that ROX-1 bound to the region indeed. These results show that ROX-1 is the key factor in the maintenance of undifferentiated state of ES cells.

Cre-loxP system can be used to conditionally regulate the gene expression. We have generated ES cell clones in which one of the alleles of *ROX-1* was disrupted and the other was changed to be regulated by Cre expression. In these cell clones, null mutation can be introduced efficiently by expressing Cre recombinase. We are now setting the condition of Cre expression to see the effect of *ROX-1* mutation. ROX-1 knockout mouse are also in preparation: we have already obtained heterozygous mice and started mating to generate homozygous mutants.

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市瀬広武．リンパ管形成−マウス発生工学を用いた研究．血管医学．4(6)：59−66，2003

Center for Experimental Medicine

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

Professor	Hiromitsu Nakauchi	教　授	中　内　啓　光
Lecturer	Atsushi Iwama	講　師	岩　間　厚　志
Research Associate	Hideo Ema	助　手	依　馬　秀　夫
Research Associate	Koji Eto	助　手	江　藤　浩　之

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 stem cell self-renewal and commitment

a. Asymmetric cell division of the hematopoietic stem cells

One of the central tasks of stem cell biology is to understand the mechanisms that regulate lineage commitment of stem cells. Despite the fact that hematopoietic stem cells (HSCs) are the best characterized stem population, how they differentiate is poorly understood. Several models have been proposed to explain HSC fate determination. Analyses of in vivo as well as in vitro colony-forming cells (CFCs), particularly CFCs obtained from blast-cell colonies, have suggested a stochastic model for HSC behavior. Consistent with this model, a permissive role for cytokines has also been suggested. The classic hematopoietic inductive microenvironment and stem cell competition models have held that extrinsic factors play an instructive role. This idea has been supported by lineage analysis of the progeny of CFCs. Because no previous study has verified these working models by directly examining HSCs, whether extrinsic signaling plays any role in HSC lineage commitment remains controversial. In order to study lineage commitment in HSCs, we therefore sought to determine the differentiation potential of the immediate progeny of HSCs at the clonal level. That HSCs are highly enriched in a population of $CD34^{-/low}$, c-Kit^{+}, Sca-1^{+} and lineage $marker^{-}$ ($CD34^{-}$KSL) cells among bone marrow cells of the adult mouse has enabled both in vitro and in vivo clonal analyses of HSCs. Cytokines such as interleukin-3 (IL-3) and thrombopoietin (TPO) together with stem cell factor (SCF) directly acted on these cells and induced their division. After $CD34^{-}$KSL cells underwent one cell division, stem cell activity became undetectable in their progeny except in limited cases where this activity was maintained in one of the two daughter cells. We assumed that lineage commitment could be responsible for loss of stem cell activity in this setting. To verify this hypothesis, we rigorously examined the differentiation potential of paired daughter cells arisen from single $CD34^{-}$KSL cells in vitro.

Despite a lack of information on lymphoid differentiation potential, in vitro colony assay permits quantitative evaluation of differentiation

potential along neutrophil (n), macrophage (m), erythroblast (E), and megakaryocyte (M) lineages at the single-cell level. In this study, using in vitro colony assays, we demonstrated that lineage commitment of multipotent $CD34^{-}KSL$ cells occurs at the initial stage of their cell divisions, and that this lineage commitment is asymmetric. Treatment with cytokines increases the chance that $CD34^{-}KSL$ cells will divide in an asymmetric rather than a symmetric manner, with one daughter cell committed to lineage-specific differentiation.

b. Glucagon-like peptide 1 (1-37) converts intestinal epithelial cells into insulin-producing cells.

Glucagon-like peptide (GLP) 1 is produced through posttranslational processing of proglucagon and acts as a regulator of various homeostatic events. Among its analogs, however, the function of GLP-1-(1-37), synthesized in small amounts in the pancreas, has been unclear. We found that GLP-1-(1-37) induces insulin production in developing and, to a lesser extent, adult intestinal epithelial cells *in vitro* and *in vivo*, a process mediated by up-regulation of the Notch-related gene *ngn3* and its downstream targets, which are involved in pancreatic endocrine differentiation. These cells became responsive to glucose challenge *in vitro* and reverse insulin-dependent diabetes after implantation into diabetic mice. Our findings suggest that efficient induction of insulin production in intestinal epithelial cells by GLP-1-(1-37) could represent a new therapeutic approach to diabetes mellitus.

c. Role for growth factors and extracellular matrix in controlling differentiation of prospectively isolated hepatic stem cells

In liver development, a number of growth factors (GFs) and components of the extracellular matrix (ECMs) lead to differentiation of liver parenchymal cells. As the liver contains many cell types, specifically investigating their functional effects on hepatic stem cell populations is difficult. Prospective isolation and clonal assays for hepatic stem cells enable the examination of direct effects of GFs and ECMs on this rare cell fraction. Using previously purified cells that fulfill the criteria for hepatic stem cells, we examined how GFs and ECMs regulate differentiation in the developing liver. We show here that hepatocyte growth factor (HGF) induced early transition of albumin (ALB)-negative stem cells to ALB-positive hepatic precursors resembling hepatoblasts and then oncostatin M (OSM) promoted their differentiation to tryptophan-2, 3dioxygenase (TO)-positive mature hepatocytes. During this transition, ECMs were necessary for the differentiation of stem cells and precursors, but their effects were only supportive. In the first step of stem cell differentiation induced by HGF, the expression of CCAAT/enhancer binding protein (C/EBP), a basic leucine zipper transcription factor, changed dramatically. When C/EBP function was inhibited in stem cells, they stopped differentiating to hepatocyte-lineage cells and proliferated actively. These are the first findings to illustrate the mechanism of hepatic stem cell differentiation in liver development.

2. Experimental models of stem cell therapy

Successful multilineage engraftment of human cord blood cells in pigs after in utero transplantation

Among analyses that measure "hematopoietic stem-cell (HSC) activity," the most useful one is a long-term competitive marrow repopulation assay (CMRA) that measures the capability of cells to reconstitute bone marrow (BM) of lethally irradiated recipient animals. The availability of CMRA, in conjunction with the development of multiparameter flow cytometry, has allowed characterization and purification of mouse HSCs. In contrast, functional analyses of human hematopoietic cells have been limited, such as colony formation assay and long-term culture (LTC). Colony formation assay detects committed and multipotent progenitor cells (colony-forming cells) but not HSCs. LTC-initiating cells (LTC-ICs), which are capable of giving rise to colony-forming cells after 5 weeks of culture on competent feeder layers, are functionally heterogeneous, and a small subpopulation of LTC-ICs seems to represent HSCs. In addition, the relationship between LTC-IC and in vivo repopulating human HSCs is not clear. In vivo functional assays for human hematopoietic stem and progenitor cells (HSPCs) based on the ability of HSPCs to survive in severe combined immunodeficient (SCID) or non-obese diabetic/SCID (NOD/SCID) mice have been reported. Transplantation of human BM or cord blood (CB) cells into these mice has resulted in an engraftment of human HSPCs in mouse BM. However, because of short life span, usefulness of these mouse models is limited. In contrast, successful engraftment of human hematopoietic stem and progenitor cells (HSPCs) in a large animal may serve not only as a model to study human hematopoiesis but also as a bioreactor to expand human HSPCs in vivo. We therefore attempted a xenotransplantation of human HSPCs into pig. Transplantation of human cord blood

cells into pig fetuses aged less than 52 days postcoitus resulted in a good engraftment rate. In one case, engraftment was detected up to 315 days posttransplantation by polymerase chain reaction. Human hematopoietic cells were detectable also by FACS in peripheral blood and BM. Furthermore, human CD34+ HSPCs were also observed in the BM of recipients. Those CD 34+ cells in BM were sorted by FACS and subjected to further analyses. First, in vitro colony formation assay resulted in formations of multilineage colonies. Second, when they were transplanted into an immunodeficient mouse they were engrafted in the mouse.

These data indicate an engraftment of human HSPCs in pig BM. In utero transplantation of human HSPCs into preimmune pig fetus resulted in a long-term engraftment of human HSPCs in the BM of recipient pig. Pig may serve as an alternative large animal for the study of human hematopoiesis and for other applications.

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