

CHRONOLOGICAL TABLE

1956 April	Institute for Virus Research, Kyoto University, was founded with two departments (Pathology and Biophysics).
1956 April	Scientific Lectures for the Public were presented commemorating the opening of the Institute (the successive Memorial Lecture Series have been presented annually hereafter).
1957 April	Department of Biochemistry and Department of Serology and Immunology were established.
1958 April	Department of Prevention and Therapeutics was established.
1958 December	"Advances in Virology", Vol. 1 (in Japanese) was published as collection of the Memorial Lectures (the successive volumes were published annually hereafter until 1960).
1958 December	"Annual Report of the Institute for Virus Research", Vol. 1, was published (the successive volumes have been published annually hereafter).
1959 July	Virus Diagnosis Center was established.
1961 October	The 1st Symposium of the Institute for Virus Research was held under the auspices of the Institute with the nationwide participants. The proceedings of the Symposium were published as the first issue of the new series of "Advances in Virology" in Japanese (the successive Symposia have been held and their proceedings published annually hereafter).
1962 April	Department of Tumor Virus was established.
1962 October	Several staff members were appointed academic staff of the Graduate School of Medicine, and students of the School were first admitted to the Institute.
1962 December	Several staff members were appointed academic staff of the Graduate School of Science, and students of the School were first admitted to the Institute.
1964 April	Virus Diagnosis Center was renamed Virological Diagnosis Center.
1965 September	Construction of the new building for the Institute commenced.
1967 March	Construction of the new building was completed.
1968 April	Department of Genetics was established.
1974 April	Department of Molecular and Cellular Virology was established.
1977 April	Department of Neurological Virus Disease was established as such that Visiting Staff be appointed.
1978 April	Animal Laboratory for Experimental Virus Infection was established.
1981 March	Construction of extension of the main building was completed. Thus the main building now constitutes five floors with a basement occupying the aggregate area of 5,410 m ² . The major part (ca. 481 m ²) of the extended area serves for researches

involving radioisotope labelling and in vitro DNA recombination experiments requiring the P3 facilities.

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| 1986 May | The memorial events for the 30th anniversary of foundation of this Institute were held on May 16-17. |
| 1986 November | Professor Yorio Hinuma was honoured as "Person of Cultural Merits (Bunkakorosha)" |
| 1987 May | Department of Biophysics and Department of Tumor Virology were reorganized to form Department of Viral Oncology which consists of 4 Laboratories. |
| 1988 April | Virological Diagnosis Center was reorganized to become Research Center for Immunodeficiency Virus which consists of Laboratory for AIDS Immunology and Laboratory of Viral Pathogenesis. |
| 1989 April | Department of Biochemistry and Department of Genetics were reorganized to form Department of Genetics and Molecular Biology which consists of 3 Laboratories. |
| 1990 March | Construction of a new building was partly completed. |
| 1990 April | Department of Pathology and Department of Molecular and Cellular Virology were reorganized to form Department of Cell Biology which consists of 3 Laboratories, while Department of Serology and Immunology, Department of Prevention and Therapeutics and Department of Neurological Virus Disease were reorganized to form Department of Biological Responses which consists of 2 laboratories and one for visiting staff. |
| 1992 April | Laboratory of Regulatory Information was established within the Department of Cell Biology to host a visiting professor as well as a research group. |
| 1993 December | Construction of the new building which accommodates three laboratories from this Institute as well as some from the Medical School and the Center for Molecular Biology and Genetics of the University was completed. |
| 1994 October | Construction of a new animal facility with some laboratories was completed. |
| 1998 April | One staff member was appointed academic staff of the Graduate School of Pharmaceutical Sciences, and students of the school were first admitted to the Institute. |
| 1998 April | Research Center for Immunodeficiency Virus was reorganized to become Research Center for Acquired Immunodeficiency Syndrome. |
| 1998 April | Laboratory of Virus Control in Research Center for Immunodeficiency Virus was established as such that Visiting Staff be appointed. |
| 1999 April | Several staff members were appointed academic staff of the Graduate School of Biostudies, and students of the school were first admitted to the Institute. |
| 2002 April | The Experimental Research Center for Infected Animals was abolished and the Experimental Research Center for Infectious Diseases was established instead. |

2005 April	Research Center for Emerging Virus was established.
2009 Jun	The Institute commenced service as a Joint Usage / Research Center for fusion of advanced technologies and innovative approaches to viral infections and life science.
2010 April	Center for Acquired Immunodeficiency Syndrome Research was reorganized to become Center for Human Retrovirus Research.

ORGANIZATION AND STAFF

(as of December, 2011)

(Numerals in parentheses indicate year of association with the Institute)

Director	Masao Matsuoka, M.D., D.Med.Sc.
Deputy Director	Yoshio Koyanagi, M.D., D.Med.Sc.
Professors Emeriti	Yoshimi Kawade, D.Sc. (1956-1988) Yorio Hinuma, M.D., D.Med.Sc. (1980-1988) Masao Hanaoka, M.D., D.Med.Sc. (1959-1989) Mutsuo Imai, D.Sc. (1965-1991) Takashi Yura, D.Sc. (1960-1993) Masakazu Hatanaka, M.D., D.Med.Sc. (1980-1995) Akinori Ishimoto, M.D., D.Med.Sc. (1964-1968, 1978-2002) Yoshiaki Ito, M.D., D.Med.Sc. (1984-2002) Masanori Hayami, D.V.M., D.Agr. (1988-2006) Koreaki Ito, D.Sc. (1971-2007) Junji Yodoi, M.D., D.Med.Sc. (1989-2010)

Department of Viral Oncology

Laboratory of Gene Analysis

Professor	Yoshinori Akiyama, D.Sc. (1988)
Associate Professor	Hiroyuki Sakai, D.Med.Sc. (1996) Hiroyuki Mori, D.Sc. (1996)
Assistant Professor	Shin-ichi Yanagawa, D.Agr. (1986)

Laboratory of Cell Regulation

Professor	Masahiko Sugita, M.D., D.Med.Sc. (2004)
Associate Professor	Isamu Matsunaga, M.D., D.Med.Sc. (2004)
Assistant Professor	Hiroataka Kuwata, D.D.S., Ph.D. (2010)

Laboratory of Tumor Biogenesis

Professor	Shin Yonehara, D.Sc. (1994) (concurrent)
Assistant Professor	Akira Murakami, D.Sc. (1979)

Laboratory of Human Tumor Viruses

Professor	Keizo Tomonaga, D.V.M., D.Vet.Med. (2011)
Associate Professor	Makoto Hijikata, D.Med.Sc. (1997)
Assistant Professor	Tomoyuki Honda, M.D., D.Med.Sc. (2011)

Department of Genetics and Molecular Biology

Laboratory of Molecular Genetics

Professor	Takashi Fujita, D.Sc. (2005)
Associate Professor	Hiroki Kato, D.Med.Sc. (2010)

Laboratory of Biochemistry

Professor	Mutsuhito Ohno, D.Sc. (2001)
Assistant Professor	Makoto Kitabatake, D.Sc. (2004) Ichiro Taniguchi, D.Sc. (2007)

Laboratory of Genetic Information Analysis

Associate Professor	Haruo Ohmori, D.Sc. (1979)
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Department of Biological Responses

Laboratory of Biological Protection

Professor	Koichi Ikuta, M.D., D.Med.Sc. (2002)
Assistant Professor	Masamichi Ueda, D.Sc. (1978) Keiko Takemoto, D.Sc. (1992) Shizue Tani-ichi, D.Health Sc. (2007)

	Takahiro Hara, D. Bio. (2008)
Laboratory of Infection and Prevention	
Associate Professor	Hiroshi Masutani, M.D., D.Med.Sc. (1992)
Bioresponse Regulation Laboratory	
Visiting Professor	Yoshihiro Kawaoka, D.V.M., D.Med.Sc. (2010)

Department of Cell Biology

Laboratory of Subcellular Biogenesis	
Professor	Fumiko Toyoshima, D.Sc. (2008)
Assistant Professor	Shigeru Matsumura, D.Bio. (2008)
Assistant Professor	Momoko Maekawa, D.Bio. (2011)
Laboratory of Growth Regulation	
Professor	Ryoichiro Kageyama, M.D., D.Med.Sc. (1997)
Associate Professor	Toshiyuki Ohtsuka, M.D., D.Med.Sc. (2000)
Assistant Professor	Taeko Kobayashi, D.Sc. (2005)
Associate Professor(Spe.)	Itaru Imayoshi, D.Bio.(2008)
Laboratory of Signal Transduction	
Associate Professor	Takayuki Miyazawa, D.V.M., D.Vet.Med. (2005)
Assistant Professor	Takeshi Kobayashi, D.V.M., D.Med.Sc. (2008)
Laboratory of Regulatory Information	
Visiting Professor	Susumu Tonegawa, Ph.D, D.Sc. (1992)

Center for Human Retrovirus Research

Laboratory of Viral Pathogenesis	
Head • Professor	Yoshio Koyanagi, M.D., D.Med.Sc. (2004)
Assistant Professor	Hirofumi Ebina, D.Med.Sc. (2009)
Laboratory of Virus Control	
Professor	Masao Matsuoka, M.D., D.Med.Sc. (1999)
Associate Professor	Junichiro Yasunaga, M.D., D.Med.Sc. (2010)
Assistant Professor	Yorifumi Satou, M.D., D.Med.Sc. (2008)
Assistant Professor	Kazuya Shimura, D.Med.Sc. (2011)
Laboratory of Viral Immunology	
Visiting Professor	Masafumi Takiguchi, M.D., D.Med.Sc. (2010)

Experimental Research Center for Infectious Diseases

Laboratory of Mouse Model	
Professor	Yoichi Shinkai, D.Med.Sc. (1998)
Associate Professor	Makoto Tachibana, D.Agr. (1998)
Assistant Professor	Toshiaki Tsubota, D.Sc.(2009)
Laboratory of Primate Model	
Head • Professor	Tatsuhiko Igarashi, D.V.M., D.Med.Sc. (2007)
Associate Professor	Tomoyuki Miura, D.V.M., D.Agr. (1988)
Assistant Professor	Takeshi Kobayashi, D.V.M., D.Med.Sc. (2008)

Center for Emerging Virus Research

Head • Professor	Yoshio Koyanagi, M.D., D.Med.Sc. (2010)
Assistant Professor	Kei Sato, D.Med.Sc. (2010)
Assistant Professor	Shin-ichiro Narita, D.Sc. (2010)
Assistant Professor	Ayano Satsuka, D.Bio (2010)

Lecturers (part time)

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Library

Committee Chairman

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Katsuaki Deguchi	Exp. Res. Cen. Inf. Dis. (Lab. Mouse Model)
Mayuko Inoue	Exp. Res. Cen. Inf. Dis. (Lab. Mouse Model)
Yutaro Yamaguchi	Exp. Res. Cen. Inf. Dis. (Lab. Mouse Model)

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Research Activities

DEPARTMENT OF VIRAL ONCOLOGY

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I. First Group

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DEPARTMENT OF VIRAL ONCOLOGY
LABORATORY OF GENE ANALYSIS

I. First Group

The research projects carried out in this group are concerned with post-translational events in the expression of genetic information. Specifically, processes of protein translation, protein translocation across and integration into the membrane, membrane protein proteolysis and extracytoplasmic stress responses are investigated by combined molecular genetic, biochemical biophysical and structural approaches.

- 1) Possible substrate binding function of a periplasmic crevasse of SecD: H. MORI, T. TSUKAZAKI¹, O. NUREKI¹, Y. AKIYAMA and K. ITO²** (¹Graduate School of Science, the University of Tokyo and ²Faculty of Life Science, Kyoto Sangyo University)

The SecYEG translocon and the SecA ATPase cooperate to facilitate protein export across the bacterial cytoplasmic membrane. In addition to these essential core components, SecDF, a pair of membrane integrated Sec factors, play important roles in efficient protein export *in vivo*. We determined the crystal structure of SecDF from *Thermus thermophilus* at 3.3Å resolution and proposed a working hypothesis based on structure-instructed biochemical and biophysical studies (1). According to the model, the first large periplasmic domain (P1) of SecD binds a substrate and undergoes functionally important conformational changes powered by the cation-motive force across the membrane. In support of this model, isolated P1 domain was capable of binding denatured polypeptides with varying affinities according to its conformational states. However, it has remained to be shown that the intact, full-length SecD indeed interacts with a translocating substrate polypeptide. To show this, we performed site-directed photo cross-linking using inverted membrane vesicles (IMVs) bearing a substrate in a state of translocation intermediate. We prepared IMVs containing *E. coli* SecD derivatives, in which a photo-reactive amino acid analogue, para-benzoyl phenylalanine (pBPA), had been placed on the molecular surface of the P1 domain. The translocation intermediate generated for this experiment was a proOmpA derivative having a disulfide loop near its C-terminus. IMVs with SecD (Arg407pBPA) produced a SecD-proOmpA cross-linked product, specifically when the proOmpA derivative was in the intermediate state. Thus, the P1 domain of intact SecD in the membrane is in close proximity to the substrate polypeptide emerging from the translocon. On the structure of TtSecDF we have determined, the residue Val228 that corresponds to Arg407 in *E.coli* SecD is located within a crevasse with an opening toward the periplasm, suggesting that this cavity in the P1 domain might have a substrate-binding function.

(1) Tsukazaki, T., Mori, H., Echizen, Y., Ishitani, R., Fukai, S., Tanaka, T., Perederina, A., Vassilyev, D. G., Kohno, T., Maturana,

2) Identification of SecD nearest neighbors by *in vivo* site-directed photo-cross-linking: Y. MACHIDA, T. TSUKAZAKI¹, O. NUREKI¹, K. ITO², Y. AKIYAMA and H. MORI.
(¹Graduate School of Science, the University of Tokyo and ²Faculty of Life Science, Kyoto Sangyo University)

Our recent structural and functional analyses of SecDF strongly suggest that SecDF forms a complex with SecYEG translocon, captures a substrate polypeptide emerging from the translocon by its P1 domain and undergoes conformational changes using the PMF (proton motive force) energy to facilitate forward movement of the polypeptide (1). However, the mode of interactions between SecDF and Sec-related factors including SecYEG remain largely unknown. To gain information on this issue, we carried out systematic *in vivo* site-directed photo-cross-linking analysis targeting SecD. This method has been successfully applied to identify SecA neighboring sites in SecY (2). Based on the *Thermus thermophilus* SecDF structure, we designed *E. coli* SecD mutations so as to introduce a photoreactive amino acid analogue, pBPA on the molecular surface of the protein. We constructed total of 50 SecD derivatives. Photo-cross-linking experiments with these mutants gave the following results. 1) When pBPA was introduced at some positions in TM4 or TM5 that are located in interface with SecF, strong cross-linked products reacting with anti-SecF antibody were observed in a UV-irradiation-dependent manner. These results support the idea that SecDF in the membrane assumes a conformation similar to the crystal structure. 2) When pBPA was introduced at a position on the membrane-proximal surface of the P1 domain, a SecY-SecD cross-linked product was generated, suggesting that the P1 domain is located near the SecYEG translocon. 3) Upon UV irradiation, SecD (R268pBPA) mutant generated a slowly migrating band, which presumably represents an intra-molecular cross-linked product and could serve as an indicator to monitor a conformational state (termed I form) of the P1 domain. Interestingly, when cells expressing this SecD derivative were treated with CCCP (a proton ionophore) to collapse PMF across the membrane, the cross-linked product dramatically reduced. Furthermore, when the mutation was combined with the SecD (Asp519Asn) or SecF(Arg247Met) mutation that abolishes both proton conductance and protein export enhancing activities of SecDF, the cross-linked product almost completely disappeared. These results are nicely consistent with our working hypothesis that the proton conductance of SecDF somehow couples with movement of the P1 domain. 4) SecD derivatives containing pBPA on an α -helical region of the P1 domain facing periplasmic space generated several slowly migrating cross-linked bands. We identified partner proteins of the some products as Skp and DegP, periplasmic chaperones that prevent newly translocated outer membrane proteins from aggregating and facilitate their delivery to outer membrane. An interesting hypothesis is that the P1 domain could act as not only a binding site for a translocating substrate but

also a docking platform for the periplasmic chaperones to promote translocation and folding of the substrate polypeptide.

(1) Tsukazaki, T., Mori, H., Echizen, Y., Ishitani, R., Fukai, S., Tanaka, T., Perederina, A., Vassilyev, D. G., Kohno, T., Maturana, A., Ito, K. and Nureki, O. (2011) *Nature* 474, 235-238.

(2) Mori, H. and Ito, K (2006) *Proc. Natl. Acad. Sci. USA*. 103, 16159-16164

3) Different expression of SecD paralogues in *Vibrio alginolyticus*: N. HASHIMOTO, S. KOJIMA¹, M. HOMMA¹, Y. AKIYAMA and H. MORI. (¹Graduate School of Science, Nagoya University)

Genome sequence analysis revealed that marine bacteria *Vibrio* species contain two sets of *secDF* genes, designated *V.secDF1* and *V.secDF2*. The former is more closely related to the *E. coli* *secDF* genes. Surprisingly, we found that V.SecDF1 can support effective protein export in a SecD-deficient *E. coli* mutant cells (*secD1*) in a Na⁺-dependent manner, providing physiological evidence for the Na⁺-coupled protein translocation mediated by V.SecDF1 (1). V.SecDF2 also promoted protein export in the *secD1* mutant cells, but in this case it did not show any Na⁺-dependence, presumably representing the H⁺-coupled function of V.SecDF2 as is the case with *E.coli* SecDF. These results raise the following questions: 1) Do both of the *Vibrio* SecDF paralogues are indeed expressed in *Vibrio* species? 2) If so, how their expressions are regulated? 3) Is there any functional difference between these two paralogues? To address these questions, we examined accumulation of the SecD paralogues in *V. alginolyticus* under various culture conditions by immunoblotting using specific antibody against each SecD paralogue. When cells were cultured in a normal rich medium containing 500 mM NaCl (pH 7.0), we detected V.SecD1, but not V.SecD2. Interestingly, when Na⁺ concentration in the medium was reduced to 50 mM, we detected a small amount of V.SecD2 and further decreasing of medium Na⁺ (to 10 mM) resulted in increased accumulation of V.SecD2. We also found that even in the presence of 500 mM NaCl, acidification of the medium to pH5.5 caused increase in the cellular amount of V.SecD2. The environmental conditions such as low Na⁺ content and/or low pH in the medium seem to be more suitable for protein translocation by H⁺-driven SecDF2 than by Na⁺-driven SecDF1. These results strongly suggest that *V. alginolyticus* possesses regulatory mechanisms for expression of SecDF paralogues to adjust to its environment.

(1) Tsukazaki, T., Mori, H., Echizen, Y., Ishitani, R., Fukai, S., Tanaka, T., Perederina, A., Vassilyev, D. G., Kohno, T., Maturana, A., Ito, K. and Nureki, O. (2011) *Nature* 474, 235-238.

4) Roles of the PDZ domains in the proteolytic function of RseP, a key protease involved in the σ^E -pathway of *E. coli* extracytoplasmic stress response: Y. HIZUKURI and Y. AKIYAMA

In the *Escherichia coli* σ^E -pathway of extracytoplasmic stress response, anti- σ^E protein RseA is sequentially processed by two membrane proteases DegS (site-1 cleavage) and RseP (site-2), resulting in the release of σ^E from the membrane and its eventual activation. Normally RseP can cleave only the DegS-cleaved intermediate of RseA. The proteolytic function of RseP is speculated to be controlled by its two PDZ domains. Recently, binding of the exposed carboxyl terminal residue (Val148) of DegS-processed RseA to the second PDZ domain of RseP has been proposed to facilitate the site-2 cleavage by RseP (1). Since this model was mainly based on *in vitro* and structural studies, we investigated whether it can be extended to physiological situations. Mutations in the ligand-binding region in the RseP PDZ domain and even the deletion of the PDZ domains little affected the cleavage of RseA and its derivative model substrates *in vivo*. Amino acid substitutions of the exposed C-terminal residue Val148 of the model substrate mimicking the DegS-processed RseA also gave minimal effect on substrate cleavage efficiencies. These results suggest that the recognition of the exposed C-terminal residue of RseA by the RseP PDZ domain makes little, if any, contribution to the cleavage of RseA *in vivo*. Moreover, we replaced the chromosomal *rseP* gene by a mutant gene encoding an RseP-derivative lacking either of the two PDZ domains and revealed that these mutants showed normal growth at any temperature examined and exhibited almost normal σ^E -activation in response to overproduction of OmpC, an outer membrane protein (OMP). These results suggest that neither of the two PDZ domains is essential for the conventional OMP-induced σ^E activation process. Together with our previous observations (2), we are speculating that the RseP PDZ domains are involved in the OMP-independent σ^E activation pathway through binding of a still unknown-ligand. To address this hypothesis, we are trying to identify a physiological ligand of RseP PDZ by using site-directed *in vivo* photo cross-linking approach targeted against the putative ligand-binding sites of the RseP PDZ domains. We have preliminarily detected some cross-linked products and their characterization is now under progress.

(1) Li, X., Wang, B., Feng, L., Kang, H., Qi, Y., Wang, J., and Shi, Y. (2009) Proc. Natl. Acad. Sci. USA, 106, 14837-14842.

(2) Inaba, K., Suzuki, M., Maegawa, K. -i., Akiyama, S., and Akiyama, Y. (2008) J. Biol. Chem., 283, 35042-35052.

5) **An attempt to identify physiological substrates of *E. coli* rhomboid protease GlpG: K. TERUSHIMA and Y. AKIYAMA**

Rhomboid proteases, a family of intramembrane cleaving proteases (I-CLiPs) that are thought to hydrolyze substrate membrane proteins within the membrane, are involved in a wide range of biological events including EGFR signaling, host cell invasion by protozoan parasites and bacterial quorum sensing. We have been studying GlpG, an *E.coli* member of rhomboid proteases. As a model rhomboid enzyme, GlpG has been extensively studied biochemically and structurally,

but its physiological substrate and cellular function remain unknown. Recently, Strisovsky *et al.*(1) showed that several residues surrounding the cleavage site are crucial for rhomboid proteases. However, the proposed motif does not completely fit with our previous data obtained from the analysis of model substrate cleavage by GlpG, suggesting that the motif recognized by GlpG somewhat deviates from the proposal consensus. To elucidate the GlpG recognition motif, we carried out systematic mutational analysis against the cleavage site region in a model substrate of GlpG. Our results showed that residues at two additional positions (P1' and P3') are crucial for cleavage by GlpG. On the other hand, as a more direct approach to identify GlpG substrates, we systematically screened single spanning membrane proteins for their GlpG-dependent cleavage and identified several candidates. These two approaches would help us to identify a physiological substrate and elucidate a cellular function and substrate cleavage mechanism of GlpG.

(1) Strisovsky, K., Sharpe, H.J, and Freeman M. (2009) Mol. Cell, 36, 1048-1059

6) Site-directed *in vivo* photo-cross-linking analysis of the membrane targeting-mediated negative regulation of *E. coli* heat shock factor σ^{32} : R. MIYAZAKI, T. YURA¹, H. MORI and Y. AKIYAMA (¹Faculty of Life Science, Kyoto Sangyo University)

Heat shock response is a major homeostatic mechanism for controlling the state of protein folding and degradation in all organisms. Expression of heat shock genes in *E. coli* is both under positive control by σ^{32} , a transcription factor dedicated to the heat shock response, and under negative feedback control (inactivation/degradation of σ^{32}) by stress-inducible molecular chaperones (DnaK/J-GrpE, GroEL/S). σ^{32} is extremely unstable *in vivo* and is degraded by membrane-localized protease FtsH. Chaperones contribute to rapid degradation of σ^{32} *in vivo*, whereas its degradation *in vitro* is very slow and not enhanced by chaperones. It is possible that some other factors are involved in degradation of σ^{32} *in vivo*.

Recent work by Yura *et al.* (unpublished results) suggests that signal recognition particles (SRP), its receptor FtsY and SecYEG translocon, which are involved in membrane protein biogenesis, are required for the chaperone-dependent feedback inhibition of σ^{32} . It has been also suggested that region 2.1 of σ^{32} is important for the negative feedback control (1). These observations raise the possibility that σ^{32} is targeted to degradation at the membrane through recognition of region 2.1 by cellular factors including chaperones, SRP, FtsY, SecYEG, and FtsH. In order to identify proteins interacting with region 2.1 *in vivo*, we employed site-directed *in vivo* photo-cross-linking approach. We constructed and expressed nineteen σ^{32} derivatives containing a photo-reactive amino acid analog (pBPA) around region 2.1. We detected several cross-linked products upon UV-irradiation. Now we are trying to identify partner proteins of the observed cross-linked products and reveal their roles in negative regulation of σ^{32} .

(1) Yura, T., Guisbert, E., Poritz, M., Lu, C.Z., Campbell, E., and Gross, C.A. (2007) Proc. Natl. Acad. Sci., USA, 104,

- 7) **Possible involvement of toxin-antitoxin systems in σ^E -dependent extracytoplasmic stress response in *Escherichia coli*: Y. DAIMON, S. NARITA¹ and Y. AKIYAMA**
(¹Center for Emerging Virus Research, IVR)

Bacteria respond to and cope with extracytoplasmic stresses that cause damage to envelope components by altering their gene expressions. In gram-negative bacteria, this 'extracytoplasmic stress response (ESR)' is required for the maintenance of cell envelope as well as for expression of virulence. σ^E (RpoE) is an alternative sigma factor that governs a major signaling pathway (σ^E pathway) of ESR in *Escherichia coli*. σ^E is activated by extracytoplasmic stresses produced under envelope-damaging conditions such as heat shock, overexpression of outer membrane proteins, and mutational alterations of chaperones and machinery required for folding and assembly of outer membrane proteins. In addition to the important role during stressed conditions, σ^E is also known to be required for growth of *E. coli* cells even under normal or non-stressed conditions. However, the essential role of σ^E for cell viability remains to be elucidated. Although a deletion of *ycdQ*, a gene for an antitoxin protein of a chromosomally-encoded toxin-antitoxin (TA) system, has been reported to suppress the lethality caused by the *rpoE* null mutation (1), the mechanism of the suppression is unknown. To gain insight into the essentiality of σ^E , we are studying possible involvement of TA systems in the σ^E -dependent stress response.

(1) Ruiz, N., Gronenberg, L.S., Kahne, D., and Silhavy, T.J. (2008) Proc. Natl. Acad. Sci. USA, 105, 5537-5542.

- 8) **X-ray crystal structural analysis of membrane bound ATP-dependent protease FtsH: R. SUNO, T. SHIMAMURA¹, T. HINO¹, A. ABE, T. ARAKAWA¹, Y. AKIYAMA, S. IWATA¹ and M. YOSHIDA²** (¹Graduate School of Medicine, Kyoto University, ²Faculty of Life Science, Kyoto Sangyo University)

ATP-dependent proteases are involved in various cellular processes including cell division, cell differentiation, signal transduction, and stress response. FtsH degrades not only misassembled subunits of membrane protein complexes for their quality control but also some short-lived cytosolic regulatory proteins for cellular regulation. FtsH comprises an N-terminal transmembrane segment and a C-terminal cytosolic region, which consists of AAA⁺ (ATPases associated with diverse cellular activities) and protease domains. Previously, we successfully crystallized and determined a soluble region of FtsH (sFtsH) containing ADP from *T. thermophilus* at 3.9Å resolution. In the hexameric structure, a substrate polypeptide can reach the active protease catalytic sites through a tunnel leading from AAA⁺ domain of the adjacent subunit, but not from the central axial region. This raises a possibility of direct delivery of a polypeptide through this tunnel. Furthermore, we succeeded in crystallizing sFtsH with several kinds of ATP analogues to

understand the molecular mechanism of FtsH in detail. These crystals diffracted at least 3.5Å resolution. Among them, we are analyzing the sample crystallized in the presence of ADP • AlFx. The primary phases were initially estimated by the molecular replacement (MR). Now, the atomic model are refining by using REFMAC and Phaser.

- 9) **Biochemical analysis of the substrate-translocating mechanism of ATP-dependent Protease FtsH: R. SUNO, M. SHIMOYAMA¹, A. ABE, N. SHIMODATE¹, Y. AKIYAMA and M. YOSHIDA²** (¹the Chemical Resources Laboratory, Tokyo Institute of Technology, ²Faculty of Life Science, Kyoto Sangyo University)

The structural analysis also suggested that several mobile regions play an important role in the operating mode of FtsH. Based on the structural information, it is conceivable that a β -hairpin and a lid-helix, which presumably form the tunnel, are involved in translocating the polypeptide. The lid-helix covering the protease catalytic site can kink at the position of the highly conserved Gly448. Substitution of this residue by other amino acids resulted in the decrease of ATPase activity and the complete loss of ATP-dependent protease activity. It was considered that these mutations impaired the flexibility of the lid-helix, leading to a more rigid FtsH with impaired functionality.

II. Second Group

- 1) **Analysis of Keratin-Associated Protein 13-Induced Activation of Canonical Wnt Signaling Pathway in vivo: S. YANAGAWA**

Using yeast two-hybrid system, I found that Keratin associated protein (Krtap) 13, a cysteine-rich cytoplasmic protein binds to Low-density lipoprotein receptor-related protein 6 (LRP6), a co-receptor for Wnt. Surprisingly, Krtap13 overexpression markedly stimulates Wnt signaling and promotes Dvl-aggregates formation. Wnt treatment is known to induce LRP6 aggregates, which contain Dvl and Axin. Thus, a possible molecular mechanism underlying Krtap13-induced activation of Wnt signaling is to induce co-clustering of LRP6 and Dvls, thereby recruiting Axin to the plasma membrane that leads to activation of Wnt signaling.

To analyze effect of ectopic overexpression of Krtap13 in vivo, I generated a Krtap13-trans-gene (Krtap13-Tg) consisting of CAG-promoter, loxp-polyA-loxp cassette, and 3XFLAG-tagged human Krtap13 cDNA and transgenic mouse lines carrying this Tg were established. This Krtap13-Tg can express Krtap13 only after Cre-induced recombination of Tg. By crossing these Krtap13-Tg mice with another transgenic mice that express Cre in a tissue-specific way, I can analyze effect of tissue specific overexpression of Krtap13 in mice. From crossing between Krtap13-Tg mice and CAG-Cre-Tg mice, 107 mice were born. However, mouse carrying

recombinant-Tg (and Cre) was never found in these neonates, suggesting that mice overexpressing Krtap13 in broad range of tissues are embryonic lethal. I am analyzing reason of this lethality. However, crossing between Krtap13 Tg mice and keratine5-Cre Tg mice, 12 mice carrying recombinant-Tg (and Cre) were born and they grew normally, suggesting that overexpression of Krtap13 in skin has little effect.

2) Analysis of CAF formation mechanism using HPV positive cells: A. SATSUKA, N. KAJITANI and H. SAKAI

In many reports, the importance of the interaction between the cancer stem cells and the microenvironments has been indicated. In the previous studies, it was suggested that HPV E6, E7, c-Myc, and H-ras were the key factors for the establishment of the cancer stem cell in the cervical cancer. These factors might alter the microenvironment to be favorable for cancer development. To examine the effect of the cancer cells in fostering the cancer-associated fibroblasts (CAFs), HPV-positive cancer cells, SiHa, HeLa, and Caski, were applied to the organotypic raft culture, and the effects on the fibroblasts were analyzed by gene-expression profiling. The expressions of CD44 and α -SMA were used as the markers for the CAF induction. In another experiment, the fibroblasts expressing an oncogene, *myc*, *src*, or *ras* were used as the transformed fibroblasts, and normal HFKs or HeLa cells were overlaid on these cells. The effect of TGF β produced by CAFs on the EMT of normal and HPV-positive keratinocytes was also examined. These inter-cellular communications might be important for the progression of the cervical cancer.

3) Identification of Novel Function of Human Papillomavirus E4: N. KAJITANI, A. SATSUKA and H. SAKAI

HPV infection begins in the basal cells of the epithelium, and as these cells divide, differentiate, and migrate toward the surface of the epithelium, the virus is able to complete its life cycle. The viral life cycle depends on the differentiation of the epithelium, but how the life cycle is controlled is not well understood. It is interesting that viral oncoproteins cause the increase of cellular proliferation and/or transformation, but terminally cellular differentiation of epithelium is required for completion of the viral life cycle.

The expression of E1^{E4} occurs in the upper layers of the HPV-infected epithelium, coordinating with the onset of viral genome amplification and the expression of viral late genes. It is known that E1^{E4} disrupts the keratin networks. It is also known that E1^{E4} induces G₂/M cell cycle arrest. But it is yet to be known well about the details of E1^{E4}. To investigate novel functions of E1^{E4}, we performed yeast two-hybrid assays and got several candidate proteins as

which interacts with E1^{E4}. We carry on the analysis about the interactions between the each candidates and E1^{E4} in vitro or in vivo. In the future, we will ascertain the function of E1^{E4} and its involvement in the viral life cycle.

4) Interaction of Human Papillomavirus E2 with E7 and Effect on Host Cells: A. KAWATE, N. KAJITANI, A. SATSUKA and H. SAKAI

HPV encodes E2 and E7. E2 is a transcription factor, and E7 is an oncoprotein interacting with many proteins such as pRb, cdk2, cyclin A. Previous studies showed that E2 formed a complex with E7. Their interaction was analyzed in immunoprecipitation method and in light scattering measurement. However, the biological role of the interaction remains to be elucidated. In order to analyze it, we investigate the effect of the interaction on the E2-mediated transcriptional regulation. We found that E7 suppressed the transcription activity of E2. The result suggests that E7 might regulate the HPV gene expression pattern by interfering the E2 function. Fine tuning of the E2-mediated gene expression of HPV by E7 could be involved in the differentiation-dependent viral lifecycle.

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DEPARTMENT OF VIRAL ONCOLOGY

LABORATORY OF GENE ANALYSIS

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^aThese authors contributed equally to this work.

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- Akiyama, Y. and Hizukuri, Y.: Function and regulation of RseP, the S2P family intramembrane protease of *E. coli*. International Union of Microbiological Societies 2011 Congress, Symposium "Bacterial Stress responses", Sapporo, Japan, September 6-10, 2011
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II. Second Group

柳川伸一：Analysis of Keratin-associated protein13-induced activation of canonical Wnt signaling pathway in vivo. 第34回日本分子生物学会年会、横浜、2011年12月13日-12月16日

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梶谷直子、佐塚文乃、川手章史、酒井博幸：HPV 18 E1^{E4}, a viral gene product encoded by the early gene region of HPV genome, interacts with vimentin intermediate filaments in vitro and in vivo. International Union of Microbiological Societies Congresses 2011, Sapporo, Sep11-16

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DEPARTMENT OF VIRAL ONCOLOGY
LABORATORY OF CELL REGULATION

The universe of antigens recognized by T lymphocytes has recently been expanded to include not only protein antigens but also lipid antigens. Unlike conventional MHC molecules that present protein-derived peptide antigens, molecules of the human group 1 CD1 family (CD1a, CD1b, CD1c) mediate presentation of lipid antigens to specific T lymphocytes. By taking lipid chemical and immunological approaches and by developing appropriate animal models (human CD1 transgenic mice, guinea pigs, and non-human primates), we aim at determining how CD1 has evolved to function critically in host defense against microbial infection and cancer. Further, inappropriate immune responses to lipids may result in induction of allergy and autoimmune diseases. These critical aspects of the newly recognized lipid-specific immunity have now been addressed in our laboratory.

- 1) **Reconstitution of the human CD1a expression and function in mice: C. KOBAYASHI, T. SHIINA¹, A. TOKIOKA, Y. HATTORI, T. KOMORI, M. KOBAYASHI-MIURA², T. TAKIZAWA³, K. TAKAHARA⁴, K. INABA⁴, H. INOKO¹, M. TAKEYA⁵, G. DRANOFF⁶ and M. SUGITA** (¹Tokai Univ., ²Shimane Univ., ³Nippon Med. Sch., ⁴Graduate Sch. Biostudies, Kyoto Univ., ⁵Kumamoto Univ., ⁶Dana-Farber Cancer Inst.)

Mice and rats are useful animals for many immunological studies, but important exceptions exist. These animals have deleted genes for group 1 CD1 family, and thus, lack the lipid recognition system that is comparable to that in humans. Given the necessity of appropriate small animal models for monitoring CD1-mediated immune responses *in vivo*, we attempted to develop two distinct, but complementary, animal systems; namely, guinea pigs and CD1 transgenic mice. We found previously that guinea pigs have evolved the CD1 system equivalent to that in humans, capable of mounting the CD1-restricted T cell response to mycobacterial lipids. On the other hand, the paucity of critical reagents often hampers detailed analysis of CD1-mediated immune responses in guinea pigs. As an alternative animal model, we generated CD1a transgenic mice carrying the human *CD1A* genome. The expression of CD1a molecules in these mice was detected exclusively in epidermal Langerhans cells and immature thymocytes, thus precisely representing CD1a distribution in humans. By establishing CD1a transgenic mice that lack the function of GM-CSF, a potent CD1a inducer *in vitro* we have demonstrated that the high-level expression of CD1a in epidermal Langerhans cells occurs independently of this cytokine. (J. Invest. Dermatol. 132: 241-244, 2012.)

- 2) **Identification of mycobacteria-derived glycolipids as a new class of target antigen for delayed-type hypersensitivity: T. KOMORI, T. NAKAMURA¹, I. MATSUNAGA, D. MORITA, Y. HATTORI, T. URAKAWA, H. KUWATA, N. FUJIWARA², K. HIROMATSU³, H. HARASHIMA¹ and M. SUGITA** (¹Hokkaido Univ., ²Osaka City Univ., ³Fukuoka Univ.)

In the guinea pig model of infection with bacillus Calmette-Guerin (BCG), an attenuated vaccine strain of *Mycobacterium bovis*, we obtained evidence for the delayed-type hypersensitivity (DTH) directed against a glycolipid antigen. Pathogenic mycobacteria produce glucose monomycolate (GMM), a glucosylated species of mycolic acids, by utilizing host-derived glucose as a substrate for mycolyltransferases. The host CD1-based immunity detects GMM and mounts potent Th1-type T cell responses. Given that Th1 cytokines, such as interferon- γ and TNF- α , are critical for host defense against mycobacterial infection, GMM is now considered as an excellent candidate of lipid-based vaccines against tuberculosis and related diseases. (J. Biol. Chem. 286: 16800-16806, 2011.)

In contrast to the Th1 dominant response induced by GMM, glycerol monomycolate (GroMM), a latent tuberculosis-associated lipid species, elicits a totally different form of hypersensitivity responses in sensitized animals. The GroMM challenge resulted in local infiltration of eosinophils, rather than mononuclear cells, associated with a skewed induction of Th2-type cytokines, such as IL-5 and IL-10. We predict that the host response to GroMM produced by dormant mycobacteria would contribute to their long-term survival in the host. (Biochem. Biophys. Res. Commun. 409: 304-307, 2011.)

- 3) **A new aspect of lipid immunity against AIDS: D. MORITA, T. IGARASHI¹, M. HORIIKE¹, N. MORI² and M. SUGITA** (¹Laboratory of Primate Model, IVR, ²Graduate Sch. Agriculture, Kyoto Univ.)

By taking advantage of IVR's superb research environments and close collaboration with Prof. Igarashi's laboratory, we have set out to how lipid immunity functions in host defense against viral infection. Viruses do not express their own lipids, but some of the viral proteins require modification with host-derived fatty acids for their critical function, suggesting the possibility that the host immunity might be able to detect lipidated viral proteins (lipoproteins). Indeed, we found that rhesus macaque monkeys infected with SIV mounted CTL responses to N-myristoylated SIV Nef lipopeptide. Given that the peptide portion of the lipopeptide is difficult to introduce amino acid mutations without disrupting the tightly regulated N-myristoylation motif, viruses would not be able to easily escape from the CTL response. Therefore, we predict that lipopeptides can function as a new class of vaccines against AIDS. (J. Immunol. 187: 608-612, 2011.)

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松永 勇：宿主にモニターされる抗酸菌脂質とその多様性 京都大学微生物科学寄付研究

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松永勇、前田伸司、中田昇、中崇、藤原永年、杉田昌彦：マイコバクテリアのポリケチド合成酵素 Pks12 によるポリメチル脂肪酸の産生. 第 84 回日本生化学会大会, 京都, 2011 年 9 月 21 日-24 日

Nef 蛋白質のミリスチン酸修飾をモニターする新たな免疫システム 第 25 回日本エイズ学会学術集会 東京 2011 年 11 月 30 日-12 月 2 日

DEPARTMENT OF VIRAL ONCOLOGY
LABORATORY OF TUMOR BIOGENESIS

Apoptosis, or programmed cell death, plays an important role in many biological processes, including embryogenesis, development of immune system, maintenance of tissue homeostasis, and elimination of virus-infected and tumor cells. We found cell surface Fas antigen (Fas), which can directly mediate apoptosis-inducing signals into cells by stimulation with agonistic anti-Fas mAbs or Fas ligand. Our main research project is to understand the intracellular signal transduction mechanism of cell death including apoptosis and caspase-independent novel types of cell death, and the biological significance/physiological role of cell death and cell death-regulating molecules. Investigations of molecular mechanisms and physiological roles of cell death are important for a better understanding of mammalian immune system, embryogenesis and tumorigenesis.

1) Bim regulates B cell receptor-mediated apoptosis in the presence of CD40 signaling in CD40-preactivated splenic B cells differentiating into plasma cells: Y. GAO, H. KAZAMA and S. YONEHARA

B cell receptor (BCR)-mediated apoptosis is critical for B cell development and homeostasis. CD40 signaling has been shown to protect immature or mature B cells from BCR-mediated apoptosis. In this study, to understand the fate of CD40-preactivated splenic B cells stimulated by BCR engagement in the presence of CD40 signaling, murine splenic B cells were cultured with anti-Igk and anti-CD40 Abs after preactivation with anti-CD40 Ab. We found that apoptosis was induced in the cultured B cells even in the presence of CD40 signaling during the 3-4 days cultivation. We detected upregulation of Bim expression followed by Bax activation in this apoptotic process, and cessation of the apoptosis in Bim-deficient B cells, indicating that Bim is a key regulator of the BCR-mediated apoptosis in the presence of CD40 signaling in CD40-preactivated B cells. Importantly, this BCR-mediated apoptosis in CD40-preactivated B cells was shown to be induced at the initiation of plasma cell differentiation at around the preplasmablast stage, and Bim-deficient B cells cultured under these conditions differentiated into plasma cells. Additionally, TGF- β was found to protect CD40-preactivated B cells from BCR-mediated apoptosis in the presence of CD40 signaling. Our identified BCR-mediated apoptosis, which is unpreventable by CD40 signaling, suggests a potential mechanism that regulates the elimination of autoreactive peripheral B cells, which should be derived from non-specific T-dependent activation of bystander B cells and continuous stimulation with self-antigens in the presence of T cell help through CD40.

2) Double-faced functions of caspase-8 in induction and protection of programmed cell death: S. KUROKI, M. KIKICHI, S. SAKAGUCHI and S. YONEHARA

Caspase-8 plays a critical role in induction of apoptosis through death receptors, such as Fas. Meanwhile, we recently found that caspase-8 has an essential role in cell survival. As a result of caspase-8 knockdown in mouse T-lymphoma cells, cell death associated with reactive oxygen species (ROS) accumulation was observed. The cell death was completely inhibited by treatment with ROS scavengers, but inhibited only in part by treatment with caspase inhibitors, expression of Bcl-xL, and knockdown of Atg-7, indicating that apoptosis and autophagy-associated cell death are simultaneously induced. Furthermore, RIP1 and RIP3, which have been reported to play an essential role in induction of necroptosis, regulate this multiple cell death as well as accumulation of ROS. Taken together, RIP1 and RIP3 were indicated to simultaneously induce not only nonapoptotic but also apoptotic cell death through ROS production in the absence of caspase-8. We also found that embryonic fibroblasts from caspase-8 KO mice are susceptible to cell death induced by IFN- γ . This cell death was not inhibited by treatment with caspase inhibitors, 3-methyladenine (inhibitor of autophagy) or necrostatin-1 (inhibitor of RIP-1), indicating that the cell death is not apoptosis, autophagic cell death and necroptosis. Interestingly, knockdown of RIP-3 expression clearly inhibited the IFN- γ -induced cell death. In addition, electron microscopic analyses indicate that the cell death is necrosis, suggesting that the cell death is a novel type of programmed necrosis mediated by RIP-3 but not by RIP-1. Thus, caspase-8 was shown to play essential roles not only in induction of death-receptor mediated apoptosis but also in protection of apoptosis, autophagic cell death, necroptosis and programmed necrosis.

3) A role of Wnt signals in the differentiation of mouse ES cells: A. MURAKAMI

ES cells are induced to differentiated cells under various culture conditions. Although many factors to maintain undifferentiated state and self-renewal are reported, the factors to induce an initial step of the differentiation are poorly known. We have been studying a role of Wnt signals in this step. Recently, we confirmed that ES cells consist of two populations, Nanog positive and negative cells. When ES cells were treated with an inhibitor of FGF or MAPK signaling pathway, which is known to repress the differentiation, the cells formed an almost homogeneous population of Nanog positive cells. Therefore, presence of the Nanog negative cell population is likely to be prerequisite for ES cells to differentiate. Preliminary experiments showed that Wnt signals are involved in transition of the cells to be Nanog negative as well as Nanog positive. Analysis of precise mechanisms of the cell transition by the Wnt signals is underway.

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DEPARTMENT OF VIRAL ONCOLOGY

LABORATORY OF TUMOR BIOGENESIS

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Masataka Someda, and Shin Yonehara. “Establishment of inducible expression and knockdown systems in mouse embryonic stem cells to analyze the functions of various genes in differentiation”, The 9th International Student Seminar. March 7-9, 2011, Kyoto.

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DEPARTMENT OF VIRAL ONCOLOGY
LABORATORY OF HUMAN TUMOR VIRUSES

I. First Group

The researches carried out in this group are focused on RNA viruses, especially negative strand RNA viruses replicating in the cell nucleus, such as bornavirus and influenza virus. All our projects aim to understand the fundamental mechanisms of the replication and pathogenesis of the viruses. In current researches we are investigating the replication and persistent mechanism of the bornavirus in the cell nucleus. The understanding the biological significance of the endogenous element of bornavirus nucleoprotein (EBLN) in mammalian genomes is one of the main focuses of bornavirus researches. We also aim to develop a novel RNA virus vector using bornavirus, which can express stably functional small RNAs. In Influenza virus researches we examine the quick response of host cells to the virus infection by analyzing the alteration of the expression profile of miRNA in infected human alveolar epithelial cells.

1) Insulin-like growth factor binding protein 3 (IGFBP3) and neurological abnormalities of transgenic mice expressing Borna disease virus phosphoprotein: T. HONDA, K. FUJINO, Y. MATSUMOTO, M. HORIE, T. DAITO and K. TOMONAGA.

In a previous study, we demonstrated that transgenic mice expressing Borna disease virus (BDV) phosphoprotein (P) in the astrocytes show striking neurobehavioral abnormalities resembling those in BDV-infected animals, and suggested that transgenic mice, P-Tg, could be a useful model to investigate the molecular mechanisms of neurobehavioral disorders. Here we report that P-Tg displays neurological deficits similar to those in autism spectrum disorders (ASD) in the features of not only neuropathological and behavioral abnormalities but also molecular disturbance in the brain. By further analyses of both the neuropathological and behavioural deficits of P-Tg, we found that P-Tg significantly reduced the density of Purkinje cells, particularly in lobule VI to VIII, of the cerebellum and showed disabled social interaction, both of which are commonly found in ASD patients. Expression profiling analyses revealed that insulin-like growth factor binding protein 3 is significantly upregulated in the cerebellum to a similar extent in a genetic factor-based animal model of ASD, the methyl-CpG-binding protein 2-null mouse. Furthermore, we demonstrated that insulin-like growth factor (IGF) signaling is suppressed in the P-Tg cerebellum and that its stimulation rescues the viability of Purkinje cells of P-Tg in the cerebellar culture system. Finally, by using database analyses we found that IGF signaling may be a commonly affected pathway in the brains of ASD patients. Our data demonstrated that IGF signaling might be a key cascade involved in ASD neuropathology. We also propose here that P-Tg may be an integral animal model

of ASD.

2) Intracellular distribution of Borna disease virus glycoprotein in living cells: T. DAITO, K. FUJINO and K. TOMONAGA.

Borna disease virus (BDV) is a non-segmented, negative-strand RNA virus that is characterized by nuclear replication and persistent infection. A unique feature of BDV is that it releases only a small number of infectious particles from infected cells. Although these characteristics might make it difficult to obtain a large amount of recombinant viruses in a reverse genetics system, the mechanism underlying the budding or assembly of BDV particle has remained largely unknown. In this study, as a first step toward understanding the virion formation of BDV, we investigated the intracellular distribution and mobility of the fluorescent marker fusion envelope glycoprotein (G) of BDV in living cells. Expression analysis revealed that fusion proteins seem to cleave into functional subunits and localize in the endoplasmic reticulum (ER)/Golgi apparatus, as well as the authentic BDV G. Furthermore, we demonstrated using fluorescence recovery after photobleaching analysis that BDV G fluorescence shows rapid recovery in both the ER/Golgi and plasma membrane regions, indicating that BDV G fusion protein may be a useful tool to investigate not only the maturation of BDV G but also the budding and assembly of BDV particles in living cells.

**3) Evolutional analysis of endogenous Borna-like nucleoprotein elements in primate species: Y. KOBAYASHI¹, M. HORIE, K. TOMONAGA and Y. SUZUKI¹.
(¹Graduate School of Natural Sciences, Nagoya City University)**

Endogenous Borna-like nucleoprotein (EBLNs) elements were recently discovered as non-retroviral RNA virus elements derived from bornavirus in the genomes of various animals. Most of EBLNs appeared to be defective, but some of primate EBLN-1 to -4, which appeared to be originated from four independent integrations of bornavirus nucleoprotein (N) gene, have retained an open reading frame (ORF) for more than 40 million years. It was therefore possible that primate EBLNs have encoded functional proteins during evolution. To examine this possibility, natural selection operating on all ORFs of primate EBLN-1 to -4 was examined by comparing the rates of synonymous and nonsynonymous substitutions. The expected number of premature termination codons in EBLN-1 generated after the divergence of Old World and New World monkeys under the selective neutrality was also examined by the Monte Carlo simulation. As a result, natural selection was not identified for the entire region as well as parts of ORFs in the pairwise analysis of primate EBLN-1 to -4 and for any branch of the phylogenetic trees for EBLN-1 to -4 after the divergence of Old World and New World monkeys. Computer simulation also indicated that the absence of premature termination codon in the present-day EBLN-1 does not necessarily support the

maintenance of function after the divergence of Old World and New World monkeys. These results suggest that EBLNs may not have been functional during this period.

4) Generation of Borna disease virus vector stably expressing foreign proteins from an intercistronic noncoding region: T. DAITO, K. FUJINO, T. HONDA, Y. MASTUMOTO and K. TOMONAGA.

Borna disease virus (BDV), a nonsegmented, negative-strand RNA virus, infects a wide variety of mammalian species and readily establishes a long-lasting, persistent infection in brain cells. Therefore, this virus could be a promising candidate as a novel RNA virus vector enabling stable gene expression in the central nervous system (CNS). Previous studies demonstrated that the 5' untranslated region of the genome is the only site for insertion and expression unit of a foreign gene. In this study, we established a novel BDV vector, in which an additional transcription cassette has been inserted into an intercistronic noncoding region between the viral phosphoprotein (P) and matrix (M) genes. The recombinant BDV (rBDV) carrying green fluorescent protein (GFP) between the P and M genes, rBDV P/M-GFP, expressed GFP efficiently in cultured cells and rodent brains for a long-period of time without attenuation. Furthermore, we generated a non-propagating rBDV, Δ GLLP/M, which lacks the envelope glycoprotein (G) and a splicing intron within the polymerase gene (L), by the trans-complementation system with either transient or stable expression of the G. Interestingly, rBDV Δ GLLP/M established a persistent infection in cultured cells with stable expression of GFP in the absence of the expression of G. Using persistently infected rBDV Δ GLLP/M-infected cells, we determined the amino acid region in the cytoplasmic tail (CT) of BDV G important for the release of infectious rBDV particles and also demonstrated that the CT region may be critical for the generation of pseudotyped rBDV having vesicular stomatitis virus G protein. Our results revealed that the newly established BDV vector constitutes an alternative tool, not only for stable expression of foreign genes in the CNS but also for understanding the mechanism of the release of enveloped virions.

II. Second Group

1) Infectious viral particle production is modulated by prostanoids in the cells: Y. ABE, T. WAKITA and M. HIJIKATA

Previously, we reported the development of three dimensional (3D) cell culture system which reproduces the lifecycle of HCV from patients. Then we found that HCV infection, replication and virus particle production were enhanced in 3D condition compared with normal culture condition (2D) of immortalized hepatocytes, HuS-E/2 cells. In this study, we compared the difference of gene expression profiles between 2D-, and 3D-cultured HuS-E/2 cells, to identify the

signal pathways related with HCV lifecycle. Microarray analysis between 2D-, and 3D-cultured HuS-E/2 cells was performed to identify signal pathways that are modulated in 3D culture condition. Then, the relationship between those signal pathways and HCV lifecycle were analyzed by using recombinant HCV (JFH1) producing cells treated with several compounds affecting the signal pathways. Microarray analysis showed differential mRNA expression levels of prostaglandin (PG) synthases in 3D-cultured cells, compared with 2D cultured-cells. Treatments with several inhibitors to the enzymes included in the arachidonic acid cascade did not largely affect the amounts of HCV RNA in the cells and culture medium, suggesting this pathway is not related with HCV genome replication and release of HCV particles. However, cyclooxygenase-1 and Thromboxane A₂ synthetase inhibitors decreased infectivity of HCV particles released in culture medium. This finding will provide new insight about the mechanism of infectious particle production as well as a new target for anti-HCV drugs.

2) IFN- λ 3 response in the early phase of the viral infected hepatocytes: Y. TSUGAWA, Y. QI, K. ONOMOTO, H. KATO, T. FUJITA and M. HIJIKATA

Several viruses are known to infect in human hepatocytes and cause the hepatitis, but the interferon (IFN) response, a first-line defense against viral infection, is not clearly defined for virus-infected hepatocytes. Here, we investigated the early innate immune responses of human hepatocytes infected with an RNA virus, Sendai virus (SV), using human primary hepatocytes, immortalized hepatocytes (HuS-E/2 cells), and hepatocellular carcinoma-derived HuH-7 cells. Within a few hours after SV infection, we observed the induction of the IFN α , β and λ 3 (also known as IL28B) genes in the primary hepatocytes and HuS-E/2 cell, but not in HuH-7 cells. We examined the contribution of retinoic acid-inducible gene (RIG)-I to those early innate immune responses using HuS-E/2 cells in which RIG-I was knocked down. The induction of IFN α 1 gene expression was similar in RIG-I knockdown and normal control HuS-E/2 cells, but expression of IFN β and λ 3 was significantly decreased in the RIG-I knockdown cells relative to the controls. These results indicated that early IFN α induction in virus-infected human hepatocytes is independent of RIG-I. Furthermore, we also found the IFN λ 3 is induced by virus infection in human hepatocytes in RIG-I dependent manner.

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LABORATORY OF HUMAN TUMOR VIRUSES

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DEPARTMENT OF GENETICS AND MOLECULAR BIOLOGY
LABORATORY OF MOLECULAR GENETICS

- 1) Dysregulation of IFN system can lead to poor response to pegylated interferon and ribavirin therapy in chronic hepatitis C: K. ONOMOTO, S. MORIMOTO, T. KAWAGUCHI, H. TOYODA, M. TANAKA, M. KURODA, K. UNO, T. KUMADA, F. MATSUDA, K. SHIMOTOHNO, T. FUJITA and Y. MURAKAMI**

Background: Despite being expensive, the standard combination of pegylated interferon (Peg-IFN)- α and ribavirin used to treat chronic hepatitis C (CH) results in a moderate clearance rate and a plethora of side effects. This makes it necessary to predict patient outcome so as to improve the accuracy of treatment. Although the antiviral mechanism of genetically altered IL28B is unknown, IL28B polymorphism is considered a good predictor of IFN combination treatment outcome. Methodology: Using microarray, we quantified the expression profile of 237 IFN related genes in 87 CH liver biopsy specimens to clarify the relationship between IFN pathway and viral elimination, and to predict patients' clinical outcome. In 72 out of 87 patients we also analyzed IL28B polymorphism (rs8099917). Principal Findings: Five IFN related-genes (IFI27, IFI 44, ISG15, MX1, and OAS1) had expression levels significantly higher in nonresponders (NR) than in normal liver (NL) and sustained virological responders (SVR); this high expression was also frequently seen in cases with the minor (TG or GG) IL28B genotype. The expression pattern of 31 IFN related-genes also differed significantly between NR and NL. We predicted drug response in NR with 86.1% accuracy by diagonal linear discriminant analysis (DLDA). Conclusion: IFN system dysregulation before treatment was associated with poor IFN therapy response. Determining IFN related-gene expression pattern based on patients' response to combination therapy, allowed us to predict drug response with high accuracy. This method can be applied to establishing novel antiviral therapies and strategies for patients using a more individual approach.

- 2) Retinoic acid-inducible gene I-inducible mir-23b inhibits infections by minor group rhinoviruses through downregulation of the very low density lipoprotein receptor: R. OUDA, K. ONOMOTO, K. TAKAHASHI, M. R. EDWARDS, H. KATO, M. YONEYAMA and T. FUJITA**

In mammals, viral infections are detected by innate immune receptors, including Toll-like receptor and retinoic acid inducible gene I (RIG-I)-like receptor (RLR), which activate the type I interferon (IFN) system. IFN essentially activates genes encoding antiviral proteins that inhibit various steps of viral replication as well as facilitate the subsequent activation of acquired immune responses. In this study, we investigated the expression of non-coding RNA upon viral infection or

RLR activation. Using a microarray, we identified several microRNAs (miRNA) specifically induced to express by RLR signaling. As suggested by Bioinformatics (miRBase Target Data base), one of the RLR-inducible miRNAs, miR-23b, actually knocked down the expression of very low density lipoprotein receptor (VLDLR) and LDLR-related protein 5 (LRP5). Transfection of miR-23b specifically inhibited infection of rhinovirus 1B (RV1B), which utilizes the low density lipoprotein receptor (LDLR) family for viral entry. Conversely, introduction of anti-miRNA-23b enhanced the viral yield. Knockdown experiments using small interfering RNA (siRNA) revealed that VLDLR, but not LRP5, is critical for an efficient infection by RV1B. Furthermore, experiments with the transfection of infectious viral RNA revealed that miR-23b did not affect post-entry viral replication. Our results strongly suggest that RIG-I signaling results in the inhibitions of infections of RV1B through the miR-23b-mediated down-regulation of its receptor VLDLR.

3) 55 Amino acid linker between helicase and carboxyl terminal domains of RIG-I functions as a critical repression domain and determines inter-domain conformation: M. KAGEYAMA, K. TAKAHASI, R. NARITA, R. HIRAI, M. YONEYAMA, H. KATO and T. FUJITA

In virus-infected cells, viral RNA with non-self structural pattern is recognized by DExD/Hbox RNA helicase, RIG-I. Once RIG-I senses viral RNA, it triggers a signaling cascade, resulting in the activation of genes including type I interferon, which activates antiviral responses. Overexpression of N-terminal caspase activation and recruitment domain (CARD) is sufficient to activate signaling; however basal activity of full-length RIG-I is undetectable. The repressor domain (RD), initially identified as a.a. 735–925, is responsible for diminished basal activity; therefore, it is suggested that RIG-I is under auto-repression in uninfected cells and the repression is reversed upon its encounter with viral RNA. In this report, we further delimited RD to a.a. 747–801, which corresponds to a linker connecting the helicase and the Cterminal domain (CTD). Alanine substitutions of the conserved residues in the linker conferred constitutive activity to full-length RIG-I. We found that the constitutive active mutants do not exhibit ATPase activity, suggesting that ATPase is required for de-repression but not signaling itself. Furthermore, trypsin digestion of recombinant RIG-I revealed that the wild-type, but not linker mutant conforms to the trypsin-resistant structure, containing CARD and helicase domain. The result strongly suggests that the linker is responsible for maintaining RIG-I in a “closed” structure to minimize unwanted production of interferon in uninfected cells. These findings shed light on the structural regulation of RIG-I function.

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DEPARTMENT OF GENETICS AND MOLECULAR BIOLOGY
LABORATORY OF BIOCHEMISTRY

In eukaryotic cells, many genes are separated by introns into multiple exons that should be joined together. In addition, the cell itself is separated by the nuclear envelope into two major compartments, the nucleus and the cytoplasm. These two types of separations necessitate specific gene expression mechanisms such as RNA splicing and nuclear transport. Prof. Mutsuhito OHNO's laboratory is studying various aspects of eukaryotic gene expression with great emphasis on "RNA" as a key molecule. In addition, Assistant Prof. Kitabatake's subgroup is focusing on quality control mechanisms of eukaryotic ribosome particles.

1) RNA distribution in the cell:

1-1) Identity elements used in mRNA export

Different RNA species, such as tRNAs, U snRNAs, mRNAs and rRNAs, utilize distinct export pathways, i.e., distinct sets of export factors. Accumulating evidence shows that the pathway of RNA export can influence the fate of a given RNA in the cytoplasm, indicating the biological importance of the choice of RNA export pathway. This means that the cellular export machinery must be able to discriminate distinct RNA species, and therefore each RNA species should have identifying features that specify its export pathway ("identity elements"). We are mainly focusing on mRNAs and performing a systematic search for identity elements used in export of mRNAs. To this end, we make various chimeric RNAs between mRNA and U1 snRNA, and look for RNA features that make the chimeric RNAs behave like an mRNA rather than a U snRNA in nuclear export process. We also look for the trans-acting factors that recognize the identity elements to elucidate the mechanisms of RNA export pathway choice. We have identified 'RNA length' as one of such identity elements and we are about to identify the responsible trans-acting factor.

1-2) Molecular mechanisms for nuclear retention of intron-containing mRNA precursors

Intron-containing pre-mRNAs are retained in the nucleus until they are spliced. This mechanism is essential for proper gene expression. Although the formation of splicing complexes on pre-mRNAs is thought to be responsible for this nuclear retention activity, the details are poorly understood. In mammalian cells in particular, very little information is available regarding the retention factors. Using a model reporter gene, we show here that U1 snRNP and U2AF but not U2 snRNP are essential for the nuclear retention of pre-mRNAs in mammalian cells, demonstrating that E complex is the major entity responsible for the nuclear retention of pre-mRNAs in mammalian cells. By focusing on factors that bind to the 3'-splice site region, we found that the 65-kD subunit of U2AF (U2AF65) is important for nuclear retention and that its multiple domains have nuclear

retention activity per se. We also provide evidence that UAP56, a DExD-box RNA helicase involved in both RNA splicing and export, cooperates with U2AF65 in exerting nuclear retention activity. Our findings provide new information regarding the pre-mRNA nuclear retention factors in mammalian cells.

2) rRNA quality control mechanisms:

How the eukaryotic cells deal with non-functional RNA molecules that were either mutated or damaged? We are searching for novel RNA quality control mechanisms in mammalian and yeast cells by mainly focusing on ribosomal RNAs.

Quality control mechanisms operate in various steps of ribosomal biogenesis to ensure the production of functional ribosome particles. It was previously reported that mature ribosome particles containing nonfunctional mutant rRNAs are also recognized and selectively removed by a cellular quality control system (nonfunctional rRNA decay; NRD). Here, we show that the NRD of 25S rRNA requires a ubiquitin E3 ligase component Rtt101p and its associated protein Mms1p, previously identified as factors involved in DNA repair. We revealed that a group of proteins associated with nonfunctional ribosome particles are ubiquitinated in a Rtt101-Mms1-dependent manner. 25S NRD was disrupted when ubiquitination was inhibited by the overexpression of modified ubiquitin molecules, demonstrating a direct role for ubiquitin in this pathway. These results uncovered an unexpected connection between DNA repair and the quality control of rRNAs. Our findings support a model in which responses to DNA and rRNA damages are triggered by a common ubiquitin ligase complex, during genotoxic stress harmful to both molecules.

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DEPARTEMENT OF GENETICS AND MOLECULAR BIOLOGY
LABORATORY OF GENE INFORMATION ANALYSIS

1) Interaction between TLS DNA polymerases and PCNA: K. MORISHITA¹, M. YAMAGUCHI¹ and H. Ohmori (¹Kyoto Institute of Technology)

Chromosomal DNA is constantly damaged by various genotoxins of both endogenous and exogenous origins. DNA damages are mostly fixed by multiple DNA repair mechanisms, but some damages escape from DNA repair and inhibit the progression of DNA replication fork by replicative DNA polymerases such as Pol δ and Pol ϵ . The replicative DNA polymerase stalled at DNA lesion site needs to be replaced with a different DNA polymerase specialized for trans-lesion DNA synthesis (TLS). Mammals have multiple TLS DNA polymerases, which include REV1, Pol η , Pol ι , Pol κ and Pol ζ . Since REV1, Pol η , Pol ι and Pol κ share multiple motifs in common, they are all classified into Y-family. Each of the four Y-family enzymes is consisted of a single subunit, whereas Pol ζ is composed of at least two subunits, the catalytic subunit REV3 and the accessory subunit REV7. Since the REV3 subunit has a high similarity to the catalytic subunit of the replicative DNA polymerase Pol δ , Pol ζ is classified into B-family, together with Pol α , Pol δ and Pol ϵ . TLS is separated into two steps, the insertion of a nucleotide opposite a lesion and the extension of the inserted nucleotide, up to several nucleotides before the replicative polymerase takes over again. In general, the Y-family polymerases function in the insertion step and Pol ζ functions in the extension step. Each of the Y-family polymerases shows a different specificity or preference for lesion to bypass; however, it remains to be clarified how each of them is selectively recruited to its cognate lesion site. REV1 is believed to play a key role during intracellular TLS, because it interacts with all of the other TLS polymerases, *via* its C-terminal domain (CTD, 1150-1251)¹.

PCNA (Proliferating Cell Nuclear Antigen) is a ring-shaped homotrimeric protein and interacts with a number of proteins involved in DNA replication, repair, recombination and checkpoint. When once loaded onto double-stranded DNA, PCNA moves along it, thereby functioning as a sliding clamp for many DNA polymerases. Recently, PCNA was shown to undergo mono-ubiquitination by the RAD6-RAD18 complex in DNA-damaged cells. The modified PCNA is considered to be a hallmark for the stalled replication fork. Consistent with this idea, all the four Y-family polymerases contain one or two copies of ubiquitin-binding domain, in addition to PCNA-binding site(s) such as PCNA-interacting protein (PIP)-box sequence. We have shown that Pol η and Pol κ have a potent PIP-box sequence at the C-terminus and Pol ι has an internal potent PIP-box sequence, in the immediate downstream of the N-terminal catalytic domain. We reported the crystal structures of the complex made of PCNA and a peptide containing each of such PIP-box sequence². Pol η and Pol κ each have another, weak PIP-box sequence in the downstream of the

N-terminal catalytic domain, similarly as in the case of Pol ϵ ¹.

It still remains controversy which region in human REV1 is responsible for PCNA binding¹. Our results obtained with yeast two-hybrid assay revealed that two separate regions of hREV1 exhibit PCNA-binding activity, for example, one (386-825) containing the central catalytic domain and the other (1085-1251) containing the CTD, while a previous paper reported that in mouse REV1, the BRCT domain near the N-terminus should be the PCNA-binding site³. A recent paper dealing with the interaction between REV1 and PCNA in yeasts indicated that PAD (polymerase-associated domain), a portion near the C-end of the catalytic domain, is responsible for PCNA binding⁴. Furthermore, the paper showed that the PAD domain of yREV1 binds to the interface of PCNA subunit, while PIP-box sequences bind to an internal region of PCNA monomer, IDCL (interdomain-connecting loop) within PCNA monomer. It seems likely that the PAD in hREV1 is one of the two PCNA-binding sites aforementioned. As to the other C-terminal PCNA-binding site in hREV1, we noticed that the sequence 1110-QKLIDGFL-1117 present ahead of the CTD is similar to the consensus sequence of PIP-box, often represented as Qxx(I, L, M)xxFF (x is any residue). However, in our yeast two-hybrid assay, we could not detect any significant PCNA-binding activity with 1102-1124 or 1150-1251 fragment of REV1, each containing the above PIP-like sequence or CTD, respectively. We are now trying to identify which sequence in the 1085-1251 region is critical for PCNA binding.

Pol ζ is considered to function at the extension step during TLS, depending on the interaction of the REV7 subunit with REV1. The C-terminal domain of REV3 was reported to contain a sequence similar to the PCNA-interacting motif sequence of the human AlkB homologue 2 (APIM)⁵. However, thus far, we have been unable to detect PCNA-binding activity with the original APIM sequence by yeast two-hybrid assay or *in vitro* pull-down assay, under the conditions where we successfully detected a potent PCNA-binding activity with the PIP-box sequence of the human p21 protein. Further experiments are necessary to clarify how Pol ζ interacts with PCNA.

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2) Intracellular interaction between REV7 and REV3 in DT40 cells: K. TAKENAKA¹, H. OHMORI and Y. MIKI¹ (¹Tokyo Medical and Dental University)

As described above, human Pol ζ is composed of at least two different subunits, hREV3

and hREV7. The accessory subunit hREV7 is also called hMAD2L2, as it has a similarity to hMAD2, a protein involved in spindle assembly checkpoint. We have shown that both hREV7 and hMAD2 bind to a 9-aa sequence within hREV3, 1877-ILKPLMSPP-1885, which we called MCS (minimum core sequence)¹. Interestingly, amino-acid substitutions in the hREV3 MCS conferred different effects on *in-vitro* interactions with hREV7 or hMAD2. For example, I1877A or L1878A substitution in the hREV3 MCS completely abolished the interaction with hMAD2, but either of the substitutions by itself showed little effect on the interaction with hREV7 while the I1877A/L1878A double substitution abolished the hREV7-interaction. On the other hand, P1880F substitution abolished the hREV7-interaction, but it did not affect the hMAD2-interaction. To further examine the effects of such mutations on *in-vivo* interaction with REV7, we decided to use DT40, a chicken pre-B cell line that is suited for gene manipulation. The chicken REV7 shows 96% sequence identity with the human REV7, and the chicken REV3 possesses the sequence identical to hREV3 MCS at the identical position. We introduced I1877A or P1880F mutation into the genomic sequence of the DT40 *REV3* gene and examined phenotypes of such mutants. The P1880F mutant showed mild sensitivities to UV-irradiation and cisplatin treatment, much weaker than those of REV3 or REV7 null mutant, while the I1877A mutant showed no difference with the wild-type DT40 cells. This result suggested that REV3 might have another REV7-binding site(s), while the REV3 MCS is the predominant REV7-binding site.

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3) Bypass of BPDE-dG in mouse cells lacking TLS polymerases: K. HASHIMOTO¹, Y. CHO¹, I-Y. YANG¹, J-I. AKAGI², E. OHASHI³, S. TATEISHI⁴, N. de WIND⁵, F. HANAOKA², H. OHMORI and M. MORIYA¹ (¹Stony Brook University, ²Gakushuin University, ³Kyushu University, ⁴Kumamoto University, ⁵Leiden University,)

The four Y-family DNA polymerases show different specificity or preference for DNA lesion to bypass *in vitro*. For example, Pol η bypasses past T-T cyclobutane pyrimidine dimer (CPD) efficiently and accurately, inserting two As opposite the lesion. Pol ι inserts mainly A opposite 3'-T of T-T (6-4) photoproduct (PP), but does not extend further. While Pol κ does not bypass T-T CPD or (6-4) PP, it is able to insert the correct C opposite BPDE-dG, a main adduct of the activated diol dioxide form of benzo[a]pyrene that is supposed to be a potent carcinogen. Furthermore, mouse cells defective for Pol κ exhibited high sensitivity to treatment with benzo[a]pyrene¹. Therefore, we were very much interested in examining how efficiently and accurately BPDE-dG could be bypassed in various MEF (mouse embryonic fibroblast) cells defective for Pol κ or other TLS polymerase. We first tested double-stranded plasmid DNAs

containing a site-specific BPDE-dG adduct, but realized that such a bulky adduct was removed soon after the plasmid was introduced into MEF cells. We thus used a gapped plasmid, in which U instead of T was inserted at multiple positions in the complementary strand, and treated the plasmid DNA with Uracil N-glycosidase and AP endonuclease just before transfection into MEF cells.

The results showed that in the wild-type and Pol κ -deficient MEF cells, bypass of BPDE-dG was extremely miscoding (>90%) with G-to-T transversions². Similar results were obtained with MEF cells deficient for Pol η or Pol ι . Furthermore, the bypass efficiencies in those MEF cells were similar, implying that any of the three Y-family polymerases was essential for bypassing the site-specific BPDE-dG adduct. In contrast, the bypass efficiency was severely reduced in MEFs defective for REV1 or REV3. Particularly, all TLS products in REV3-deficient MEF cells were error-free. We interpret these results as indicating that the replicative DNA polymerase (Pol δ or Pol ϵ), which is the enzyme first encountered the BPDE-dG adduct in the gapped template, inserts mostly A before dissociating from the primer terminus, by an activity similar to terminal transferase, and the inserted A is extended by Pol ζ (REV3-REV complex) with the help of REV1. When the replicative DNA polymerase dissociates from the primer terminus upon encountering the BPDE-dG adduct, Pol κ has a chance to insert mainly the correct C opposite the lesion and extend further. During bypass of the BPDE-dG adduct in this experiment system, such an error-free bypass is dominant in REV3- or REV1-deficient MEF cells, but it plays a minor role in the wild-type MEF cells.

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- W. Ito, M. Yokoi, H. Mitani, H. Ohmori and F. Hanaoka : Stalled Pol η at its cognate substrate emerged an alternative TLS pathway via interaction with REV1. 第34回日本分子生物学会年会、横浜、12月13～17日、2011

DEPARTMENT OF BIOLOGICAL RESPONSES
LABORATORY OF BIOLOGICAL PROTECTION

Our laboratory has made two major achievements. First, we have found that fetal and adult hematopoietic stem cells have different developmental potential to differentiate into lymphocytes. Second, we have demonstrated that interleukin-7 (IL-7) controls DNA recombination of lymphocyte antigen receptor genes by changing chromatin structure. Both of them are related with fundamental questions in medicine and biology.

Based on these findings, we are now pursuing research on development and regulation of the immune system, focusing on the following questions: (1) function of IL-7 receptor (IL-7R) in immune system; (2) control mechanism of lymphocyte antigen receptor genes by IL-7; (3) regulation of immune response by IL-7R expression; and (4) distribution and function of IL-7-producing cells in lymphoid organs.

1) The pre-TCR signal induces transcriptional silencing of the TCR γ locus by reducing the recruitment of STAT5 and Runx to transcriptional enhancers: S. TANI-ICHI and K. IKUTA

The mouse TCR γ locus is positively regulated by the transcription factors STAT5 and Runx. While the locus undergoes frequent rearrangements in T lymphocytes, TCR γ transcription is repressed in $\alpha\beta$ T cells. This phenomenon, known as TCR γ silencing, depends on pre-TCR-induced thymocyte proliferation. The molecular basis for TCR γ silencing, however, is largely unknown. Here, we show that pre-TCR signaling reduces transcription and histone acetylation of the TCR γ locus irrespective of V-J rearrangements. We also demonstrate that Runx is recruited to E γ and HsA enhancer elements of the TCR γ locus, primarily at the CD4⁻CD8⁻ double-negative stage, and that Runx binding to these elements decreases at later stages of thymocyte development. Importantly, anti-CD3 Ab treatment decreased IL-7R expression levels, STAT5 phosphorylation, and recruitment of STAT5 and Runx to E γ and HsA elements in RAG2-deficient thymocytes, suggesting that pre-TCR signaling triggers reduced binding of STAT5 and Runx to the enhancer elements. Furthermore, we observed that misexpression of STAT5 or Runx in the CD4⁺CD8⁺ double-positive cell line DPK induces TCR γ gene transcription. Finally, we showed that TCR γ transcription is induced in $\alpha\beta$ T cells from Runx3 transgenic mice, suggesting that Runx3 counteracts TCR γ silencing in $\alpha\beta$ T cells *in vivo*. Our results suggest that pre-TCR signaling indirectly inactivates TCR γ enhancers by reducing recruitment of STAT5 and Runx and imply that this effect is an important step for TCR γ silencing in $\alpha\beta$ T cells.

2) STAT5 controls the rearrangement of TCR J γ gene segments through STAT-binding motifs in the J γ promoters: K. WAGATSUMA, S. TANI-ICHI, B. LIANG, T. HARA and K. IKUTA

Mouse TCR γ locus consists of four clusters, and each cluster contains V γ , J γ and C γ segments. The γ 1 cluster has four V γ gene segments (V γ 5, V γ 2, V γ 4 and V γ 3), and they are preferentially rearranged with J γ 1 gene segment within the same cluster. From *in vitro* and *ex vivo* analyses, we previously showed that STAT5 activated by IL-7R binds to STAT motifs in J γ promoters and increases histone acetylation, germline transcription and chromatin accessibility of the J γ gene segments. However, it remains unclear whether the STAT motifs in the J γ promoters play a critical role in the rearrangements of the TCR γ locus *in vivo*. To address this issue, we generated two kinds of J γ 1 promoter mutant mice. One carries point mutations in STAT motifs in the J γ 1 promoter (J γ 1P stat-mut), and the other has a 940-bp deletion of the J γ 1 promoter including the STAT motifs (Δ J γ 1P). Flow cytometric analysis showed that V γ 2⁺ and V γ 5⁺ T cells of the γ 1 cluster were severely decreased in thymus and small intestine of these mutant mice. V γ 3⁺ T cells were also decreased in the skin and in fetal thymus. In contrast, V γ 1.1⁺ T cells of the γ 4 cluster were unchanged in the mutant mice. Importantly, V–J rearrangements of the γ 1 cluster were drastically reduced in these mutant mice, while the rearrangements of other clusters were unchanged. It is noteworthy that the two mutant mice showed similar reduction in the rearrangements. Furthermore, germline transcription at the J γ 1 gene segment was severely reduced in the J γ 1P stat-mut mice. These results demonstrate that the STAT motifs in the J γ 1 promoter are essential for V–J recombination of the J γ 1 gene segment *in vivo*, and support the idea that STAT motifs control local accessibility of the J γ gene segments by recruiting STAT5.

3) IL-7R controls differentiation of CD8 T cells and maintenance of peripheral T cells: S. TANI-ICHI, A. ABE, T. HARA and K. IKUTA

The IL-7R is essential for differentiation and survival of T cells. We previously showed that IL-7R α -deficient mice have severely reduced numbers of $\alpha\beta$ T cells and completely lack $\gamma\delta$ T cells. However, the role of the IL-7R was not precisely determined in late stages of T cell development, because IL-7R α -deficient mice have profound detrimental effects on early thymocytes. To address this question, we established IL-7R α -floxed mice and crossed with CD4-Cre transgenic mice. In the thymus, total cell numbers of CD4-Cre IL-7R $\alpha^{\text{flox/flox}}$ mice were similar to control mice. Whereas differentiation of CD4⁺CD8⁺, CD4⁺CD8⁺, CD4 single positive (SP) cells and $\gamma\delta$ T cells were not affected, the numbers of mature CD8 SP cells were markedly reduced in CD4-Cre IL-7R $\alpha^{\text{flox/flox}}$ thymus. In addition, the development of NKT cells and regulatory T cells were partially impaired in the thymus of CD4-Cre IL-7R $\alpha^{\text{flox/flox}}$ mice. The expression of anti-

apoptotic factor Bcl-2, a major target gene of IL-7 signal, was reduced in CD4 and CD8 T cells, and the development of CD8 T cells was rescued by introduction of a Bcl-2 transgene. In the periphery, although CD4-Cre IL-7R $\alpha^{\text{flox/flox}}$ mice have comparable numbers of lymph nodes and Peyer's patches to control mice, there were a selective loss of CD4 and CD8 T cells and a selective gain of $\gamma\delta$ T cells. These data demonstrate that the IL-7R is required for differentiation of CD8 T cells, NKT cells and regulatory T cells in thymus.

4) Distribution of IL-7-expressing cells in lymphoid tissues: T. HARA, S. SHITARA, G. CUI, S. TANI-ICHI and K. IKUTA

IL-7 is an essential cytokine for lymphocyte development and survival produced by mesenchymal and epithelial cells in lymphoid organs. However, little is known about the precise nature and distribution of IL-7-expressing cells in vivo. To address this question, we established IL-7-GFP knock-in mice. We found that the majority of thymic epithelial cells (TECs) express GFP in the cortex and medulla. A large number of cortical TECs express GFP at high levels, while most medullary TECs express GFP at low levels. Their expression levels decrease gradually with aging. In the lymph node paracortex, fibroblastic reticular cells (FRCs) express GFP at intermediate levels. In addition, we detected high levels of GFP expression in lymphatic endothelial cells of lymph nodes, intestines, and skin. In the spleen, FRCs scattered in the white pulp express GFP at low levels. Moreover, we found intermediate levels of GFP expression in the stromal cells lining the marginal zone and surrounding central arterioles. In the bone marrow, some VCAM-1⁺ stromal cells express GFP at high levels. In the colon, some epithelial cells express high levels of GFP. After induction of acute colitis with DSS, GFP expression was elevated in the intestinal epithelial cells. Thus, the IL-7-GFP knock-in mouse reveals unreported types of IL-7-expressing cells and provides a powerful tool to analyze the IL-7-niche in the lymphoid organs under physiological and pathological conditions.

5) Evidence for the thymic origin of $\gamma\delta$ intestinal intraepithelial lymphocytes: S. SHITARA, B. LIANG, T. HARA, K. WAGATSUMA, S. TANI-ICHI and K. IKUTA

Intestinal intraepithelial lymphocytes (IELs) are composed of TCR $\alpha\beta^+$ and TCR $\gamma\delta^+$ IELs and play a key role in host mucosal immunity. A fate-mapping experiment showed that $\alpha\beta$ IELs originate from the thymus. Because $\gamma\delta$ IELs develop in reduced but substantial numbers in nude mice, the issue on thymic versus extrathymic generation of $\gamma\delta$ IELs is still a matter of debate. IL-7^{-/-} mice totally lack $\gamma\delta$ T cells in thymus and intestine, suggesting that IL-7 is essential for $\gamma\delta$ IEL development. To elucidate the origin of $\gamma\delta$ IELs, we crossed IL-7-floxed mice with FoxN1-Cre transgenic mice to obtain the conditional knockout mice deficient in IL-7 production from thymic

epithelial cells. FoxN1-Cre IL-7^{flox/flox} mice showed 20-fold and 100-fold reduced numbers of $\alpha\beta$ and $\gamma\delta$ T cells, respectively, in the thymus. In small intestine, cell numbers of both $\alpha\beta$ and $\gamma\delta$ IELs were significantly reduced at 4 weeks in FoxN1-Cre IL-7^{flox/flox} mice. The numbers of $\alpha\beta$ IELs gradually increased to reach the levels of control mice at 12 weeks, while those of $\gamma\delta$ IELs remained at low levels. Next, we crossed the IL-7-floxed mice with villin-Cre (Vil-Cre) transgenic mice to obtain the conditional knockout mice deficient in IL-7 production from intestinal epithelial cells. Vil-Cre IL-7^{flox/flox} mice showed similar numbers of $\alpha\beta$ and $\gamma\delta$ IELs compared with control mice. These results collectively demonstrate that IL-7 produced in the thymus is essential for $\gamma\delta$ IEL development. Thus, this study presents strong evidence for the thymic origin $\gamma\delta$ IELs.

6) ELISA kit system for detecting calreticulin: M. UEDA, S. KAGEYAMA¹ and T. YOSHIKI² (¹Department of Urology, Shiga University of Medicine, ²Department of Clinical Oncology, Kyoto Pharmaceutical University)

Calreticulin (CRT) is the protein found in human urogenital organs. We have found that CRT exhibited three polypeptide forms; normal spliced form, alternative spliced forms and non-spliced full-length form. The immunoblot analysis of urogenital tissue with PVDF membrane showed that the amount of full-length form of CRT correlates to urogenital cancers (Kageyama et al. Clin Chem 2004). For diagnosis of urogenital cancers, we constructed the assay kit by ELISA method with HRP. We prepared a monoclonal antibody (mAb) against full-length form of CRT reactive in the native and denaturated forms by SDS. The specificity and sensitivity of ELISA system with this mAb and seven other mAbs are under investigation.

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DEPARTMENT OF BIOLOGICAL RESPONSES
LABORATORY OF INFECTION AND PREVENTION

The research projects carried out in this group are studies on α -arrestin family proteins including thioredoxin binding protein-2 (TBP-2) also referred as thioredoxin interacting protein (Txnip) or Vitamin D3 up-regulated protein 1 (VDUP1), especially focusing on their important medical and biological aspects such as cancer suppression and the regulation of energy metabolism.

1) Thioredoxin binding protein (TBP)-2/Txnip and α -arrestin proteins in cancer and diabetes mellitus: H. MASUTANI, E. YOSHIHARA and S. MASAKI

Thioredoxin binding protein (TBP)-2/thioredoxin interacting protein (Txnip) is an α -arrestin protein that has attracted much attention as a multifunctional regulator. TBP-2 expression is downregulated in tumor cells and the level of TBP-2 is correlated with clinical stage of cancer. Mice with mutations or knockout of the TBP-2 gene are much more susceptible to carcinogenesis than wild-type mice, indicating a role for TBP-2 in cancer suppression. Studies have also revealed roles for TBP-2 in metabolic control. Enhancement of TBP-2 expression causes impairment of insulin sensitivity and glucose-induced insulin secretion, and β -cell apoptosis. Since these changes are important characteristics of type 2 diabetes mellitus (T2DM), TBP-2 is an attractive target for the development of drugs against T2DM. TBP-2 regulates transcription of metabolic regulating genes. TBP-2-like inducible membrane protein (TLIMP)/arrestin domain containing 3 (ARRDC3) regulates endocytosis of receptors such as the β_2 -adrenergic receptor. The α -arrestin family possesses PPXY motifs and may function as an adaptor/scaffold for NEDD family ubiquitin ligases. Elucidation of the molecular mechanisms of α -arrestin proteins would provide a new pharmacological basis for developing approaches against cancer and T2DM.

2) Deficiency of thioredoxin binding protein (TBP)-2 enhances TGF- β signaling and contributes to TGF- β -induced epithelial to mesenchymal transition: S. MASAKI and H. MASUTANI

Transforming growth factor beta (TGF- β) has regulatory roles in cell growth, differentiation, apoptosis, invasion and epithelial-mesenchymal transition (EMT) of various cancer cells. TGF- β -induced EMT is an important step for the progression of carcinoma cells to the invasion state. Thioredoxin binding protein-2 (TBP-2), also called as Txnip or VDUP1, is downregulated in various types of human cancer and is considered to be a tumor suppressor. However, it remains unclear how TBP-2 is involved in the regulation of the invasion and metastasis of cancer. We demonstrated that TBP-2 interacts with Smad ubiquitin regulatory factor 2 (Smurf2), a regulator of

TGF- β signaling. TBP-2 deficiency increased TGF- β -induced transcriptional activation and the expression level of TGF- β target genes. TBP-2 deficiency also caused sustained Smad2 phosphorylation. Knockdown of TBP-2 resulted in up-regulation of transcriptional factors related to TGF- β -mediated induction of EMT and led to accelerate TGF- β -induced EMT in A549 and 253J cells. These results show that TBP-2 is a novel regulator of TGF- β signaling and EMT, and suggest that TBP-2 is a potential therapeutic target and a prognostic indicator of cancer.

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DEPARTMENT OF CELL BIOLOGY
LABORATORY OF SUBCELLULAR BIOGENESIS

- 1) **ABL1 regulates spindle orientation in adherent cells and mammalian skin: S. MATSUMURA, M. HAMASAKI, M. EBISUYA¹, T. YAMAMOTO², E. NISHIDA³ and F. TOYOSHIMA** (¹ICDO, Career-Path Promotion Unit, Kyoto University, ²iCeMS, Kyoto University, ³Graduate School of Biostudies, Kyoto University)

Despite the growing evidence for the regulated spindle orientation in mammals, a systematic approach for identifying the responsible genes in mammalian cells has not been established. We performed a kinase-targeting RNAi screen in HeLa cells and identified ABL1 as a novel regulator of spindle orientation. Knockdown of ABL1 causes the cortical accumulation of LGN, an evolutionarily conserved regulator of spindle orientation, which results in the LGN-dependent spindle rotation and spindle misorientation. In vivo inactivation of ABL1 by a pharmacological inhibitor or by ablation of the *abl1* gene causes spindle misorientation and LGN mislocalization in mouse epidermis. Furthermore, ABL1 directly phosphorylates NuMA, a binding partner of LGN, on tyrosine 1774. This phosphorylation maintains the cortical localization of NuMA during metaphase, and ensures the LGN/NuMA-dependent spindle orientation control. This study provides a novel approach to identify genes regulating spindle orientation in mammals and uncovers new signaling pathways for this mechanism.

- 2) **Roles of cholesterol metabolites in the control of cell division: M. HAMASAKI, S. MATSUMURA and F. TOYOSHIMA**

Cholesterol is a precursor of steroid hormones and is required for the maintenance of homeostasis. However, little is known about the function of cholesterol metabolites during mitosis. We found that the RNAi-mediated knockdown of Cyp11a1, which catalyzes the cleavage of cholesterol side chain to produce pregnenolone, induced multipolar spindles in mitotic HeLa cells. Introduction of pregnenolone, but not progesterone or 17-hydroxy-pregnenolone, into the cells transfected with Cyp11a1 siRNA restored the proper spindle formation in these cells. We further show that the centriole disengagement, which occurs in anaphase/telophase in normal condition, takes place in prometaphase/metaphase in the Cyp11a1-depleted cells. This precocious centriole disengagement is again suppressed by the introduction of pregnenolone into these cells. These results demonstrate that pregnenolone is required for the maintenance of centriole engagement during prometaphase/metaphase. We are now investigating how pregnenolone regulates centriole engagement.

3) Regulation of the early endosomes during mitosis: K. IKAWA, S. MATSUMURA, ¹M. FUKUDA and F. TOYOSHIMA (¹Department of Developmental Biology and Neurosciences, Tohoku University)

Membrane fusion of early endosomes is a critical step in endocytic trafficking. However in mitosis, an endosomal fusion is known to be inhibited. The mechanisms as well as biological significance of this negative regulation for the endosomal fusion during mitosis are poorly understood. We have found that one of the mitotic regulators plays an essential role in this mechanism.

LIST OF PUBLICATIONS

DEPARTMENT OF CELL BIOLOGY

LABORATORY OF SUBCELLULAR BIOGENESIS

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- S. Iwano, S. Matsumura and F. Toyoshima: Analysis of a novel spindle orientation regulator. Exciting Biology Series "Cellular Development: Biology at the Interface", Sep.29-Oct.1, 2011, Kobe, Japan (poster)
- M. Hamasaki, S. Matsumura and F. Toyoshima: Cholesterol metabolites pregnenolone is required for centriole engagement. EMBO Conference "Centrosomes and Spindle Pole Bodies", October 2-6, 2011, Barcelona, Spain (Poster)
- S. Matsumura, M. Hamasaki, T. Yamamoto, M. Ebisuya, M. Sato, E. Nishida and F. Toyoshima: Genome-wide survey of kinases identified ABL1 as a novel regulator of spindle orientation in adherent cells and mammalian skin. EMBO Conference "Centrosomes and Spindle Pole Bodies", October 2-6, 2011, Barcelona, Spain (oral)
- S. Matsumura, M. Hamasaki, T. Yamamoto, M. Ebisuya, M. Sato, E. Nishida and F. Toyoshima: ABL1 regulates spindle orientation in adherent cells and mammalian skin. Cold Spring Harbor Laboratory Meeting, "Stem Cell Biology", Sep. 20-24, 2011, New York, USA (poster)
- 濱崎真弓、松村繁、豊島文子：コレステロール側鎖切断酵素 CYP11A1 は中心小体のエンゲージメントを制御する。第 84 回 日本生化学会大会、京都、2011 年 9 月 22 日 (口頭)

松村繁、濱崎真弓、山本拓也、戎家美紀、佐藤みずほ、西田栄介、豊島文子：ABL1 による細胞分裂軸方向の制御機構. 第 63 回 日本細胞生物学会大会、札幌、2011 年 6 月 27-29 日（ポスター）

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DEPARTMENT OF CELL BIOLOGY
LABORATORY OF GROWTH REGULATION

The research interest of this laboratory is to understand the molecular mechanism of cell differentiation and organogenesis. Particularly, we are interested in basic helix-loop-helix (bHLH) transcription factors that regulate various developmental processes including neural development and somite formation. We are characterizing the functions of bHLH genes by misexpressing the genes with retrovirus and electroporation (gain-of-function study) and by generating knock-out mice (loss-of-function study). We previously showed that bHLH proneural genes such as *Mash1* and *Math3* promote neuronal versus glial fate determination whereas the bHLH genes *Hes1* and *Hes5* regulate maintenance of neural stem cells by repressing proneural gene expression. Interestingly, in neural stem cells, *Hes1* expression oscillates with a period of about 2-3 hours, while *Hes1* oscillations drive cyclic expression of the proneural gene *Neurogenin2* (*Ngn2*) and the Notch ligand gene *Deltalike1* (*Dll1*). In contrast, the expression of *Ngn2* and *Dll1* is sustained (non-oscillatory) in postmitotic differentiating neurons. Our data suggest that depending on the expression mode (oscillatory versus sustained), *Ngn2* can lead to two opposite outcomes: *Ngn2* maintains neural stem cells when the expression oscillates, whereas it induces neuronal differentiation when the expression is sustained. It seems that *Dll1* oscillation is advantageous for keeping a group of cells undifferentiated by mutual activation of Notch signaling, which induces *Hes1* expression. We also found that expression of the bHLH gene *Hes7* oscillates in the presomitic mesoderm, and that this oscillation regulates the periodic somite formation. By making and evaluating mathematical modeling, we are now studying how the dynamics of gene expression are controlled in these cells.

It has been shown that new neurons are continuously born from neural stem cells in the adult brain, and that this continuous neurogenesis plays an important role in many brain activities. We found that adult neurogenesis is essential for the innately programmed olfactory-dependent behaviors as well as spatial memory. We are now developing new research tools to identify the neural circuit responsible for such behaviors that integrates newly born neurons. We also found that in the absence of Notch signaling, neural stem cells are depleted in the adult brain, suggesting that the Notch pathway is essential for maintenance of adult neural stem cells and for continuation of adult neurogenesis. These results raised the possibility that Notch signaling genes are therapeutic targets to cure many brain disorders by activation of adult neurogenesis.

- 1) **Intronic delay is essential for oscillatory expression in the segmentation clock: Y. TAKASHIMA, T. OHTSUKA, A. GONZALEZ, H. MIYACHI and R. KAGEYAMA**

Proper timing of gene expression is essential for many biological events, but the molecular

mechanisms that control timing remain largely unclear. It has been suggested that introns contribute to the timing mechanisms of gene expression, but this hypothesis has not been tested with natural genes. One of the best systems for examining the significance of introns is the oscillator network in the somite segmentation clock, because mathematical modeling predicted that oscillating expression depends on negative feedback with a delayed timing. The basic helix-loop-helix repressor gene *Hes7* is cyclically expressed in the presomitic mesoderm (PSM) and regulates the somite segmentation. Here, we found that introns lead to an ~19-min delay in the *Hes7* gene expression, and mathematical modeling suggested that without such a delay, *Hes7* oscillations would be abolished. To test this prediction, we generated mice carrying the *Hes7* locus whose introns were removed. In these mice, *Hes7* expression did not oscillate but occurred steadily, leading to severe segmentation defects. These results indicate that introns are indeed required for *Hes7* oscillations and point to the significance of intronic delays in dynamic gene expression.

2) Cooperative functions of Hes/Hey genes in auditory hair cell and supporting cell development: T. TATEYA, I. IMAYOSHI, I. TATEYA, J. ITO and R. KAGEYAMA

Notch-mediated lateral inhibition has been reported to regulate auditory hair cell and supporting cell development from common precursors. While the Notch effector genes *Hes1*, *Hes5* and *Hey1* are expressed in the developing cochlea, inactivation of either of them causes only mild abnormality, suggesting their functional redundancy. To explore the roles of Hes/Hey genes in cochlear development, we examined compound heterozygous or homozygous mutant mice that lacked *Hes1*, *Hes5* and *Hey1* alleles. We found that a reduction in Hes/Hey gene dosage led to graded increase of hair cell formation. However, if at least one allele of *Hes1*, *Hes5* or *Hey1* was intact, excessive hair cells were accompanied by overproduction of supporting cells, suggesting that the hair cell increase does not occur at the expense of supporting cells, and that each Hes/Hey gene functions to induce supporting cells. By contrast, when all alleles of *Hes1*, *Hes5* and *Hey1* were inactivated, the number of hair cells increased more drastically, whereas that of supporting cells was unchanged compared with control, suggesting that supporting cell formation was balanced by their overproduction and fate conversion into hair cells. The increase of the cell numbers seemed to occur after the prosensory domain formation in the mutants because the proliferation state and the size of the prosensory domain were not affected. Thus, *Hes1*, *Hes5* and *Hey1* cooperatively inhibit hair cell formation, and one allele of *Hes1*, *Hes5* or *Hey1* is sufficient for supporting cell production probably by lateral inhibition in the sensory epithelium. Strikingly, Hes/Hey mutations lead to disorganized cell alignment and polarity and to hearing loss despite hair cell overproduction. These results suggest that Hes/Hey gene dosage is essential not only for generation of appropriate numbers of hair cells and supporting cells by controlling cell proliferation and lateral inhibition but

also for the hearing ability by regulating the cell alignment and polarity.

3) Continuous neurogenesis in the adult forebrain is required for innate olfactory responses: M. SAKAMOTO, I. IMAYOSHI, T. OHTSUKA, M. YAMAGUCHI, K. MORI and R. KAGEYAMA

Although the functional significance of adult neurogenesis in hippocampal-dependent learning and memory has been well documented, the role of such neurogenesis in olfactory activity is rather obscure. To understand the significance of adult neurogenesis in olfactory functions, we genetically ablated newly born neurons by using tamoxifen-treated Nestin-CreER(T2);neuron-specific enolase-diphtheria toxin fragment A (NSE-DTA) mice. In these mice, tamoxifen-inducible Cre recombinase allows the NSE (Eno2) gene to drive DTA expression in differentiating neurons, leading to the efficient ablation of newly born neurons in the forebrain. These mutant mice were capable of discriminating odors as competently as control mice. Strikingly, although control and mutant mice frequently showed freezing behaviors to a fox scent, a predator odor, mutant mice approached this odor when they were conditioned to associate the odor with a reward, whereas control mice did not approach the odor. Furthermore, although mutant males and females showed normal social recognition behaviors to other mice of a different sex, mutant males displayed deficits in male-male aggression and male sexual behaviors toward females, whereas mutant females displayed deficits in fertility and nurturing, indicating that sex-specific activities, which are known to depend on olfaction, are impaired. These results suggest that continuous neurogenesis is required for predator avoidance and sex-specific responses that are olfaction dependent and innately programmed.

4) Different types of oscillations in Notch and Fgf signaling regulate the spatiotemporal periodicity of somitogenesis: Y. NIWA, H. SHIMOJO, A. ISOMURA, A. GONZALEZ, H. MIYACHI and R. KAGEYAMA

Somitogenesis is controlled by cyclic genes such as Notch effectors and by the wave front established by morphogens such as Fgf8, but the precise mechanism of how these factors are coordinated remains to be determined. Here, we show that effectors of Notch and Fgf pathways oscillate in different dynamics and that oscillations in Notch signaling generate alternating phase shift, thereby periodically segregating a group of synchronized cells, whereas oscillations in Fgf signaling released these synchronized cells for somitogenesis at the same time. These results suggest that Notch oscillators define the prospective somite region, while Fgf oscillators regulate the pace of segmentation.

5) Gene expression profiling of neural stem cells and identification of regulators of neural differentiation during cortical development: T. OHTSUKA, H. SHIMOJO, M. MATSUNAGA, N. WATANABE, K. KOMETANI, N. MINATO and R. KAGEYAMA

During mammalian brain development, neural stem cells transform from neuroepithelial cells to radial glial cells and finally remain as astrocyte-like cells in the postnatal and adult brain. Neuroepithelial cells divide symmetrically and expand the neural stem cell pool; after the onset of neurogenesis, radial glial cells sequentially produce deep layer neurons and then superficial layer neurons by asymmetric, self-renewing divisions during cortical development. Thereafter, gliogenesis supersedes neurogenesis, while a subset of neural stem cells retain their stemness and lurk in the postnatal and adult brain. Thus, neural stem cells undergo alterations in morphology and the capacity to proliferate or give rise to various types of neural cells in a temporally regulated manner. To shed light on the temporal alterations of embryonic neural stem cells, we sorted the green fluorescent protein-positive cells from the dorsolateral telencephalon (neocortical region) of pHes1-d2EGFP transgenic mouse embryos at different developmental stages and performed gene expression profiling. Among dozens of transcription factors differentially expressed by cells in the ventricular zone during the course of development, several of them exhibited the activity to inhibit neuronal differentiation when overexpressed. Furthermore, knockdown of Tcf3 or Klf15 led to accelerated neuronal differentiation of neural stem cells in the developing cortex, and neurospheres originated from Klf15 knockdown cells mostly lacked neurogenic activities and only retained gliogenic activities. These results suggest that Tcf3 and Klf15 play critical roles in the maintenance of neural stem cells at early and late embryonic stages, respectively.

6) Six1 is indispensable for production of functional progenitor cells during olfactory epithelial development: K. IKEDA, R. KAGEYAMA, Y. SUZUKI and K. KAWAKAMI

The rodent olfactory epithelium (OE) is a good model system for studying the principles of stem and progenitor cell biology, because of its capacity for continuous neurogenesis throughout life and relatively well-characterized neuronal lineage. The development of mouse OE is divided into two stages, early and established neurogenesis. In established neurogenesis, which starts at embryonic day (E) 12.5, sustentacular cells and olfactory receptor neurons (ORNs) are produced from apical and basal progenitors, respectively. We previously reported that Six1(-/-) shows a lack of mature ORNs throughout development and disorganization of OE after E12.5. However, the molecular bases for these defects have not been addressed. Here, we show that Six1 is expressed in

both apical and basal progenitors. In Six1(-/-) mice, apical proliferating cells were absent and no morphologically identifiable sustentacular cells were observed. Consistently, the expression of Notch2 and Jagged1 in the apical layer was absent in Six1(-/-) mice. On the other hand, basal proliferating cells were observed in Six1(-/-) animals, but the expression of Ngn1, NeuroD, Notch1, and Jagged2 in the basal layer was absent. The expression of Mash1, the determination gene for ORNs, and Hes genes was enhanced in Six1(-/-) mice. The present findings suggest that Six1 regulates production of functional apical and basal progenitors during OE development, through the regulation of various genes, such as neuronal basic helix-loop-helix (bHLH), neuronal repressor bHLH, and genes involved in the Notch signaling pathway.

7) Fbxw7-dependent degradation of Notch is required for control of "stemness" and neuronal-glia differentiation in neural stem cells: A. MATSUMOTO, I. ONOYAMA, T. SUNABORI, R. KAGEYAMA, H. OKANO and K.-I. NAJAYAMA

Control of the growth and differentiation of neural stem cells is fundamental to brain development and is largely dependent on the Notch signaling pathway. The mechanism by which the activity of Notch is regulated during brain development has remained unclear, however. Fbxw7 (also known as Fbw7, SEL-10, hCdc4, or hAgo) is the F-box protein subunit of an Skp1-Cul1-F-box protein (SCF)-type ubiquitin ligase complex that plays a central role in the degradation of Notch family members. We now show that mice with brain-specific deletion of Fbxw7 (Nestin-Cre/Fbxw7(F/F) mice) die shortly after birth with morphological abnormalities of the brain and the absence of suckling behavior. The maintenance of neural stem cells was sustained in association with the accumulation of Notch1 and Notch3, as well as up-regulation of Notch target genes in the mutant mice. Astrogenesis was also enhanced in the mutant mice *in vivo*, and the differentiation of neural progenitor cells was skewed toward astrocytes rather than neurons *in vitro*, with the latter effect being reversed by treatment of the cells with a pharmacological inhibitor of the Notch signaling pathway. Our results thus implicate Fbxw7 as a key regulator of the maintenance and differentiation of neural stem cells in the brain.

8) Notch-Hes1 pathway is required for the development of IL-17-producing $\gamma\delta$ T cells: K. SHIBATA, H. YAMADA, T. SATO, T. DEJIMA, M. NAKAMURA, T. IKAWA, H. HARA, S. YAMASAKI, R. KAGEYAMA, Y. IWAKURA, H. KAWAMOTO, H. TOH and Y. YOSHIKAI

Unlike conventional T cells, which are exported from the thymus as naive cells and acquire effector functions upon antigen encounter in the periphery, a subset of $\gamma\delta$ T cells differentiates into effectors that produce IL-17 within the fetal thymus. We demonstrate here that

intrathymic development of the naturally occurring IL-17-producing $\gamma\delta$ T cells is independent of STAT3 and partly dependent on ROR γ t. Comparative gene-expression analysis identified Hes1, one of the basic helix-loop-helix proteins involved in Notch signaling, as a factor specifically expressed in IL-17-producing $\gamma\delta$ T cells. Hes1 is critically involved in the development of IL-17-producing $\gamma\delta$ T cells, as evidenced by their severe decrease in the thymi of Hes1-deficient fetal mice. Delta-like 4 (Dll4)-expressing stromal cells support the development of IL-17-producing $\gamma\delta$ T cells in vitro. In addition, conditional Hes1 ablation in peripheral $\gamma\delta$ T cells decreases their IL-17 production but not their IFN- γ production. These results reveal a unique differentiation pathway of IL-17-producing $\gamma\delta$ T cells.

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DEPARTMENT OF CELL BIOLOGY
LABORATORY OF SIGNAL TRANSDUCTION

1) Comparison of two quantitative assays for xenotropic murine leukemia virus-related virus: E. SATO, R. YOSHIKAWA and T. MIYAZAWA

Xenotropic murine leukemia virus-related virus (XMRV), a novel gammaretrovirus in humans, was found in patients with prostate cancer (PC) and chronic fatigue syndrome (CFS). However, there has been controversy whether XMRV is directly associated with human diseases. In this study, we developed a LacZ marker rescue assay using human embryonic kidney 293T cells and a focus assay using a feline fibroblastic sarcoma-positive leukemia-negative QN10S cells. XMRV induced prominent foci in QN10S cells and the viral titer determined by the focus assay was as high as that by the LacZ marker rescue assay. Because the focus assay is simple and sensitive, it will be useful for monitoring infectious XMRVs in CFS and PC patients and virological studies for XMRV.

2) Identification of functional receptors for RD-114 virus in dogs: R. YOSHIKAWA, T. KOBAYASHI and T. MIYAZAWA

The genomes of mammalian species contain enormous copies of endogenous retroviruses (ERVs). In general, many ERVs have lost their infectivity. However, several ERVs have maintained their infectivity. All domestic cats have an infectious ERV, termed RD-114 and several feline cell lines produce infectious RD-114 viruses. Recently, we found that several feline and canine live attenuated vaccines were contaminated with infectious RD-114 viruses (Miyazawa *et al.*, *J. Virol.* (2010); Yoshikawa *et al.*, *Biologicals* (2011)). In this study, we confirmed that the RD-114 virus efficiently infected and proliferated well in canine primary cells as well as canine cell line (a fibroblast cell line derived from canine thymus). In addition, we identified canine ASCT1 and ASCT2, sodium-dependent neutral amino acid transporters, as RD-114 virus receptors. The canine ASCT2 also is a functional receptor for simian retrovirus type 2, a pathogenic simian which induces immunodeficiency in rhesus macaques. Identification of canine receptor for RD-114 virus will help for evaluating the risk of contamination of the virus in vaccines.

3) Mapping of a neutralizing epitope in the surface envelope protein of porcine endogenous retrovirus subgroup B: Y. NAKAYA, S. HOSHINO, J. YASUDA¹ and T. MIYAZAWA (¹Department of Emerging Infectious Diseases, Nagasaki University)

Pigs are thought to be the most suitable donor animal for xenotransplantation. However,

pigs harbour potentially hazardous infectious agents, termed porcine endogenous retroviruses (PERVs), in its genome. In this study, we generated a mAb against PERV-B surface (SU) envelope protein (Env), designated KRT1. KRT1 binding was detected by an indirect immunofluorescence assay and flow cytometric analysis on cells infected with PERV-B. KRT1 neutralized PERV-B pseudotype virus and specifically recognized PERV-B SU Env, but not PERV-A SU Env by immunoblotting analysis. The peptide-ELISA revealed that KRT1 recognized a linear peptide sequence (ALEPPHNLPVP) residing in a proline-rich region that is one of the subdomains of SU Env. In conclusion, the KRT1 antibody will serve as a useful tool for the study of PERV-B and, more importantly, it may provide new protective strategies against PERV-B infection in xenotransplantation.

- 4) Identification and characterization of feline UBE1L gene: S. SHIMODE, T. MIYAZAWA, T. KOBAYASHI, H. SATO¹ and T. TANABE¹** (¹Laboratory of Veterinary Microbiology, Faculty of Veterinary Medicine, School of Veterinary Medicine, Kitasato University)

Interferon-stimulated gene 15 (ISG15) is one of the type I interferon-inducible proteins expressed after bacterial or viral infection. ISG15 has two ubiquitin (UB)-like domains and is capable of conjugating to intracellular proteins (ISGylation). Addition of ISG15 known as ISGylation is an ubiquitin-like posttranslational modification. ISG15 and/or ISGylation play an important role in antiviral activity. Addition of ISG15, known as ISGylation, is an ubiquitin-like posttranslational modification. Coexpression of ISG15 and ubiquitin-activating enzyme E1-like protein (UBE1L) is required to induce ISGylation *in vitro*, but these enzymes of felis have not been described. Previously, we identified feline ISG15 gene and found that the capsid protein of feline immunodeficiency virus was ISGylated *in vitro* by treatment with feline interferon- ω . In this study, we cloned feline UBE1L (FeUBE1L) gene to further study the mechanism of the antiviral activities induced by ISGylation. Sequencing analysis revealed that active sites of FeUBE1L were highly conserved. These data suggest that FeUBE1L has an enzymatic activity. Further, expression of FeUBE1L was induced in feline cell lines by treatment with feline interferon- ω and ovine interferon- τ .

- 5) Analysis of newly identified KoRV-related sequences: S. HOSHINO, T. KOBAYASHI and T. MIYAZAWA** (¹Laboratoy of Primate Model, IVR)

Retroviral sequences are present in mammalian genomes and called endogenous retroviruses (ERVs). ERVs are remnants of ancestral retroviruses, which had invaded into host genomes. Koala retrovirus (KoRV) was isolated from koalas, showing leukemia and

immunodeficiency, and it has been pointed out that KoRV might be related to these diseases. In 2006, Tarlinton *et al.* reported that KoRV had been invading koala genomes in only 200 years. In their report, KoRV was detected in all northern and some western population of koalas in Australia, although it was not detected in the population of Kangaroo island. In Japan, the infection status of KoRV has not been investigated, whereas nine zoos have reared many koalas. To know the infection status of KoRV in koalas kept in Japanese zoos, genomic DNAs were isolated from buffy coat cells and we analyzed them by PCR. We found that all Queensland koalas and four out of 11 Victorian koalas harbored KoRV proviruses. Seven out of 11 Victorian koalas did not have any known KoRV; however, we also found that these KoRV-free koalas harbored a long terminal repeat (LTR) which was similar to that of the KoRV. Then, we cloned and sequenced whole genome containing the 5'- and 3'-LTR, corresponding to KoRV, in KoRV-free koalas by long range PCR. By sequencing analysis, the LTR that we sequenced was nearly identical to the KoRV LTR. We also found that the obtained 5'-*gag* sequences (1-135 nt) were nearly identical to 5'-KoRV *gag* sequences (1-135 nt), and the obtained 3'-*env* sequences partially matched 3'-KoRV *env* sequence, respectively. Furthermore, we examined the integration site and copy numbers of the newly identified KoRV-related sequence (KRRS) in the koalas and the presence of the virus in other marsupials. Now we are trying to reveal the importance of existence of KRRS in koala genomes.

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仲屋友喜、越勝男、木崎景一郎、馬場健司、今川和彦、橋爪一善、宮沢孝幸：ウシ胎盤形成過程において機能を発揮する新規内在性レトロウイルス由来遺伝子の同定 第 151 回日本獣医学会学術集会、東京、2011 年 3 月 30 日-4 月 1 日

吉川禄助、佐藤英次、下島昌幸、前田健、宮沢孝幸：動物用生物製剤への内在性レトロウイルスの迷入とその危険性評価 第 151 回日本獣医学会学術集会、東京、2011 年 3 月 30 日-4 月 1 日

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福岡藍子、森川裕子、宮沢孝幸、安田二郎：ワクチン製造用ネコ培養細胞の樹立 第 48 回日本ウイルス学会九州支部総会、北九州、2011 年 8 月 26 日-27 日

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仲屋友喜、小林剛、宮沢孝幸：ウシ内在性レトロウイルス K1 エンベロープタンパクの開裂に重要な領域の探索 第 152 回日本獣医学会学術集会、堺、2011 年 9 月 19 日-21 日

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宮沢孝幸：哺乳類の胎盤形成における内在性レトロウイルスの進化的役割 日本遺伝学会

第 83 回大会、京都、2011 年 9 月 20 日-22 日

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CENTER FOR HUMAN RETROVIRUS RESEARCH LABORATORY OF VIRAL PATHOGENESIS

Goal of our research group: What is the molecular mechanism of viral infection and pathogenesis? The subjects are human viruses, human immunodeficiency virus type 1 (HIV-1), herpes simplex virus type 1 (HSV-1) and Epstein-Barr virus (EBV).

1) Interaction of HIV Protein and Host Restriction Factor: P. GEE, H. EBINA, K. SATO, N. MISAWA, Y. KANEMURA, N. KASAI and Y. KOYANAGI

The introduction of an SIV or HIV-2 accessory protein, known as viral protein x (VPX), into myeloid cells before the addition of HIV-1 has been shown to target SAMHD1 for ubiquitin-dependent proteasome degradation, resulting in augmented HIV-1 infection. It has been also known that SAMHD1 is a player in host innate immunity. In Aicardi-Goutieres Syndrome (AGS), a rare disease characterized by hereditary encephalopathy, mutations in the SAMHD1 gene have been linked to elevated cytokine responses, most likely due to inefficient clearing of cellular nucleic acids. Both clearance of cellular nucleic acids and the inhibition of HIV-1 are presumed to be dependent on a nucleotidase and/or phosphodiesterase activity, predicted by a conserved HD domain that is responsible for divalent metal ion binding and is highly conserved in homologous enzymes. We successfully cloned, expressed, and purified recombinant SAMHD1 from *E. coli* and characterized its enzymatic activity in terms of its divalent metal ion preference and substrate usage. We found that SAMHD1 is a metal-dependent enzyme that it is active against a wide range of ribonucleoside 5'-mono-, di-, and triphosphates, indicating that this protein may be an important player in cells to regulate intracellular nucleotide metabolism during AGS and HIV infection.

2) HIV-1 Pathogenesis: K. SATO, N. MISAWA and Y. KOYANAGI

While human cells express potent antiviral proteins as part of the host defense repertoire, viruses have evolved their own arsenal of proteins to antagonize them. BST2 was identified as an inhibitory cellular protein of HIV-1 replication, which tethers virions to the cell surface to prevent their release *in vitro* culture system. On the other hand, the HIV-1 accessory protein, Vpu, has the ability to downregulate and counteract BST2. Vpu also possesses the ability to downmodulate cellular CD4 molecules expressed on infected cells. However, the role of Vpu in HIV-1 infection *in vivo* remains unclear. We generated NOG-hCD34 mice by transplanting newborn NOD/SCID/IL2R γ^{null} mice with human CD34 $^{+}$ cells and using this model, we found that Vpu contributes to the efficient spread of HIV-1 *in vivo* during the acute phase of infection. The level of viral protein expression, the amount of cell-free virions in vpu-deficient HIV-1-infected mice was

profoundly lower than that in wild-type (WT) HIV-1-infected mice. We provide a novel insight suggesting that Vpu concomitantly downregulates BST2 and CD4 from the surface of infected cells. Our findings suggest that Vpu augments the initial burst phase of HIV-1 replication *in vivo* by downmodulating BST2 and CD4 in infected cells.

3) HIV Integration and Latency: H. EBINA, Y. KANEMURA, Y. SUZUKI, K. URATA and Y. KOYANAGI

HIV-1 possesses a viral protein, integrase (IN), which is necessary for its efficient integration in target cells. However, it has been reported that an IN-defective HIV strain is still capable of integration. We assessed the ability of WT HIV-1 to establish infection in the presence of IN inhibitors. We observed a low, yet clear infection of inhibitor-incubated cells infected with WT HIV which was identical to cells infected with IN-deficient HIV, D64A. Furthermore, the IN-independent integration could be enhanced by the pretreatment of cells with DNA-damaging agents suggesting that integration is mediated by a DNA repair system. Moreover, significantly faster viral replication kinetics with augmented viral DNA integration was observed after infection in irradiated cells treated with IN inhibitor compared to nonirradiated cells. Altogether, our results suggest that HIV DNA has integration potential in the presence of an IN inhibitor and may serve as a virus reservoir.

4) APOBEC1-Mediated Attenuation of Herpes Simplex Virus 1 Indicate That Neurons Have an Antiviral Role during Herpes Simplex Encephalitis: P. GEE, H. EBINA, Y. KANEMURA and Y. KOYANAGI

APOBEC1 (A1) is a cytidine deaminase involved in the regulation of lipids in the small intestine. HSV-1 is a ubiquitous pathogen that is capable of infecting neurons in the brain, causing encephalitis. We show that A1 is induced during encephalitis in neurons of rats infected with HSV-1. In cells stably expressing A1, HSV-1 infection resulted in significantly reduced virus replication compared to that in control cells. Infectivity could be restored to levels comparable to those observed for control cells if A1 expression was silenced by specific A1 short hairpin RNAs. Moreover, cytidine deaminase activity appeared to be essential for this inhibition and led to an impaired accumulation of viral mRNA transcripts and DNA copy numbers. The sequencing of viral gene UL54 DNA, extracted from infected A1-expressing cells, revealed G-to-A and C-to-T transitions, indicating that A1 associates with HSV-1 DNA. Taken together, our results demonstrate a model in which A1 induction during encephalitis in neurons may aid in thwarting HSV-1 infection.

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小柳義夫細胞性ウイルス抑制因子: ヘルペスウイルスとレトロウイルスの共通メカニズム

平成 23 年度北海道大学遺伝子病制御研究所研究集会 「感染、免疫、炎症、発癌」
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**CENTER FOR HUMAN RETROVIRUS RESEARCH
LABORATORY OF VIRUS CONTROL**

- 1) Pathogenesis of HTLV-1 bZIP factor (HBZ) *in vivo*: J. YASUNAGA, P. MIYAZATO, J. TANABE, K. SUGATA, N. TAGUCHI, Y. MITOBE, Y. MITAGAMI, M. TANABE and M. MATSUOKA.**

Human T-cell leukemia virus type 1 (HTLV-1) is the first retrovirus that induces diseases in human. HTLV-1 causes a neoplastic disease, adult T-cell leukemia (ATL), and the inflammatory diseases, such as HTLV-1 associated myelopathy/tropical spastic paraparesis and uveitis. In addition, it is clinically known that HTLV-1 induces cellular immunodeficiency in the infected subjects, although its molecular mechanism was obscure. HTLV-1 belongs to complex retrovirus, which encodes regulatory genes (*tax* and *rex*) and several accessory genes, such as *p30*, *p12*, *p13* and *HTLV-1 bZIP factor (HBZ)*. Among them, it is suggested that Tax and HBZ play important roles in HTLV-1-induced pathogenesis. Whereas Tax expression is frequently silenced in ATL cells, transcription of the *HBZ* gene is detected in all of the ATL cell lines and primary ATL cases, indicating that HBZ is a critical factor for ATL leukemogenesis. We have established HBZ transgenic mice (HBZ-Tg) that express *HBZ* gene in CD4⁺ T-cells. Recently, we reported that HBZ-Tg developed T-cell lymphomas and systemic inflammatory diseases, such as dermatitis and alveolitis. Immunological analyses revealed that the population of regulatory T cells (Tregs) was increased in HBZ-Tg, and T-lymphoma tissues in HBZ-Tg frequently expressed Foxp3, a master molecule of Treg. Interestingly, the suppressive function of Tregs from HBZ-Tg was impaired compared with non-Tg littermates, suggesting that HBZ expression increases dysfunctional Tregs resulting in malignant transformation and inflammatory disorders *in vivo*. We have also reported that HBZ impairs production of Th1 cytokines inducing cellular immunodeficiency in HBZ-Tg. Those phenotypes of HBZ-Tg, namely, lymphoma development, inflammatory diseases, and immunodeficiency, are very similar to those of HTLV-1 carriers. Our observations imply that *HBZ* has a crucial role in HTLV-1-associated pathogenesis.

- 2) Molecular functions of HBZ in ATL leukemogenesis: J. YASUNAGA, P. MIYAZATO, T. ZHAO, J. FAN, K. HAGIYA, J. TANABE, A. TANAKA-NAKANISHI, G. MA, Y. MITOBE, M. MIURA, N. SONO, A. KAWATSUKI, Y. MITAGAMI, M. TANABE and M. MATSUOKA.**

Tax and HBZ are considered to play the important roles in leukemogenesis of ATL, although the precise mechanism has not been clarified. Interestingly, these two proteins have the opposite functions in various signaling pathways. HBZ specifically suppresses the classical NF- κ B

pathway by targeting p65, whereas Tax activates both classical and alternative pathways. Tax is known to suppress TGF- β signaling through inhibition of Smad proteins. Recently, we have reported that HBZ can form a complex with Smad2/3 and p300 to activate the transcription of TGF- β -responsive genes, such as *Foxp3*. On the other hand, HBZ represses the activity of Foxp3 by forming complex with Foxp3 and NFAT; those findings can explain why functionally impaired Tregs are increased in HBZ-Tg. In addition, we reported that HBZ interacts with activating transcription factor 3 (ATF3) and interferes with the activation of p53 by ATF3, suggesting an anti-apoptotic effect of HBZ. Our findings suggest that HBZ complicatedly regulates the cellular signaling pathways together with Tax, and finally leads T-cells to malignant transformation. We also identified other cellular targets of HBZ. We are analyzing their significances in leukemogenesis of HTLV-1-infected cells.

3) Characterization of DNA repair proteins involved in retroviral integration: Y. SAKURAI and M. MATSUOKA.

Retrovirus synthesizes viral dsDNA by reverse transcription and inserts the DNA into the host genome by integration. Some viruses strongly prefer specific genomic regions for their integration. Mouse leukemia virus (MLV) prefers the regions near transcriptional start sites, CpG islands and DNase hyper sensitive sites for its integration, while the molecular mechanism for this preference is unknown. In this study, we analyzed a large number of the integration sites by massively parallel sequencing, and found that human mutant cells lacking a DNA repair protein NBS1 and NBS1-knockout MEFs showed decreased MLV integration frequency near transcriptional start sites, CpG islands and DNase hyper sensitive sites. NBS1-deficient human cells also showed decreased integration within H3K4me3, H3K9ac and H3K36ac regions, which are histone modifications strongly detected around active promoters. In contrast, the integration frequency increased surrounding regions rich in H4K20me3, which is known to be associated with heterochromatin. Moreover, we demonstrated physical interaction of NBS1 and viral DNA before integration in MLV-infected cells by using ChIP assay. This study indicates that NBS1 is a host factor regulating MLV integration targeting.

4) Novel resistance mechanism to HIV-1 fusion inhibitors: K. SHIMURA and M. MATSUOKA.

Enfuvirtide (T-20), an HIV-1 gp41-derived peptide, efficiently inhibits HIV infection by blocking the fusion between viral envelope proteins and the plasma membrane. We have developed several potential second-generation fusion inhibitors (FIs), such as SC34 and SC34EK, which are active against T-20-resistant variants. Resistant HIV-1 to SC34EK contained several mutations in

gp41, and about half of them were located in the C-terminus of gp41, specifically called cytoplasmic tail (CT). This region is believed to be essential for efficient viral infection and replication, while there is no report that FIs selected mutations in this region so far. We observed that mutations in CT conferred resistance to FIs, and impaired viral infection. These results indicate that FI-selected mutations in CT involved in the drug susceptibility by influencing the viral infection steps.

5) Development of new small-molecule inhibitors for HIV: K. SHIMURA, H. TOGAMI, and M. MATSUOKA.

Recent anti-retroviral therapy (ART) potently suppresses HIV-1 replication, and improves prognosis of HIV-1 infected individuals. However, long-term antiviral therapies induce drug resistant viruses, and this is a major obstacle of efficient therapies. In order to develop new small-molecule anti-HIV drugs, we screened tens of thousands of compounds and several with anti-HIV activity were identified. Among them, some compounds seem to inhibit HIV replication by a novel mode of action. We are going to identify the mechanism of action and reveal antiviral spectrum.

LIST OF PUBLICATIONS

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Sugata K, Satou Y, Yasunaga JI, Hara H, Ohshima K, Utsunomiya A, Mitsuyama M, Matsuoka M. HTLV-1 bZIP factor impairs cell-mediated immunity by suppressing production of Th1 cytokines. *Blood* 119(2):434-444, 2011.

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EXPERIMENTAL RESEARCH CENTER FOR INFECTIOUS DISEASES

LABORATORY OF MOUSE MODEL

Our research objective is to understand the molecular mechanisms that control chromatin function and genome diversity & stability in mammals. To address this question, we are currently analyzing functional molecules which are expressed in the nucleus.

1) **Roles of the histone lysine demethylases *Jmjd1a* and *Jmjd1b* in murine embryonic development: M. TACHIBANA, S. KUROKI and Y. SHINKAI**

Histone H3 lysine 9 (H3K9) methylation is a repressive epigenetic mark for heterochromatin formation and transcriptional silencing. Our research purpose is to understand the dynamics of H3K9 methylation in mammalian development and to identify the molecule(s) that regulate H3K9 methylation. We previously showed that coordinated expression of the H3K9 methyltransferase G9a and the H3K9 demethylase *Jmjd1a* dynamically regulate H3K9 methylation levels during male meiosis in mice (Tachibana et al., 2007). To further elucidate molecular function of *Jmjd1a* during murine development, we have established *Jmjd1a* knockout (KO) mice (Inagaki et al., 2009). *Jmjd1a* is dispensable for embryonic development since *Jmjd1a*-KO offspring were delivered normally. *Jmjd1a*-related protein *Jmjd1b* can also catalyze H3K9 demethylation. *Jmjd1b*-KO mice were born at sub-Mendelian ratio. To investigate a redundant role of *Jmjd1a* and *Jmjd1b* on mouse embryogenesis, mice carrying both *Jmjd1b*^{+/-} and *Jmjd1b*^{+/-} alleles were intercrossed. We could not obtain offspring carrying both *Jmjd1a*^{-/-} and *Jmjd1b*^{-/-} alleles until E7.5, suggesting that *Jmjd1a/b* double KO (DKO) mice are embryonic lethal.

To further investigate the function of *Jmjd1a/b*, we established ES cells in which *Jmjd1a* and *Jmjd1b* were conditionally disrupted by 4-hydroxytamoxifen (OHT) treatment. *Jmjd1a/b* conditional KO ES cells could not grow in the presence of OHT, whereas either *Jmjd1a*- or *Jmjd1b*-KO ES cells grow normally, indicating *Jmjd1a* and *b* were redundantly required for ES cell growth. Propidium iodide (PI) staining analysis indicated PI-positive cells were dramatically increased when both *Jmjd1a* and *b* alleles were mutated. These facts indicate cell death is induced in *Jmjd1a/b*-depleted ES cells. Importantly, levels of dimethyl H3K9 (H3K9me₂) was drastically increased when both *Jmjd1a* and *Jmjd1b* were mutated. In contrast, levels of H3K9me₂ were only slightly elevated when either *Jmjd1a* or *Jmjd1b* was mutated. These facts suggest *Jmjd1a* and *b* are redundantly required not only for ES cell survival but also for H3K9 demethylation.

2) **Analysis of epigenetic regulation of mammalian sex differentiation: M. TACHIBANA**

Sex differentiation is the process of development of the differences between males and

females from an undifferentiated zygote. This event is essential for sexually reproducing organisms to pass a combination of genetic material to offspring, resulting in increased genetic diversity. In mammal, *Sry* is a key transcription factor that switches the developmental program into testes in the bipotential fetal gonads (Koopman et al., 1991). However, it is unknown how epigenetic change occurs during the differentiating process from bipotential gonads into the differentiated male/female gonads. To understand epigenetic change in this processes, we established *Ad4BP/SF1-LNGFR* transgenic (TG) mice that express human low-affinity nerve growth factor receptor (LNGFR) in gonadal somatic cells. In these mice, LNGFR was successfully expressed specifically in gonadal somatic cells the TG lines. Next, we performed the purification of gonadal somatic cells using anti-LNGFR antibodies and magnetic separation system. More than 95% cells purified were positive for Ad4BP/SF1 protein, indicating purification was achieved successfully. Currently we are planning to analyze epigenome structure and gene expression profile of bipotential E11.5 gonads using the purified cells described above.

3) Roles of endogenous retroviruses repressor, ESET, in DNA repair/genome integrity: T.TSUBOTA and Y. SHINKAI

About forty percent of the mammalian genome is derived from retroelements, of which ~10% are endogenous retroviruses (ERVs). Since these retroelements could potentially cause diseases including cancer, it is critical to suppress their transposon activities. Recently, it has been reported that histone H3-lysine 9 (H3K9) methyltransferase, ESET, is required for the repression of ERVs in the mouse embryonic stem (mES) cells.

When *Eset* is conditionally knockout (*Eset* CKO) in mES cells by hydroxytamoxifen (OHT), the growth is rapidly inhibited. To understand the mechanism of this growth retardation, first the cell cycle analysis was performed and revealed that *Eset* CKO cells show the G1/S and G2/M phase arrest. Additionally, deletion of *Eset* also causes the apoptotic cell death. As expected from these results, the expression level of p53 was increased suggesting a decrease of genomic stability. Indeed, gamma(γ)-H2AX level in the mutant was higher than that of WT cells. Deletion of *Eset* also causes the abnormal nuclear structure including micronuclei which is a biomarker of genotoxic stress. From these results, it is strongly suggested that ESET is required for genome stability in the mES cells.

Since one of the retrotransposons, Line-1, has been reported to cause DNA damages, the expression level of Line-1 was investigated and showed that it is clearly reactivated in the *Eset* CKO cells. Therefore, it suggests that at least, in part, derepression of Line-1 likely causes DNA damages. Interestingly, although most of the γ -H2AX foci of WT cells were co-localized with DNA repair protein, 53BP1, which is recruited to damage sites via di-methylation of histone H4-lysine 20 (H4K20), some of the damage foci in the *Eset* CKO cells were not. Therefore, the other possibility

is that *Eset* deletion causes the reduction of 53BP1 recruitment to repair the spontaneous DNA damages, leading to genome destabilization. Currently, the molecular mechanism of this process is investigating.

LIST OF PUBLICATIONS

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EXPERIMENTAL RESEARCH CENTER FOR INFECTIOUS DISEASES LABORATORY OF PRIMATE MODEL

It has been 28 years since human immunodeficiency virus (HIV-1), the causative agent of acquired immune deficiency syndrome (AIDS) was first identified. Since then, our knowledge on HIV-1 and the pathophysiology of AIDS has grown enormously. Unfortunately, however, we have not yet developed an effective prophylactic measure or a thorough therapeutic intervention, and AIDS remains top priority among global public health agenda.

To develop effective preventive or therapeutic measures against AIDS, we need an experimental model system that recapitulates HIV-1 infection in humans. From the beginning of AIDS epidemic, HIV-1 has been known for its narrow host range. To overcome the narrow host range of HIV-1 and develop a dependable animal model for AIDS, our laboratory, first in the world, generated a chimeric simian-human immunodeficiency virus (SHIV), that carries HIV-1 derived *tat*, *rev*, *vpu* and *env* genes in the backbone of simian immunodeficiency virus, a closely related simian virus to HIV-1. Since then, SHIV/macaque model has been further developed and there are currently several SHIV strains available in the field and some of them cause acute disease followed by AIDS-like clinical manifestations.

We have been pursuing the following subjects,

1. Development and improvement of SHIV/macaque models,
2. SHIV-induced pathogenesis,
3. Development of novel vaccines and evaluation using SHIV/macaque system,
4. Identification of virus reservoir in HIV-1 infected individuals under highly active anti-retroviral therapy (HAART) using SIV infected monkeys as a model.

In addition to the abovementioned projects, we have been making efforts to establish non-human primate disease model for flavivirus infection, especially, dengue hemorrhagic fever.

1) T cells monitor N-myristoylation of the nef protein in simian immunodeficiency virus-infected monkeys: D. MORITA, T. IGARASHI, M. HORIIKE, N. MORI and M. SUGITA

The use of the host cellular machinery is essential for pathogenic viruses to replicate in host cells. HIV and SIV borrow the host-derived N-myristoyl-transferase and its substrate, myristoyl-CoA, for coupling a saturated C(14) fatty acid (myristic acid) to the N-terminal glycine residue of the Nef protein. This biochemical reaction, referred to as N-myristoylation, assists its targeting to the plasma membrane, thereby supporting the immunosuppressive activity proposed for the Nef protein. In this study, we show that the host immunity is equipped with CTLs capable of sensing N-myristoylation of the Nef protein. A rhesus macaque CD8(+) T cell line was established

that specifically recognized N-myristoylated, but not unmodified, peptides of the Nef protein. Furthermore, the population size of N-myristoylated Nef peptide-specific T cells was found to increase significantly in the circulation of SIV-infected monkeys. Thus, these results identify N-myristoylated viral peptides as a novel class of CTL target Ag.

2) Dominant induction of vaccine antigen-specific cytotoxic T lymphocyte responses after simian immunodeficiency virus challenge: Y. TAKAHARA, S. MATSUOKA, T. KUWANO, T. TSUKAMOTO, H. YAMAMOTO, H. ISHII, T. NAKASONE, A. TAKEDA, M. INOUE, A. IIDA, H. HARA, T. SHU, M. HASEGAWA, H. SAKAWAKI, M. HORIIKE, T. MIURA, T. IGARASHI, T. K. NARUSE, A. KIMURA and T. MATANO

Cytotoxic T lymphocyte (CTL) responses are crucial for the control of human and simian immunodeficiency virus (HIV and SIV) replication. A promising AIDS vaccine strategy is to induce CTL memory resulting in more effective CTL responses post-viral exposure compared to those in natural HIV infections. We previously developed a CTL-inducing vaccine and showed SIV control in some vaccinated rhesus macaques. These vaccine-based SIV controllers elicited vaccine antigen-specific CTL responses dominantly in the acute phase post-challenge. Here, we examined CTL responses post-challenge in those vaccinated animals that failed to control SIV replication. Unvaccinated rhesus macaques possessing the major histocompatibility complex class I haplotype 90-088-Ij dominantly elicited SIV non-Gag antigen-specific CTL responses after SIV challenge, while those induced with Gag-specific CTL memory by prophylactic vaccination failed to control SIV replication with dominant Gag-specific CTL responses in the acute phase, indicating dominant induction of vaccine antigen-specific CTL responses post-challenge even in non-controllers. Further analysis suggested that prophylactic vaccination results in dominant induction of vaccine antigen-specific CTL responses post-viral exposure but delays SIV non-vaccine antigen-specific CTL responses. These results imply a significant influence of prophylactic vaccination on CTL immunodominance post-viral exposure, providing insights into antigen design in development of a CTL-inducing AIDS vaccine.

3) Isolation of potent neutralizing monoclonal antibodies from an SIV-infected rhesus macaque by phage display: T. KUWATA, Y. KATSUMATA, K. TAKAKI, T. MIURA and T. IGARASHI

The humoral immune response is a mechanism that potently suppresses or prevents viral infections. However, genetic diversity and resistance to antibody-mediated neutralization are serious obstacles in controlling HIV-1 infection. In this study, we isolated monoclonal antibodies

from an SIV-infected macaque by using the phage display method to characterize antibodies in SIV infection. Variable regions of immunoglobulin genes were amplified by rhesus macaque-specific primers and inserted into the phagemid pComb3X, which produced the Fab fragment. Antibodies against SIV proteins were selected by biopanning using an SIV protein-coated 96-well plate. A total of 20 Fab clones obtained included 14 clones directed to gp41, four clones to gp120, and two clones to p27. The anti-gp120 Fab clones completely neutralized the homologous neutralization-sensitive SIVsmH635FC and the genetically divergent SIVmac316, and showed at least 50% inhibition against the neutralization-resistant strain, SIVsmE543-3. Competition ELISA revealed that these anti-gp120 Fab clones recognize the same epitope on gp120 including the V3 loop. Identification of antibodies with potent neutralizing activity will help to elucidate the mechanisms for inducing broadly neutralizing antibodies.

4) Recombination Mediated Changes in Coreceptor Usage Confers an Augmented PATHOGENIC PHENOTYPE IN A NON-HUMAN PRIMATE MODEL OF HIV-1 INDUCED AIDS: Y. NISHIMURA, M. SHINGAI, W. R. LEE, R. SADJADPOUR, O. K. DONAU, R. WILLEY, J. M. BRENCHELEY, R. IYENGAR, A. BUCKLER-WHITE, T. IGARASHI and M. A. MARTIN

Evolution of the env gene in transmitted R5-tropic human immunodeficiency virus type 1 (HIV-1) strains is the most widely accepted mechanism driving coreceptor switching. In some infected individuals, however, a shift in coreceptor utilization can occur as a result of the reemergence of a cotransmitted, but rapidly controlled, X4 virus. The latter possibility was studied by dually infecting rhesus macaques with X4 and R5 chimeric simian simian/human immunodeficiency viruses (SHIVs) and monitoring the replication status of each virus using specific primer pairs. In one of the infected monkeys, both SHIVs were potently suppressed by week 12 postinoculation, but a burst of viremia at week 51 was accompanied by an unrelenting loss of total CD4⁺ T cells and the development of clinical disease. PCR analyses of plasma viral RNA indicated an env gene segment containing the V3 region from the inoculated X4 SHIV had been transferred into the genetic background of the input R5 SHIV by intergenomic recombination, creating an X4 virus with novel replicative, serological, and pathogenic properties. These results indicate that the effects of retrovirus recombination in vivo can be functionally profound and may even occur when one of the recombination participants is undetectable in the circulation as cell-free virus.

5) Major histocompatibility complex class I-restricted cytotoxic T lymphocyte responses during primary simian immunodeficiency virus infection in Burmese rhesus macaques: M. NAKAMURA, Y. TAKAHARA, H. ISHII, H. SAKAWAKI, M.

**HORIIKE, T. MIURA, T. IGARASHI, T. K. NARUSE, A. KIMURA, T. MATANO
and S. MATSUOKA**

Major histocompatibility complex class I (MHC-I)-restricted CD8(+) cytotoxic T lymphocyte (CTL) responses are crucial for the control of human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) replication. In particular, Gag-specific CTL responses have been shown to exert strong suppressive pressure on HIV/SIV replication. Additionally, association of Vif-specific CTL frequencies with in vitro anti-SIV efficacy has been suggested recently. Host MHC-I genotypes could affect the immunodominance patterns of these potent CTL responses. Here, Gag- and Vif-specific CTL responses during primary SIVmac239 infection were examined in three groups of Burmese rhesus macaques, each group having a different MHC-I haplotype. The first group of four macaques, which possessed the MHC-I haplotype 90-010-Ie, did not show Gag- or Vif-specific CTL responses. However, Nef-specific CTL responses were elicited, suggesting that primary SIV infection does not induce predominant CTL responses specific for Gag/Vif epitopes restricted by 90-010-Ie-derived MHC-I molecules. In contrast, Gag- and Vif-specific CTL responses were induced in the second group of two 89-075-Iw-positive animals and the third group of two 91-010-Is-positive animals. Considering the potential of prophylactic vaccination to affect CTL immunodominance post-viral exposure, these groups of macaques would be useful for evaluation of vaccine antigen-specific CTL efficacy against SIV infection.

LIST OF PUBLICATIONS

EXPERIMENTAL RESEARCH CENTER FOR INFECTIOUS DISEASES

LABORATORY OF PRIMATE MODEL

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- Morita, D., Igarashi, T., Horiike, M., Mori, N. and Sugita, M. Cutting edge: T cells monitor N-myristoylation of the Nef protein in simian immunodeficiency virus-infected monkeys. *J. Immunol.* 187, 608-612, 2011.
- Boehme, K.W., Ikizler, M., Kobayashi, T. and Dermody, T.S. Reverse genetics for mammalian reovirus. *Methods* 55, 109-113, 2011.
- Nishimura, Y., Shingai, M., Lee, W.R., Sadjadpour, R., Donau, O.K., Willey, R., Brenchley, J.M., Iyengar, R., Buckler-White, A., Igarashi, T. and Martin, M. A. Recombination-mediated changes in coreceptor usage confer an augmented pathogenic phenotype in a nonhuman

- primate model of HIV-1-induced AIDS. *J. Virol.* 85, 10617-10626, 2011.
- Reiter, D.M., Frierson, J.M., Halvorson, E.E., Kobayashi, T., Dermody, T.S. and Stehle, T. Crystal structure of reovirus attachment protein sigma1 in complex with sialylated oligosaccharides. *PLoS Pathog.* 7, e1002166, 2011.
- Nakamura, M., Takahara, Y., Ishii, H., Sakawaki, H., Horiike, M., Miura, T., Igarashi, T., Naruse, T.K., Kimura, A., Matano, T. and Matsuoka, S. Major histocompatibility complex class I-restricted cytotoxic T lymphocyte responses during primary simian immunodeficiency virus infection in Burmese rhesus macaques. *Microbiol. Immunol.* 55, 768-773, 2011.
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- Watanabe, T., Shinya, K., Watanabe, S., Imai, M., Hatta, M., Li, C., Wolter, B.F., Neumann, G., Hanson, A., Ozawa, M., Yamada, S., Imai, H., Sakabe, S., Takano, R., Iwatsuki-Horimoto, K., Kiso, M., Ito, M., Fukuyama, S., Kawakami, E., Gorai, T., Simmons, H. A., Schenkman, D., Brunner, K., Capuano, S. V., 3rd, Weinfurter, J. T., Nishio, W., Maniwa, Y., Igarashi, T. Makino, A., Travanty, E. A., Wang, J., Kilander, A., Dudman, S. G., Suresh, M., Mason, R. J., Hungnes, O., Friedrich, T. C. and Kawaoka, Y. Avian-type receptor-binding ability can increase influenza virus pathogenicity in macaques. *J. Virol.* 85, 13195-13203, 2011.
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- Iwami, S., Beauchemin, C., Tada, T., Igarashi, T., Miura, T. : Quantification system of viral dynamics in vitro -the dynamics of SHIV on HSC-F-. 8th ESMTB Conference. Poland. June 28-July 2. 2011.
- Iwami, S., Beauchemin, C., Tada, T., Igarashi, T., Miura, T. : Quantifying viral dynamics based on in vitro experiments and mathematical modeling. IUMS. Japan. September 11-16. 2011.
- 川岸崇裕、日向亮輔、加藤文博、好井健太朗、高島郁夫、三浦智行、五十嵐樹彦、小林剛 : 新規相同組換え技術による組換えダニ媒介性脳炎ウイルスの構築 第152回日本獣医学会学術集会、大阪、 2011年9月19-21日
- 仲屋友喜、小林剛、宮沢孝幸 : ウシ内在性レトロウイルスK1エンベロープタンパクの開裂に重要な領域の探索 第152回日本獣医学会学術集会、大阪、 2011年9月19-21日
- 加藤文博、日向亮輔、川岸崇裕、大石真也、藤井信孝、三浦智行、五十嵐樹彦、小林剛 : 抗デングウイルス活性を有する薬剤の探索 第18回トガ・フラビ・ペスチウイルス

ス研究会、第 18 回トガ・フラビ・ペスチウイルス研究会、東京、2011 年 11 月 11 日

川岸崇裕、日向亮輔、加藤文博、三浦智行、五十嵐樹彦、小林剛：ダニ媒介性脳炎ウイルス IR99 株の感染性 cDNA クローンの構築 第 18 回トガ・フラビ・ペスチウイルス研究会、第 18 回トガ・フラビ・ペスチウイルス研究会、東京、2011 年 11 月 11 日
日向亮輔、川岸崇裕、加藤文博、好井健太朗、高島郁夫、三浦智行、五十嵐樹彦、小林剛：フラビウイルスにおける宿主 DNA 修復機構を用いた新規リバーシジェネティクス系の開発 第 18 回トガ・フラビ・ペスチウイルス研究会、第 18 回トガ・フラビ・ペスチウイルス研究会、東京、2011 年 11 月 11 日

大附寛幸、三浦智行、小林剛、吉村和久、玉村啓和、松下修三、五十嵐樹彦：中和抵抗性のサル/ヒト免疫不全ウイルスの作製と in vitro における立体構造変化誘導剤による中和感受性増強効果の評価 第 25 回日本エイズ学会学術集会、東京、2011 年 11 月 30 日-12 月 2 日

中村碧、高原悠佑、阪脇廣美、堀池麻里子、三浦智行、五十嵐樹彦、成瀬妙子、木村彰方、俣野哲朗、松岡沙織：サルエイズモデル感染初期における MHC クラス I ハプロタイプ別の CTL 反応優位パターンの解析 第 25 回日本エイズ学会学術集会、東京、2011 年 11 月 30 日-12 月 2 日

張陰峰、五十嵐樹彦、松尾和浩、堀端重男、横溝香里、三浦智行、大橋貴、山本直樹、志田壽利：高病原性 SIV に対する組換え BCG と弱毒ワクシニア (m8Δ) エイズワクチンの防御効果 第 25 回日本エイズ学会学術集会、東京、2011 年 11 月 30 日-12 月 2 日

森田大輔、五十嵐樹彦、堀池麻里子、森直樹、杉田昌彦：Nef 蛋白質のミリスチン酸修飾をモニターする新たな免疫システム 第 25 回日本エイズ学会学術集会、東京、2011 年 11 月 30 日-12 月 2 日

CENTER FOR EMERGING VIRUS RESEARCH

1) **HIV and EBV Pathogenesis: K. SATO, N. MISAWA and Y. KOYANAGI**

Epstein-Barr virus-associated hemophagocytic lymphohistiocytosis (EBV-HLH) is a rare yet devastating disorder caused by Epstein-Barr virus (EBV) infection in humans. However, the mechanism of this disease has yet to be elucidated due to a lack of appropriate animal models. Here, we utilized a human CD34⁺ cell-transplanted humanized mouse model and reproduced pathological conditions resembling EBV-HLH in humans. By 10 weeks postinfection, two thirds of the infected mice died after exhibiting high and persistent viremia, leukocytosis, IFN- γ cytokinemia, normocytic anemia, and thrombocytopenia. EBV-infected mice also showed systemic organ infiltration by activated CD8⁺ T cells and prominent hemophagocytosis in bone marrow, spleen, and liver. Notably, the level of EBV load in plasma correlated directly with both the activation frequency of CD8⁺ T cells and the level of IFN- γ in plasma. Moreover, high levels of EBER1 were detected in plasma of infected mice, reflecting what has been observed in patients. These findings suggest that our EBV infection model mirrors virological, hematological, and immunopathological aspects of EBV-HLH. Furthermore, in contrast to CD8⁺ T cells, we found a significant decrease of NK cells, MDCs, and PDCs in spleen of infected mice, suggesting that the collapse of balanced immunity associates with the progression of EBV-HLH pathogenesis.

2) **Role of cell surface proteases in the outer membrane protein assembly: S. NARITA and Y. AKIYAMA¹** (¹Department of Viral Oncology, IVR)

The aim of research in this group is to clarify the survival strategy of gram-negative bacteria. Various species in this phylum have been identified as causative microorganisms of many infectious diseases. It is of great importance, therefore, to understand their survival strategy to cope with emerging infectious diseases. A characteristic feature of the gram-negative bacteria's cell structure is the presence of the outer membrane surrounding the cytoplasmic membrane and the periplasmic space. These envelope structure functions as a permeability barrier against toxic compounds and serves to maintain homeostasis of the periplasm and cytoplasm. Because the outer membrane is essential for the growth of gram-negative bacteria, knowledge of the biosynthesis, assembly and quality control systems of the outer membrane components would contribute to development of new drugs against gram-negative pathogenic bacteria. We study these systems using *Escherichia coli*, the model organism that has ever been most extensively studied.

The σ^E stress response system senses misfolded outer membrane proteins (OMPs) in the periplasmic space and regulates expression of a set of genes that function to cope with envelope stresses. Upon activation of σ^E , expression of genes for periplasmic chaperones/proteases and

components of the machineries for OMP and lipopolysaccharide assemblies are up-regulated while those for OMPs are down-regulated, both contributing to reducing the threat to periplasmic accumulation of misfolded OMPs. Although many genes have been identified as constituents of the σ^E regulon, their functions are still not fully understood. We characterized *yfgC*, a σ^E -regulated gene encoding a putative periplasmic protease. An *E. coli* $\Delta yfgC$ mutant showed increased sensitivity to detergents and antibiotics, suggesting that the loss of the *yfgC* function compromises integrity of the outer membrane. Consistently, we found that folding of LptD, an outer membrane protein involved in the transport and assembly of lipopolysaccharide to the cell surface, became defective in this strain. The defective outer membrane function caused by the $\Delta yfgC$ mutation was further aggravated by additional disruption of genes encoding periplasmic chaperones or subunits of the BAM complex that are required for assembly of outer membrane proteins. These results suggest that YfgC assists proper assembly of outer membrane proteins.

LIST OF PUBLICATIONS

CENTER FOR EMERGING VIRUS RESEARCH

1) First Group

- Sato, K., Koyanagi, Y. The mouse is out of the bag: insights and perspectives on HIV-1-infected humanized mouse models. *Exp Biol Med*, 236: 977-985, 2011.
- Sato, K., Misawa, N., Nie, C., Satou, Y., Iwakiri, D., Matsuoka, M., Takahashi, R., Kuzushima, K., Ito, M., Takada, K., Koyanagi, Y. A novel animal model of Epstein-Barr virus-associated hemophagocytic lymphohistiocytosis in humanized mice. *Blood*, 117: 5663-5673, 2011.
- Kobayashi, T., Ode, H., Yoshida, T., Sato, K., Gee, P., Yamamoto, S.P., Ebina, H., Strebel, K., Sato, H., Koyanagi, Y. Identification of amino acids in the human tetherin transmembrane domain responsible for HIV-1 Vpu interaction and susceptibility. *J. Virol*, 85: 932-945, 2011.
- 佐藤佳, 小柳義夫. ヒト化マウスの利用: EBウイルス関連血球貪食性リンパ球組織球症動物モデル. *血液内科*, 63(6), 627-633, 2011.

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- Sato K, Misawa N, and Koyanagi Y. Dynamics of human-specific virus infection in humanized mice, T lymphocyte dynamics in acute and chronic viral infection – Infectious Disease Research Network, poster #23, London, England, 2011 年 1 月 24 日
- Iwami S, Sato K, Misawa N, Kobayashi T, De Boer R, Koyanagi Y, DNA labeling system by peripheral blood of humanized mouse, T lymphocyte dynamics in acute and chronic viral

infection, London, England, 2011 年 1 月 24 日

Iwami S, Sato K, Misawa N, Kobayashi T, De Boer R, Koyanagi Y, DNA labeling system by peripheral blood of humanized mouse, The 1st International Symposium on Innovative Mathematical Modeling, P-215, Tokyo, 2011年3月1日

佐藤佳. 生体内 HIV-1 複製における Vpr タンパク質の意義, 第 13 回白馬シンポジウム, oral #9, 札幌, 2011 年 5 月 19 日

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佐藤佳, 三沢尚子, 小柳義夫. ヒト化マウスモデルを用いた HIV-1 感染病態の解析, 第 25 回近畿エイズ研究会, 京都, 2011 年 6 月 18 日

Iwami S, Sato K, Koyanagi Y, Mathematical modeling and *in vitro* experiments in virology, Korean Society for Mathematical Biology 2011 annual meeting, Ulsan, Korea, 2011年8月25日.

Sato K, Misawa N, Ito M, and Koyanagi Y. HIV-1 Vpr protein accelerates HIV-1 replication during acute phase in vivo, XV International Congress of Virology, VI-PO21-12, 札幌, 2011 年 9 月 13 日

岩見真吾, 佐藤佳, Rob de Boer, 小柳義夫, 細胞ダイナミクスの定量化 - 動物実験と数理解析の融合 -, 日本数学会2011年度秋季総合分科会, 長野, 2011年10月1日

Sato K, Misawa N, Ito M, and Koyanagi Y. HIV-1 Vpr protein accelerates HIV-1 replication during acute phase in vivo, 3rd International Workshop on Humanized mice, Pittsburgh, USA, 2011 年 10 月 29 日

Iwami S, Sato K, Koyanagi Y, Mathematical modeling of wild type and Vpr -deleted HIV-1 infection *in vitro*, CBI/JSBi (情報計算法学生物学会/日本バイオインフォマティクス学会合同大会) 2011, 神戸, 2011年11月8日

佐藤佳, 三沢尚子, 佐藤賢文, 松岡雅雄, 伊藤守, 小柳義夫. 急性感染期のHIV-1増殖における制御性T細胞とVprの寄与, 第25回日本エイズ学会, WS1-006, 東京, 2011年12月2日

岩見真吾, 佐藤佳, 小柳義夫, 培養細胞実験の数理解モデリングを用いたウイルスダイナミクスにおけるVpr機能の解明, 第25回日本エイズ学会, 東京, 2011年12月2日

2) Second Group

Narita, S., Tokuda, H. Overexpression of LolCDE allows the deletion of the *Escherichia coli* gene encoding apolipoprotein N-acyltransferase. J. Bacteriol., 193: 4832-4830, 2011.

Narita, S. ABC transporters involved in the biogenesis of the outer membrane in gram-negative bacteria. Biosci. Biotechnol. Biochem., 75: 1044-1054, 2011.

埜和之, 成田新一郎, 徳田元. Rcsリン酸リレー系によるリポ蛋白質特異的シャペロンLolAの発現制御, 日本農芸化学会2011年度大会, 3C26p07, 京都, 2011年3月27日

成田新一郎. 大腸菌リポ蛋白質の外膜局在化に関わるABCトランスポーターの解析, 2010年度国立遺伝学研究所研究会「単細胞システムの細胞構築と増殖制御の研究」, 三島, 2011年3月31日

成田新一郎, 秋山芳展. 大腸菌表層タンパク質の品質管理に関わる新規プロテアーゼホモログの解析, 第8回21世紀大腸菌研究会, 南木曾, 2011年5月19日

REPRODUCTIVE ENGINEERING TEAM

Reproductive engineering team is a support unit for generating transgenic mouse (Tg) and knockout mouse (KO) under the animal committee of our institute. We also perform cryopreservation of mouse fertilized eggs. Current staffs are Kitano and Miyachi. Results of last three years are as follows.

1) Freezing embryos

2009	75 strains	20,337 embryos
2010	101 strains	18,620 embryos
2011	117 strains	25,130 embryos

2) Introduction of mouse strains from outside

	Frozen embryos	Live mice
2009	7 strains	2 strains
2010	4 strains	6 strains
2011	1 strain	3 strains

3) Transgenic mouse production with cloned DNAs

	No of constructs	No of embryos injected	No of transgenic pups obtained
2009	94	33,821	190 (0.6%)
2010	90	32,875	124 (0.3%)
2011	81	29,031	227 (0.8%)

4) Production of chimeric mouse

	No of ES clones	No of embryos injected	No of coatcolor chimera obtained
2009	52	4,587	242 (5.3%)
2010	106	7,106	394 (5.5%)
2011	107	5,828	324 (5.5%)

LIST OF PUBLICATIONS

REPRODUCTIVE ENGINEERING TEAM

Takashima, Y., Ohtsuka, T., González, A., Miyachi, H., Kageyama, R. Intronic delay is essential for oscillatory expression in the segmentation clock. *Proc. Natl. Acad. Sci. USA* . 108, 3300-3305, 2011

Niwa, Y., Shimojo, H., Isomura, A., González, A., Miyachi, H., Kageyama, R. Different types of oscillations in Notch and Fgf signaling regulate the spatiotemporal periodicity of somitogenesis. *Genes & Dev.* 25, 1115-1120, 2011

Yamazaki, D., Tabara, Y., Kita, S., Hanada, H., Komazaki, S., Nitou, D., Mishima, A., Nishi, M., Yamamura, H., Yamamoto, S., Kakizawa, S., Miyachi, H., Yamamoto, S., Miyata, T., Kawano, Y., Kamide, K., Ogihara, T., Hata, A., Umemura, S., Soma, M., Takashashi, N., Imaizumi, Y., Miki, T., Iwamoto, T., Takeshima, H. TRIC-A Channels in Vascular Smooth Muscle Contribute to Blood Pressure Maintenance. *Cell Metabolism*.14, 231-241, 2011

COMPUTER NETWORK OF INSTITUTE FOR VIRUS RESEARCH

Institute for Virus Research LAN system (IVR-LAN) has administrated by the network committee consisted of four staffs (Prof. Toyoshima, Prof. Akiyama, Associate Prof. Mori and Instructor Takemoto). IVR-LAN service has covered for researchers of some medical departments as well as IVR, and the primary purpose of IVR-LAN is to offer accessibility to the Internet in support of their studies. IVR-LAN has provided a variety of network services, including E-Mail, WEB-mail, WWW, File-sharing, SSH and all Outgoing TCP services except for P2P. Main services are working on Sun Sparc platform with Solaris 10 and DELL with Linux.

In an effort to make net life as smooth as possible, we replaced old pop server with Linux platform new ones to save NFS conflicts and improve response time. Next, we created the online room booking system. All IVR-LAN users can check the availability of the seminar rooms or make a booking on WEB page. This year we created a subnet for isolating some computers which were used with experimental equipments and subjected to computer virus infection. The subnet ensured a more secure network for us. User's sample files are saved in a NAS of the subnet, that protected by an anti-virus application, and users who login IVR-LAN could access via router to the NAS only .

However IVR-LAN has adequately equipped, we must have a responsibility for sending/getting data. A few accidents have occurred in this year. IVR-LAN users need to get certifications of training of e-learning course which is provided by Institute for Information Management and Communication of Kyoto university.

In addition to the administration of network, Takemoto began to analyze RNA-Seq and ChIP-Seq coupled with high throughput DNA sequencing to find epigenetic changes which might be controlled during differentiation.

STAFF CHANGES OF THE INSTITUTE

Appointments

During the period of January to December 2011, the following new staffs were appointed; Dr. Keizo Tomonaga as a Professor of Department of Viral Oncology, Dr. Yasushi Kawaguchi as a Visiting Associate Professor of Department of Biological Responses, Dr. Momoko Maekawa as an Assistant Professor of Department of Cell Biology, Dr. Tomoyuki Honda as an Assistant Professor of Department of Viral Oncology, Dr. Kazuya Shimura as an Assistant Professor of Center for Human Retrovirus Research, Drs. Kenji Nakahigashi, Yasuhiko Horiguchi, Sho Yamasaki and Yasuhito Tanaka as a Lecturer (part time) of Department of Viral Oncology, Drs. Kazufumi Matsushita and Yutaro Kumagai as a Lecturer (part time) of Department of Genetics and Molecular Biology, Dr. Yoshiyuki Suzuki as a Lecturer (part time) of Department of Cell Biology, Drs. Yoichiro Iwakura, Osamu Takeuchi, Tatsuo Shioda, Yukihiro Nishiyama, Hirofumi Akari, Tsuneo Morishima and Tatsuya Tsurumi as a Lecturer (part time) of Center for Human Retrovirus Research, Drs. Ikuo Wada and Koki Taniguchi as a Lecturer (part time) of Experimental Research Center for Infectious Diseases.

Departure

Drs. Katsuji Sugie, Toru Kiyono, Yasuhito Tanaka, Yoshiharu Matsuura, Hisashi Arase, Junji Takeda, Hiroaki Takeuchi, Yusuke Yanagi, Takeshi Noda, Kyoko Shinya, Michinori Kohara and Koichi Morita left the Institute. 2011

THE SCIENTIFIC LECTURES OF THE INSTITUTE FOR VIRUS RESEARCH

The annual scientific lecture of this Institute was held on July 5, 2011 at the Kyoto University Shirankaikan Yamauchi Hall.

Program

Opening Remarks: Masao Matsuoka

1. Bornavirus: A new development of RNA virus research, Keizo Tomonaga, this Institute
2. Measles virus: Towards better understanding of virus-induced membrane fusion and establishment of a new mouse model for measles, Yusuke Yanagi, Kyushu University
3. The roles of histone lysine methylation in biological processes, Yoichi Shinkai, this Institute
4. The roles of cohesin acetylation; Identification of Hdac8 mutations in Cornelia de Lange syndrome patients, Katsuhiko Shirahige, The University of Tokyo

SEMINARS OF THE INSTITUTE FOR VIRUS RESEARCH

Eighteen seminars were held at the Institute for Virus Research under the auspices of the Institute in 2011. Nine lectures were from abroad and nine others were from Japan.

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| February 25 | Dr. Masanobu Satake, Tohoku University, Japan. “ Involvement of Runx transcription factor in the maintenance of T lymphocytes naivety ”. |
| March 8 | Dr. Ruth Sperling, The Hebrew University, Israel . “ Pre-mRNA splicing - a network of interactions within the pre-mRNA processing machine ”. |
| April 4 | Dr. Yoshihisa Yamano, St. Marianna University, School of Medicine, Japan. “ Dysregulation of immune system in HAM/TSP ”. |
| May 25 | Dr. Kazunari Miyamichi, Stanford University, USA. “ Cortical representations of olfactory input by transsynaptic tracing ”. |
| June 1 | Dr. Tomoharu Sugiyama, University of Tsukuba, Japan. “ mRNA decay in meiosis - A evolutionarily conserved mechanism for differentiation repression? ”. |
| June 6 | Dr. Yasuyuki Fujita, Hokkaido University, Japan. “ Interface between normal and transformed epithelial cells ”. |
| June 14 | Dr. Katsura Asano, Kansas State University, USA. “ Translation regulation mechanisms by initiation factors eIF4G and eIF3e/Int6 ”. |
| June 24 | Dr. Shigeki Yoshiura, RIKEN, Japan. “ Non-cell-autonomous control of the orientation of stem cell polarity and divisions ”. |

- June 28 Dr. Masahiro Yamashita, The Aaron Diamond AIDS Research Center, USA “ Hiv-1 infection of non-dividing cells ”.
- July 14 Dr. Kosuke Miyauchi, RIKEN, Japan. “ Mechanism of HIV entry and a novel eliminating system for HIV-infected cells by activation of CASP3 ”.
- July 29 Dr. Kenji Nakahigashi, Keio University, Japan, “ Systematic phenome analysis of *Escherichia coli* multiple-knockout mutants reveals hidden reactions in central carbon metabolism ”.
- September 7 Dr. John L.R. Rubenstein, University of Carifornia, USA.
“ Transcriptional control of interneuron development ”.
- September 9 Dr. Carol A. Gross, University of California, USA “ Using systems approaches to dissect central bacterial cellular processes ”.
- October 5 Dr. Dong-Yan Jin, The University of Hong Kong, China. “ Roles of group I p21-activated kinases and LKB1/SIK1 kinases in Tax-mediated activation of human T cell leukemia virus type 1 long terminal repeats ”.
- October 14 Dr. Yasuhiko Horiguchi, Osaka University Japan. “ Attempts to understand how pathogenic bacteria of the genus *Bordetella* exert specific pathogenicity in specific host ”.
- October 24 Dr. Mineki Saito, University of the Ryukyus, Japan. “ Pathogenesis of neurotoxicity by chronic viral infection ”.
- November 16 Dr. Christos Delidakis, University of Crete, Greece. “ DSL protein ubiquitylation and signalling in *Drosophila* ”.

December 22 Dr. Tomomi Kiyomitsu, Whitehead Institute, USA. “ Chromosome and spindle pole-derived signals generate an intrinsic code for spindle position and orientation ”.