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FOR VIRUS RESEARCH 2012**

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Cover:

Borna disease virus (BDV)-infected Vero cells stained by anti-BDV envelope glycoprotein (G) antibody (red). The cells were visualized by fluorescence and DIC microscopy (Top). BDV particles captured by electron microscopy (middle). The cerebellum of transgenic mouse expressing BDV P protein in glial cells. The brain section was stained by anti-calbindin (green) and BDV P (red) antibodies (bottom, left). The actin filaments of BDV-infected C6 cells were visualized with fluorescently labeled phalloidin (bottom, right).

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.

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.
2010 April	Research Center for Emerging Virus was reorganized to become Center for Emerging Virus Research.

ORGANIZATION AND STAFF

(as of December, 2012)

(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)
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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) Asako McCloskey (2012)

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

Professor	Osamu Takeuchi, M.D., Ph.D. (2012)
Associate Professor	Hiroshi Masutani, M.D., D.Med.Sc. (1992)

Assistant Professor	Takashi Mino, D.Eng. (2012)
Bioresponse Regulation Laboratory	
Visiting Professor	Yoshihiro Kawaoka, D.V.M., D.Med.Sc. (2010)
Visiting Assistant Professor	Takao Masuda (2012)

Department of Cell Biology

Laboratory of Subcellular Biogenesis

Professor	Fumiko Toyoshima, D.Sc. (2008)
Assistant Professor	Shigeru Matsumura, D.Bio. (2008)
	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)
Associate Professor (Spe.)	Itaru Imayoshi, D.Bio. (2008)
Assistant Professor	Taeko Kobayashi, D.Sc. (2005)
Assistant Professor (Spe.)	Tomoko Tateya, M.D., D.Med.Sc. (2008)

Laboratory of Signal Transduction

Associate Professor	Takayuki Miyazawa, D.V.M., D.Vet.Med. (2005)
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Laboratory of Regulatory Information

Visiting Professor	Susumu Tonegawa, Ph.D, D.Sc. (1992)
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Center for Human Retrovirus Research

Laboratory of Viral Pathogenesis

Professor	Yoshio Koyanagi, M.D., D.Med.Sc. (2004)
Assistant Professor	Hirofumi Ebina, D.Med.Sc. (2009)
	Kei Sato, D.Med.Sc. (2012)

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)
	Kazuya Shimura, D.Med.Sc. (2011)

Laboratory of Viral Immunology

Visiting Professor	Hiroaki Mitsuya, M.D., Ph.D. (2012)
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Experimental Research Center for Infectious Diseases

Laboratory of Mouse Model

Associate Professor	Makoto Tachibana, D.Agr. (1998)
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Laboratory of Primate Model

Professor	Tatsuhiko Igarashi, D.V.M., D.Med.Sc. (2007)
Associate Professor	Tomoyuki Miura, D.V.M., D.Agr. (1988)

Center for Emerging Virus Research

Head • Professor	Yoshio Koyanagi, M.D., D.Med.Sc. (2010)
Assistant Professor (Spe.)	Shin-ichiro Narita, D.Sc. (2010)
	Yosuke Yamaoka, Pharm.D. (2012)
	Akiko Makino, D.V.M.Ph.D (2012)

Lecturers (part time)

Kenji Inaba
 Tohru Minamino
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 Naoto Ito
 Kouji Moriishi
 Yosiharu Matsuura
 Kouichi Watashi
 Gen Yamada

Fumitoshi Ishino
Akio Adachi
Eiji Morita
Toshiki Watanabe
Shinichi Oka

Library

Committee Chairman

Hiroyuki Mori, D.Sc. (1996)

Administration Office

Chief Officer
General Affairs
Finance

Kazumi Inui (2010)
Hiroyuki Matsunaga (2011)
Satoshi Matsushita (2011)

Research Fellows

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Fumihiko Kato	Exp. Res. Cen. Inf. Dis. (Lab. Primate Model)
Takahiro Kawagishi	Exp. Res. Cen. Inf. Dis. (Lab. Primate Model)
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Miyuki Nishi	Dept. Cell Biol. (Lab. Growth Regul.)
Kyoko Hirano	Dept. Cell Biol. (Lab. Growth Regul.)
Yuki Maeda	Dept. Cell Biol. (Lab. Growth Regul.)
Mitsuko Fukuhara	Ctr. HuRetro. Res. (Lab. Viral Pathogenesis)
Naoki Sono	Ctr. HuRetro. Res. (Lab. Virus Control.)
Yuichi Mitobe	Ctr. HuRetro. Res. (Lab. Virus Control.)
Haruka Kinosada	Ctr. HuRetro. Res. (Lab. Virus Control.)
Katsuaki Deguchi	Exp. Res. Cen. Inf. Dis. (Lab. Mouse Model)
Mayuko Inoue	Exp. Res. Cen. Inf. Dis. (Lab. Mouse Model)

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Research Activities

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) Identification of a substrate contact site in SecD by site-directed *in vivo* photo-cross-linking: K. MITO, Y. MACHIDA, T. TSUKAZAKI¹, O. NUREKI¹, K. ITO², Y. AKIYAMA and H. MORI (¹Univ. Tokyo, ²Kyoto Sangyo Univ.)

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 3.3 Å resolution and proposed a working hypothesis based on structure-instructed biochemical and biophysical studies. According to the model, 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) to facilitate forward movement of the polypeptide (1). However, the mode of interaction between SecDF and Sec-related factors including SecYEG remain largely unknown. To gain information on this issue, we performed systematic site-directed *in vivo* photo-cross-linking analysis targeted to *E. coli* SecD and identified SecD-amino acid residues, located in close proximity to the P1 head sub-domain in a SecD conformational state termed form I, to SecF or to periplasmic chaperones (DegP and Skp) (See the last issue of Annual Report of IVR).

To examine the effect of the PMF on molecular interactions involving SecD, we carried out *in vivo* photo-cross-linking experiments using $\Delta atpE$ mutant cells. Because this strain lacks functional F₀F₁ ATPase, CCCP (a proton ionophore) treatment of the cells specifically collapses the PMF across the cytoplasmic membrane without affecting intracellular ATP concentrations. Under such conditions, we observed dramatic reduction in a product of intra-molecular cross-linking that takes place when the P1 domain is in the form I conformational state as well as in some of SecD-SecF cross-linked products formed within the intramembrane interfaces. These results nicely support the notion that the movement of the P1 domain is coupled with SecDF-mediated conductance of protons. We also observed that some of cross-linked adducts formed normally between the P1 domain of SecD and the periplasmic chaperones were reduced markedly by CCCP treatment, suggesting that the

SecDF P1 domain interacts with the periplasmic chaperones in PMF-dependent manners.

Interestingly, we noted a maltose-dependent crosslinking involving SecD (Ser295pBPA). Using strains deleted for a series of the *mal* operon genes and antibodies against maltose-binding protein (MBP), we were able to show that this crosslinking had occurred between SecD and MBP. Although it remains to be shown whether this crosslinking represents an event of translocation of newly synthesized MBP, our results raise a possibility that the region near the position 295 in P1 domain forms a path for translocating substrate polypeptide chains.

(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) Up-regulation of V. SecD2 expression by protein export defect in *Vibrio alginolyticus*: H. MORI, N. HASHIMOTO, S. KOJIMA¹, M. HOMMA¹ and Y. AKIYAMA (¹Nagoya Univ.)

Marine bacteria *Vibrio* species contain two sets of *secDF* genes, designated *V.secDF1* (more closely related to the *E. coli secDF* genes) and *V.secDF2* and these gene products seem to utilize Na⁺ and H⁺ to support effective protein export, respectively. Immunoblotting analysis using specific antibodies against the V. SecD paralogues reveals constitutive expression of V.SecD1 and regulated expression of V.SecD2 in *Vibrio alginolyticus* VI05 cells. The V.SecD2 expression is dramatically induced by reduction of Na⁺ concentration and/or acidification in the growth medium (See the last issue of Annual Report of IVR).

To understand physiological significance of V.SecD2 and the molecular mechanism of the V.SecD2 expression, we constructed a $\Delta V.secDF2$ mutant strain and a V.SecDF1 depletable-strain in which the *V.secDF1* genes were placed under the control of arabinose promoter on the chromosomal DNA and examined their *in vivo* growth phenotypes and their protein export abilities. Deletion of *V.secDF2* genes resulted in severe decrease in protein export activity that coincided with the growth retardation by decreasing Na⁺ concentration in the medium, suggesting that V.SecDF2 plays a crucial role in protein export when the Na⁺ motive force across the cytoplasmic membrane is limited. Surprisingly, we found that depletion of V.SecDF1 affected neither protein export nor cell growth. The V.SecDF1 depletion allowed the *Vibrio* cells to accumulate V.SecD2 protein even under the condition at which V.SecD2 expression in the wild type cell was tightly repressed. Furthermore, we strikingly observed drastic induction of V.SecD2 by NaN₃ (a specific SecA ATPase inhibitor) treatment of the wild type *Vibrio* cells. These results indicate that V.SecD2 expression is induced by reduction of cellular ability for protein export. Now we are trying to clarify the molecular mechanism how *Vibrio* cells monitors cellular activity for protein export and up-regulates V.SecD2 expression.

3) Construction and Characterization of a $\Delta VsecDF1$ Strain: N. HASHIMOTO, S. KOJIMA¹, M. HOMMA¹, Y. AKIYAMA and H. MORI (¹Nagoya Univ.)

As we described in the previous paragraph, the V.SecDF1-depletable strain exhibited no apparent defective phenotypes even under the depletion condition, strongly suggesting that V.SecDF1 is dispensable for cell viability. To prove our consideration, we decided to construct a *VsecDF1* deletion strain. First, we constructed a plasmid carrying both 5' and 3' flanking regions (about 1 kbp) of *VsecDF1* genes between which *Km^r* gene was sandwiched, and obtained a kanamycin resistant *Vibrio* cell by a double cross-over homologous recombination. Disruption of *VsecDF1* genes was confirmed by DNA sequencing of its genomic DNA and immunoblotting analysis of the obtained strain using the anti-V.SecD1 antibody. We found that the *VsecDF1* deletion strain grew healthy under various culture conditions including reduction of Na⁺ concentration and/or acidification, indicating that V.SecDF1 is not essential for cell growth. Immunoblotting analysis of the $\Delta VsecDF1$ strain showed that V.SecD2 protein stably accumulated in all experimental conditions that we examined. These results are consistent with our idea that V.SecDF2 expression is up-regulated when cellular function for protein export is compromised.

4) Roles of the PDZ domains and their ligand-bindings in the proteolytic function of RseP, a key regulator involved in the *E. coli* extracytoplasmic stress response: Y. HIZUKURI, T. SUZUKI¹, N. DOHMAE¹ and Y. AKIYAMA (¹RIKEN)

The *Escherichia coli* σ^E extracytoplasmic stress response monitors and responds to folding stress in the cell envelope. A protease cascade directed at RseA, a membrane-spanning anti- σ that inhibits σ^E activity, controls this critical signal-transduction system. Stress cues activate DegS to cleave RseA; a second cleavage by RseP releases RseA from the membrane, enabling its rapid degradation. Stress control of proteolysis requires that RseP cleavage is dependent on DegS cleavage. From our previous works the proteolytic function of RseP is speculated to be controlled by its two PDZ domains in the periplasmic region through binding of a still unknown-ligand.

Recent *in vitro* and structural studies found that RseP cleavage requires binding of RseP PDZ-C to the newly exposed C-terminal residue (Val148) of RseA, generated by DegS cleavage, explaining dependence (1). We tested this mechanism *in vivo*. Neither mutation in the putative PDZ ligand-binding regions nor even deletion of entire RseP PDZ domains had significant effects on RseA cleavage *in vivo*, and the C-terminal residue of DegS-processed RseA also little affected RseA cleavage. Indeed, strains with a chromosomal *rseP* gene deleted for either PDZ domain and strains with a chromosomal *rseA* V148 mutation grew normally and exhibited almost normal σ^E activation in response to stress signals. We concluded that recognition of the cleaved amino acid by the RseP PDZ

domain is not essential for sequential cleavage of RseA and σ^E stress response *in vivo* (2).

To identify possible ligands of the RseP PDZ domains, we carried out site-directed *in vivo* photo-cross-linking experiments targeted against the RseP PDZ domains. We detected some cross-linked products upon irradiation with UV, when a photo-reactive amino acid analog (pBPA) was introduced into the putative ligand-binding region of PDZ-N. We purified these cross-linked products by immobilized metal ion affinity chromatography using polyhistidine-tagged RseP. LC-MS/MS analysis of the purified cross-linked products identified some periplasmic proteins as the candidates for cross-linking partners. We are now investigating the possible interaction between RseP and these periplasmic proteins and its physiological meaning in the RseP function.

(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) Hizukuri, Y., and Akiyama, Y. (2012) Mol. Microbiol., 86, 1232-1245.

5) Analysis of a membrane-inserted β -hairpin motif of RseP, the S2P family intramembrane protease of *E. coli*: K. AKIYAMA, T. NOGI¹ and Y. AKIYAMA (¹Yokohama City Univ.)

The S2P family of intramembrane proteases plays critical roles in transmembrane signaling from bacteria to higher eukaryotic cells. However, the molecular mechanism of substrate recognition and intramembrane proteolysis remains largely unknown.

Sequence comparison of the S2P family proteases suggests that they share a core domain composed of three transmembrane segments (TMSs); the first and the third TMSs of the core domain contain the HEXxH metalloprotease motif and the conserved Asp residue, respectively, which together constitute a proteolytic active site. Recent elucidation of the X-ray crystal structure of archaeal S2P protease (mjS2P) (1) revealed that a hydrophobic region in the cytoplasmic domain between the first and the second TMS of the core domain loops into the membrane as a β -hairpin structure. Interestingly this unique structure appears to be conserved among S2P proteases including *E. coli* RseP, *B. subtilis* SpoIVFB and mammalian S2P. We are now investigating possible roles of this β -hairpin loop in the proteolytic function of RseP.

(1) Feng, L. Yan, H., Wu, Z., Yan, N., Wang, Z., Jeffrey, P. D., and Shi, Y. (2007) Science, 318, 1609-1612.

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 (¹Kyoto Sangyo Univ.)

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 particle (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 SRP, FtsY, SecYEG, FtsH and chaperones. In order to identify proteins interacting with region 2.1 *in vivo*, we employed a 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 have tried to identify partner proteins of the observed cross-linked products. Our results showed that some cross-linked products contained DnaJ, DnaK and Ffh (the protein component of SRP), suggesting that these proteins interact with region 2.1 or its neighboring region *in vivo*.

(1) Yura, T. Guisbert, E., Poritz, M., Lu, C. Z. Campbell, E., and Gross, C. A. (2007) Proc. Natl. Acad. Sci, USA, 104, 17638-17643.

7) Mechanism by which toxin-antitoxin systems confer suppression of σ^E essentiality in *Escherichia coli*: Y. DAIMON, S. NARITA¹ and Y. AKIYAMA (¹Center Emerg. Virus Res., 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.

The toxin-antitoxin (TA) system is a set of two closely linked genes that together encode a toxin and a cognate antitoxin. TA systems are ubiquitous in bacterial chromosomes and have versatile roles such as stabilization of genomes, regulation of programmed cell death, persister cell or biofilm formation, and stress responses. Deletion of *hicB*, a gene for an antitoxin of a TA system, has been

reported to suppress the lethality of the *rpoE*-null mutation (1). However, the mechanism of the suppression remains an enigma. In this study, using a transcriptome profiling, we revealed that the *hicB*-null mutation caused down-regulation of the genes involved in stress-defense. We also found an *E. coli* strain in which the lethality of *rpoE*-null mutation is not suppressed by *hicB*-null mutation. These findings should provide us with a deeper understanding of the suppression mechanism of the σ^E essentiality by TA system.

(1) Button, J. E., Silhavy, T. J., and Ruiz. N. 2007. J. Bacteriol. 189:1523-1530.

8) *In vitro* analysis of YfgC, a putative periplasmic protease of *Escherichia coli*: C. MASUI, S. NARITA¹, Y. AKIYAMA (¹Center Emerging Virus Res., IVR)

To maintain the function of the outer membrane as a permeability barrier with selectivity, gram-negative bacteria are equipped with quality control systems that sense and combat against defects of outer-membrane constituents. When misfolding of outer membrane proteins occurs, σ^E pathway, one of the extracytoplasmic stress response system, is activated, which results in the activation of σ^E and the transcription of multiple genes involved in the extracytoplasmic stress responses. We characterized a member of the σ^E regulon, *yfgC*, which encodes a putative periplasmic protease.

The *Escherichia coli* $\Delta yfgC$ mutant showed increased sensitivity to detergents and antibiotics. Accordingly, proper folding of LptD, which is involved in the transport and assembly of lipopolysaccharide to the outer membrane, was also affected. These results indicate that YfgC may contribute to maintain the quality of the outer membrane by governing the assembly of outer membrane proteins. However the substrate of YfgC has not been identified yet, and *in vitro* analysis of YfgC has not been performed. So the purposes of this study are to analyze chemical properties of YfgC *in vitro* and to identify the substrate of YfgC.

First, to prepare pure YfgC at high concentration for *in vitro* assays, we overexpressed His-tagged YfgC in *E. Coli* from a plasmid, converted cells into spheroplasts, and subjected the periplasm fraction to Ni-NTA or TALON metal affinity chromatography. As a result, we could purified YfgC to near homogeneity. We also purified YfgC(E137Q), a variant in which glutamate at the putative protease active-site of YfgC was replaced by glutamine, for the control experiments. With these purified proteins, we are analyzing the protease activity of YfgC.

9) Structures of the cytosolic region of ATP-dependent protease FtsH with ADP-Aluminum fluoride and AMPPNP: R. SUNO, T.SHIMAMURA¹, T. HINO¹, A. ABE, Y.-H. WATANABE², N. SHIMODATE³, Y. AKIYAMA, S. IWATA¹ and M. YOSHIDA⁴ (¹Kyoto Univ., ²Konan Univ., ³Tokyo Inst. Tech., ⁴Kyoto Sangyo Univ.)

AAA+ protease are involved in various cellular activities including the protein quality control. Most of AAA+ protease can form the hexameric form and translocate a polypeptide through the central pore into the chamber of the protease complex where protease catalytic sites exist at the surface of the chamber wall. The translocation is coupled with the ATPase cycle, accompanying large conformational change of the AAA+ complex. FtsH is the member of AAA+ super family, and is membrane-bound protein. Previously, we showed the crystal structure of hexameric cytosolic region of FtsH bound ADP, and the structural information revealed that FtsH had the putative polypeptide translocation tunnel but the proteolytic chamber. We proposed the novel model of the ATP-dependent substrate translocation mechanism. Here, We newly solved the structure of soluble region of FtsH binding to ADP-aluminum fluoride or AMPPNP to 3.5 Å or 3.7 Å, respectively. We reported that FtsH could form the two kinds of conformations, the open and closed form, and we also proposed that FtsH could be reciprocated between the open form and the closed form. The superimposition of these structures showed that the relative orientation of the AAA+ domains against the protease domain of FtsH bound to ADP • AlFx or AMPPNP was the intermediate state between the open and the closed form. It was conceivable that ATPase domain moved to the protease domain and FtsH became the half-closed form after ATP binding. Some of AAA+ proteins were reported to be able to degrade the substrate polypeptide with ATP binding. In fact, FtsH could also degrade the unfolded substrate protein in the presence of AMPPNP, ATPγS, but not ADP. Our previous model of the substrate polypeptide degradation mechanism proposed that one subunit of FtsH sent the substrate polypeptides into the protease catalytic site of the adjacent subunit with ATP hydrolysis. But, this movement of FtsH with ATP binding permitted to send the substrate polypeptide into the protease catalytic site of the adjacent subunit, if the substrate polypeptide bound to FtsH in the open form. We revised the model of the ATP-dependent protease mechanism in the consideration of the new structures, and will discuss about the model in the session.

10) Biochemical analysis of the polypeptide-translocating mechanism of ATP-dependent Protease FtsH: R. SUNO, M. SHIMOYAMA¹, A. ABE, N. SHIMODATE¹, Y. AKIYAMA and M. YOSHIDA² (¹Tokyo Inst. Tech. ²Kyoto Sangyo Univ.)

The previous 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 Glycine 448. We generated a series of mutants that reduce propensity to form a kink in the lid helix. Most of these mutants retain small but sizable ATPase activities, bind the substrate protein normally, but lose protease activity, suggesting that the kink formation of the lid helix is essential for the function of FtsH.

II. Second Group

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

I found that Keratin associated protein (Krtap) 13 binds to LRP6, a co-receptor for Wnt. Surprisingly, Krtap13 overexpression markedly stimulates Wnt signaling. Krtap13 induces 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 mice 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 vivo. From crossing between Krtap13-Tg mice and keratin5-Cre Tg mice, skin-specific expression of Krtap13 was achieved. About 6 month after birth, these mice showed symptom similar to atopic dermatitis and cataract. In contrast, 15% of mice born from crossing between Krtap13-Tg mice and CAG-Cre Tg mice, generated lymphoma/leukemia 1~1.5 years after birth. I am pathologically analyzing these mice.

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

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 although viral oncoproteins cause the increase of cellular proliferation and/or transformation, 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}. As the results, it is suggested that E1^{E4} associates with the Aggresome compartment that is one of cellular inclusion body systems. 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 investigated 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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Xue, Y., Chowdhury, S., Liu, X., Akiyama, Y., Ellman, J., and Ha, Y. The conformational change in rhomboid protease GlpG induced by inhibitor binding to its S'-subsites. *Biochemistry* 51, 3723-31, 2012.

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伊藤維昭、茶谷悠平、中森健太、千葉志信、秋山芳展、阿保達彦：Nascentome：合成途上鎖の解析. 第1回 RIBOSOME MEETING、東広島、2012年3月15日-16日

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- 檜作洋平、禾 晃和、田畑早苗、川上-田村恵子、小田 隆、佐藤 衛、高木淳一、秋山芳展：大腸菌 RIP プロテアーゼ RseP のタンデム PDZ ドメインによる新たな機能制御メカニズム. 第85回日本生化学会大会、福岡、2012年12月14日-16日
- 森 博幸、橋本成祐、小嶋誠司、本間道夫、秋山芳展：ビブリオ菌のタンパク質分泌マシナリー: SecDF パラログの発現制御機構. 第85回日本生化学会大会シンポジウム「運動超分子マシナリーの機能メカニズム」福岡、2012年12月14日-16日
- 寿野良二：膜結合型 AAA+プロテアーゼ FtsH の ATP 結合による基質ポリペプチド分解メカニズム. 第85回日本生化学会大会、福岡、2012年12月14日-16日

II. Second Group

- Ma, G., Yasunaga, J.-I., Fan, J., Yanagawa, S.-I., Matsuoka, M. HTLV-1bZIP factor dysregulates the Wnt pathways to support proliferation and migration of adult T-cell leukemia cells. *Oncogene*, 2012 Oct8. doi: 10.1038/onc.2012.450

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DEPARTMENT OF VIRAL ONCOLOGY
LABORATORY OF CELL REGULATION

The universe of antigens recognized by T lymphocytes has now 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, a novel immune pathway directed against microbial lipopeptide antigens has recently been identified. On the basis of these discoveries, we now address the possibility for “lipid-based” vaccines against tuberculosis and AIDS.

- 1) T cell response to mycobacteria-derived glycolipids in rhesus macaques: D. MORITA, Y. HATTORI, T. NAKAMURA¹, T. IGARASHI², H. HARASHIMA¹ and M. SUGITA**
(¹Hokkaido Univ., ²Laboratory of Primate Model, IVR.)

Upon entry into the host, pathogenic mycobacteria produce glucose monomycolate (GMM), a glucosylated species of mycolic acids, by utilizing host-derived glucose as a substrate for mycolyltransferases. In the guinea pig model vaccinated with *Mycobacterium bovis* bacillus Calmette-Guerin (BCG), we obtained evidence for the delayed-type hypersensitivity (DTH) directed against GMM. The host CD1-based immunity detects GMM and mounts potent Th1-type T cell responses. Given that Th1 cytokines, such as IFN- γ and TNF- α , are critical for efficient 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)

To extend this study further, we set out to analyze responses to GMM in non-human primates. GMM-specific, CD1-restricted T cells were detected in the circulation of rhesus macaque monkeys after but not before vaccination with BCG. The circulating GMM-specific T cells were found in both CD4⁺ and CD8⁺ T cell populations, and upon antigenic stimulation, a majority of the GMM-specific T cells produced IFN- γ and TNF- α , two major host protective cytokines functioning against infection with mycobacteria. These T cells extravasated and penetrated deeply into the site of infection where CD1⁺ macrophages accumulated. On the basis of these observations, we are now inspired to test whether vaccination with GMM in the purified form may elicit the specific response observed in BCG-vaccinated animals. (*Infect. Immun.* 81: 311-316, 2013)

- 2) Lipopeptide-specific T cell immunity against AIDS: D. MORITA, Y. YAMAMOTO, J.**

SUZUKI¹, N. MORI², T. IGARASHI³, and M. SUGITA (¹Primate Research Inst., Kyoto Univ., ²Graduate Sch. Agriculture, Kyoto Univ., ³Laboratory of Primate Model, IVR,)

By taking advantage of IVR's superb research environments and close collaboration with Prof. Igarashi's laboratory, we are encouraged to address a naive question as to how lipid immunity functions in host defense against viral infections as viruses do not express their own lipids. Given that some of the viral proteins require modification with host-derived fatty acids for their critical function, we hypothesized 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 5-mer lipopeptide (C14nef5). (*J. Immunol.* 187: 608-612, 2011)

Functional studies with C14nef5-derived structural analogues revealed that the putative lipopeptide antigen-presenting molecule might have two separate antigen-binding sites, one for interaction with a C14 saturated acyl chain and the other for anchorage of the C-terminal serine residues. Furthermore, amino acid residues recognized preferentially by the T cell receptor were identified, allowing us to propose a molecular model for lipopeptide antigen presentation. (*J. Virol.* 87: 482-488, 2013)

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LABORATORY OF CELL REGULATION

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森田大輔、五十嵐樹彦、森直樹、杉田昌彦：ウイルス蛋白質のミリスチン酸修飾を検知する新たなT細胞応答 第23回日本生体防御学会学術集会 東京 2012年7月9日

-11 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) Fas-deficiency in mice with the Balb/c background induces blepharitis with allergic inflammation and hyper IgE production in conjunction with severe autoimmune disease: S. TAKAHASHI, A. FUKUOKA, S. FUTATSUGI-YUMIKURA, T. YOSHIMOTO, K. NAKAHISHI and S. YONEHARA**

Fas (CD95) is a cell surface death receptor belonging to the tumor necrosis factor receptor superfamily, which mediates apoptosis-inducing signaling when activated by Fas ligand or its agonistic antibody. *lpr* mice with a loss of apoptosis-inducing function mutation in *Fas* gene develop systemic autoimmune disease and lymphadenopathy but not allergic inflammation. In the case of Fas mutations including *lpr* and knockout (KO), background genes determine the incidence and severity of lymphadenopathy and histopathological manifestation of systemic autoimmunity: MRL-*lpr/lpr* mice, and C57BL/6-*lpr/lpr* or C57BL/6 Fas KO mice develop severe and minimum disease, respectively. We generated Fas KO mice with the Balb/c background, that show severer autoimmune phenotypes than MRL-*lpr/lpr* mice, such as critical infiltration of mononuclear cells into lung, liver and spleen, elevated serum levels of autoantibody (ANA and anti-SSA), and a decreased life span. To our astonishment, Balb/c Fas KO mice spontaneously develop blepharitis with not only autoimmune inflammation with deposition of autoantibody but also allergic inflammation with infiltration of eosinophil and mast cell, and show the capacity to strongly increase serum level of IgE and IgG1 along with their aging. Thus, Fas expression regulates development of not only autoimmune disease but also allergic inflammation.

- 2) Protease activity of procaspase-8 is essential for cell survival by inhibiting both apoptotic and nonapoptotic cell death dependent on receptor interacting protein kinase**

1 (RIP1) and RIP3: M. KIKUCHI, S. KUROKI, M. KAYAMA, S. SAKAGUCHI, K.K. LEE and S. YONEHARA

Caspase-8 has an important role as an initiator caspase during death receptor- mediated apoptosis. Moreover, it has been reported to contribute to the regulation of cell fate in various types of cells including T-cells. In this paper, we show that caspase-8 has an essential role in cell survival in mouse T-lymphoma-derived L5178Y cells. The knockdown of caspase-8 expression decreased the growth rate and increased cell death, both of which were induced by the absence of protease activity of procaspase-8. The cell death was associated with reactive oxygen species (ROS) accumulation, caspase activation and autophagosome formation. The cell death was inhibited completely by treatment with ROS scavengers, but only partly by treatment with caspase inhibitors, expression of Bcl-xL, and knockdown of caspase-3 or Atg-7 which completely inhibits apoptosis or autophagosome formation, respectively, indicating that apoptosis and autophagy-associated cell death are induced simultaneously by the knockdown of caspase-8 expression. Further analysis indicated that RIP1 and RIP3 regulate this multiple cell death, because the cell death as well as ROS production was completely inhibited by not only treatment with the RIP1 inhibitor necrostatin-1, but also by knockdown of RIP3. Thus, in the absence of protease activity of procaspase-8, RIP1 and RIP3 simultaneously induce not only nonapoptotic cell death conceivably including autophagic cell death and necroptosis but also apoptosis through ROS production in mouse T-lymphoma cells.

3) Identification of mechanism that couples multisite phosphorylation of Yes-associated protein (YAP) with transcriptional coactivation and regulation of apoptosis: K.K. LEE and S. YONEHARA

The transcriptional coactivator Yes-associated protein (YAP) has been implicated in tumorigenesis by regulating cell proliferation and apoptosis. YAP interacts with the transcription factor TEAD and is essential in mediating TEAD-dependent gene expression. Here we show that YAP is hyperphosphorylated and activated in response to genotoxic stress such as UV irradiation and cisplatin treatment. Using high resolution mobility shift assay for phosphorylated proteins, we identified multiple sites of phosphorylation induced by UV irradiation. Pretreatment with p38 and JNK inhibitors completely suppressed the mobility retardation of phosphorylated YAP in UV-irradiated cells. Co-immunoprecipitation experiments showed that the physical interaction of YAP with TEAD was markedly enhanced by UV irradiation or CDDP treatment but suppressed by pretreatment with p38 and JNK inhibitors. Similarly, pretreatment with p38 and JNK inhibitors suppressed the expression of YAP/TEAD target genes, which were elevated on exposure to genotoxic stress. Using phosphomimetic and phosphorylation-deficient YAP mutants, we showed that the coactivator activity of YAP correlated with its state of phosphorylation and sensitivity to

cisplatin-induced apoptosis. Our results demonstrate that multisite phosphorylation of YAP induces YAP/TEAD-dependent gene expression and provides a mechanism by which YAP regulates apoptosis differently depending on cellular context.

4) A role of Wnt signals in the self-renewal and the differentiation of mouse ES cells: A. MURAKAMI

ES cells are maintained in an undifferentiated state or are induced to differentiated cells under various culture conditions. Many signaling pathways or factors have been identified to be involved in those processes. Among them, we are currently interested in the Wnt signaling pathway, which is likely to contribute to both processes. An activation of the Wnt signal keeps ES cells in undifferentiated state. On the other hand, there are some Wnt signals that are involved in an induction of differentiation.

In ES cells, expression of several members of Wnt family were detected, such as Wnt1, Wnt3, Wnt6, Wnt8a and Wnt10b. The expression of Wnt6 and Wnt10b were down regulated in differentiated cells, and knock down of the expression of either gene resulted in poor proliferation. Thus, they are suggested to play a role in maintenance of self-renewal. The expression of Wnt3 and Wnt8a were up regulated as cells differentiated, and knock down of the expression of either gene inhibited mesoderm induction, suggesting their involvement in the differentiation process. An analysis of the difference between the Wnt signals in these processes is underway.

LIST OF PUBLICATIONS

DEPARTMENT OF VIRAL ONCOLOGY

LABORATORY OF TUMOR BIOGENESIS

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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) Intranuclear persistence of bornavirus reveals a new life cycle of RNA virus closely associated with host chromosome: Y. MATSUMOTO, T. HONDA, T. DAITOA, M. HORIE, K. FUJINO, S. NAKAMURA and K. TOMONAGA**

Bornaviruses are nonsegmented negative-strand RNA viruses that establish a persistent infection in the nucleus and integrate occasionally a DNA genome copy into the host chromosomal DNA. However, how these viruses achieve intranuclear infection remains unclear. We show that Borna disease virus (BDV), a mammalian bornavirus, closely associates with the cellular chromosome to ensure intranuclear infection. BDV generates viral factories within the nucleus using host chromatin as a scaffold. In addition, the viral ribonucleoprotein (RNP) interacts directly with the host chromosome throughout the cell cycle, using core histones as a docking platform. HMGB1, a host chromatin-remodeling DNA architectural protein, is required to stabilize RNP on chromosomes and for efficient BDV RNA transcription in the nucleus. During metaphase, the association of RNP with mitotic chromosomes allows the viral RNA to segregate into daughter cells and ensure persistent infection. Thus, bornaviruses likely evolved a chromosome-dependent lifecycle to achieve stable intranuclear infection.

- 2) Interaction between the phosphoproteins and X proteins of bornaviruses from different vertebrate species reveals evolutionarily conserved functions of the viral proteins: K. FUJINO, M. HORIE, T. HONDA, S. NAKAMURA, Y. MATSUMOTO and K. TOMONAGA**

Bornavirus, a non-segmented, negative-strand RNA viruses, is currently classified into several genetically distinct genotypes, such as Borna disease virus (BDV) and avian bornaviruses (ABVs). Recent studies revealed that bornavirus genotypes show unique sequence variability in the putative 5' untranslated region (5' UTR) of X/P mRNA, which is a bicistronic mRNA for X and phosphoprotein (P). In this study, to understand the evolutionary relationship among the bornavirus genotypes, we investigated the functional interaction between X and P proteins of four bornavirus genotypes, BDV, ABV genotype 4 and 5 and reptile bornavirus (RBV), of which the putative 5' UTRs exhibit variation in the length. Immunofluorescence and immunoprecipitation analyses using mammalian and avian cell lines revealed that the X proteins of bornaviruses conserve the ability to facilitate the export of P from the nucleus to the cytoplasm via interaction with P. Furthermore, we showed that inter-genotypic interactions may occur between X and P among the genotypes, except for X of RBV. In addition, BDV minireplicon assay demonstrated that the X and P proteins of ABVs, but not RBV, can affect the polymerase activity of BDV. This study demonstrates that bornaviruses may conserve the fundamental function of a regulatory protein during their evolution, whereas RBV have evolved distinctly from the other bornavirus genotypes.

3) Detection and sequence analysis of avian bornavirus genotype 5 in *Eclectus roratus* with feather picking disorder: Y. M. HORIE, T. HONDA and K. TOMONAGA

Avian bornavirus (ABV) was discovered recently in parrots with proventricular dilatation disease (PDD), a fatal neurological disease. Although ABV has been shown to be a causative agent of PDD, the virological characteristics of ABV are largely unknown. Here we report the detection of ABV genotype 5 RNA in *Eclectus roratus* with feather picking disorder (FPD). Interestingly, the bird was persistently infected with ABV5 at least for 8 months without clinical signs of PDD. Although it remains unclear whether ABV5 is associated with FPD, these findings raise the importance of epidemiological studies of birds with diseases other than PDD.

4) Generation of human bronchial epithelial cell lines stably expressing GALNT3 wild-type and mutant clones: S. NAKAMURA, M. HORIE, K. FUJINO, Y. MATSUMOTO, T. HONDA and K. TOMONAGA

As a tool to understand the role of mucins in the infection of respiratory viruses, we established cell lines stably expressing inactive mutants of UDP-GalNAc:polypeptide N-acetylgalactosaminyltransferase 3 (GALNT3), which initiates O-glycosylation of mucins. We introduced single amino acid mutations into the regions essential for the enzyme activity of GALNT3 using the expression plasmid of human GALNT3 and transfected the mutant constructs into a human bronchial epithelial cell line, BEAS-2B. We showed that although the mutants of

GALNT3 exhibit an authentic localization at the Golgi apparatus, the glycosylation pattern of the expressing cell lines appeared to be different from that of the cells expressing wild-type GALNT3. These results suggested that the established cell lines express inactive forms of GALNT3 and might be useful in investigation of the significance of O-glycosylation of mucins in respiratory virus infections.

II. Second Group

1) Infectious viral particle production is modulated by thromboxane A₂ synthase in the cells: Y. ABE, H. H. ALY, M. IMAMURA, T. WAKITA, K. SHIMOTOHNO, K. CHAYAMA, M. HIJIKATA

Previously, we developed the three-dimensional (3D) cell culture system, using human immortalized hepatocytes (HuS-E/2 cells), supporting the lifecycle of blood-borne hepatitis C virus (bbHCV), and found that 3D-culture condition offers a better environment for the HCV lifecycle. This study was designed to reveal signaling pathways supporting HCV lifecycle under the 3D-culture condition in order to identify targets for development of drugs against HCV. The gene expression profiles between 2D-, and 3D-cultured HuS-E/2 cells were compared. After identification of signaling pathways showing differential activation in 3D-cultured cells, the contribution of those pathways to HCV lifecycle was analyzed in the recombinant HCV production cell culture system by using siRNAs and chemical reagents. Anti-HCV effect of the reagents modulating the signaling pathway was investigated in chimeric mouse with humanized liver system. The microarray comparison showed the differential gene expression of prostaglandin (PG) synthases under 3D condition. Results of siRNA and shRNA treatments suggested the contribution of thromboxane A₂ synthase (TXAS) to infectious HCV-like particle production independently of the RNA genome replication and the particle egression. Treatment of TXAS inhibitor resulted in a similar outcome, indicating a key role of its enzyme activity in this event. TXAS inhibitor did not cause accumulation of infectious virus-like particles in the cells. These data suggested that TXAS activity is required for formation of the infectivity. Finally, the inhibitory effect of TXAS inhibitor on proliferation of bbHCV was observed in the chimeric mouse system, suggesting the potential of TXAS inhibitor as an anti-HCV drug.

2) Constitutively produced interferon α 1 functions in prevention of viral infection in human hepatocytes: Y. TSUGAWA, H. KATO, T. FUJITA, K. SHIMOTOHNO, M. HIJIKATA

Several viruses are known to elicit innate immune responses in human liver after infection. However, the detail of interferon (IFN) system in human hepatocytes has not been clarified yet. In

this study, the immortalized human hepatocytes (HuS-E/2 cells) was used, as the cells showed similar innate immune responses against RNA virus infection to primary human hepatocyte (PHH). Constitutive expression of IFN α 1 gene, but not IFN β and IFN λ s genes, was observed in both PHH and HuS-E/2 cells without viral infection albeit at the low level. To investigate the role of this constitutively produced IFN α 1 in innate immune response in the cells, the expression profiles of innate immunity-related genes after Sendai virus (SeV) infection were compared between the cells with and without pretreatment of the neutralizing antibody against IFN α or IFN α 1 receptor 2 to inhibit the cell signaling induced by preexisting IFN α . The results observed in the cells with pretreatment were as follows: 1. The basal mRNA levels of IFN-stimulated genes (ISGs) were reduced, although that of IFN α 1 gene was not affected; 2. The expression of IFN β , IFN λ s genes and some ISGs induced by SeV infection was significantly decreased at the immediate early phase of infection; 3. Accelerated viral proliferation was found immediately after SeV infection. These results suggested that IFN α 1 constitutively produced in human hepatocytes enhanced the anti-viral potential of the cells through the preactivation of positive feedback system for IFN system.

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LABORATORY OF HUMAN TUMOR VIRUSES

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- Matsumoto Y., Fujino K., Horie M., Nakamura S., Honda T., Schwemmle M., Tomonaga K. American Society for Virology 31th Annual Meeting. Wisconsin, USA. 21-25 July 2012
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II. Second Group

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- 津川陽司、加藤博己、藤田尚志、下遠野邦忠、土方 誠：ヒト肝細胞における抗ウイルス自然免疫応答機構の解析、第60回日本ウイルス学会学術集会、大阪 2012年11月13-15日
- Tsugawa Y., Kato H., Fujita T., Shimotohno T., Hijikata M.: Constitutively produced Interferon α 1 functions in prevention of viral infection in human hepatocytes、第35回日本分子生物学会年会、福岡 2012年12月11-14日

DEPARTMENT OF GENETICS AND MOLECULAR BIOLOGY
LABORATORY OF MOLECULAR GENETICS

- 1) **Critical Role of an Antiviral Stress Granule Containing RIG-I and PKR in Viral Detection and Innate Immunity: ONOMOTO, K., JOGI, M., YOO, J-S., NARITA, R., MORIMOTO, S., TAKEMURA, A., SAMBHARA, S., KAWAGUCHI, A., OSARI, S., NAGATA, K., MATSUMIYA, T., NAMIKI, H., YONEYAMA, M. AND FUJITA T.**

Retinoic acid inducible gene I (RIG-I)-like receptors (RLRs) function as cytoplasmic sensors for viral RNA to initiate antiviral responses including type I interferon (IFN) production. It has been unclear how RIG-I encounters and senses viral RNA. To address this issue, we examined intracellular localization of RIG-I in response to viral infection using newly generated anti-RIG-I antibody. Immunohistochemical analysis revealed that RLRs localized in virus-induced granules containing stress granule (SG) markers together with viral RNA and antiviral proteins. Because of similarity in morphology and components, we termed these aggregates antiviral stress granules (avSGs). Influenza A virus (IAV) deficient in non-structural protein 1 (NS1) efficiently generated avSGs as well as IFN, however IAV encoding NS1 produced little. Inhibition of avSGs formation by removal of either the SG component or double-stranded RNA (dsRNA)-dependent protein kinase (PKR) resulted in diminished IFN production and concomitant enhancement of viral replication. Furthermore, we observed that transfection of dsRNA resulted in IFN production in an avSGs-dependent manner. These results strongly suggest that the avSG is the locus for non-self RNA sensing and the orchestration of multiple proteins is critical in the triggering of antiviral responses.

- 2) **Identification of germicidal compound against picornavirus in bamboo pyroligneous acid. Journal of Agricultural and Food Chemistry: MARUMOTO, S., YAMAMOTO, S., NISHIMURA, H., ONOMOTO, K., YATAGAI, M., YAZAKI, K., FUJITA, T. AND WATANABE, T.**

The germicidal activity of pyroligneous acid (PA) against a picornavirus, encephalomyocarditis virus (EMCV), was analyzed, and the component responsible for its disinfectant activity was identified. Bamboo PA (BPA) inactivated EMCV, but neutralization of BPA abolished this activity. Using liquid-liquid phase extraction and silica gel column chromatography, the hydrophobic active fraction of BPA was separated and its 12 major components were identified. The active fraction was reconstructed by mixing synthetic chemicals at the determined concentrations, and a subtraction series of one chemical from the complete mixture was prepared. An in vitro virus assay demonstrated that phenol was the sole germicidal component,

and acetic acid augmented the phenol's inactivating activity resulting in >5-log decrease in EMCV infectivity. Considering the low environmental risk of PA, these findings suggest that BPA is a potentially useful agent for preventing viral epidemics in agricultural and human environments.

3) Impairment of interferon regulatory factor-3 activation by hepatitis C virus core protein basic amino acid region 1: INOUE, K., TSUKIYAMA-KOHARA, K., MATSUDA, C., YONEYAMA, M., FUJITA, T., KUGE, S., YOSHIBA, M. AND KOHARA, M.

Interferon regulatory factor-3 (IRF-3), a key transcriptional factor in the type I interferon system, is frequently impaired by hepatitis C virus (HCV), in order to establish persistent infection. However, the exact mechanism by which the virus establishes persistent infection has not been fully understood yet. The present study aimed to investigate the effects of various HCV proteins on IRF-3 activation, and elucidate the underlying mechanisms. To achieve this, full-length HCV and HCV subgenomic constructs corresponding to structural and each of the nonstructural proteins were transiently transfected into HepG2 cells. IFN- β induction, plaque formation, and IRF-3 dimerization were elicited by Newcastle disease virus (NDV) infection. The expressions of IRF-3 homodimer and its monomer, Ser386-phosphorylated IRF-3, and HCV core protein were detected by immunofluorescence and western blotting. IFN- β mRNA expression was quantified by real-time PCR (RT-PCR), and IRF-3 activity was measured by the levels of IRF-3 dimerization and phosphorylation, induced by NDV infection or polyribonucleosinic:polyribocytidylic acid [poly(I:C)]. Switching of the expression of the complete HCV genome as well as the core proteins, E1, E2, and NS2, suppressed IFN- β mRNA levels and IRF-3 dimerization, induced by NDV infection. Our study revealed a crucial region of the HCV core protein, basic amino acid region 1 (BR1), to inhibit IRF-3 dimerization as well as its phosphorylation induced by NDV infection and poly (I:C), thus interfering with IRF-3 activation. Therefore, our study suggests that rescue of the IRF-3 pathway impairment may be an effective treatment for HCV infection.

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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, 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.

1-2) The hnRNP C tetramer as a molecular ruler

Specific RNA recognition is usually achieved by specific RNA sequences or structures, but we have recently reported a molecular mechanism by which the formation of export RNP complexes is specified by RNA length. RNA polymerase II (Pol II) synthesizes not only mRNAs but also shorter RNAs, including spliceosomal U snRNAs. Although the key U snRNA export factor, PHAX, can bind to mRNA *in vitro*, PHAX is excluded from mRNA *in vivo*. The heterotetramer of the heterogeneous nuclear RNP (hnRNP) C1/C2 specifically binds Pol II transcripts longer than 200–300nt, and funnels them into the mRNA export pathway by inhibiting their binding by PHAX, whereas shorter transcripts not bound by the heterotetramer are committed to the U snRNA export pathway.

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.

Although we have clarified that 25S NRD requires an E3 ubiquitin ligase complex, which is involved in ribosomal ubiquitination, the degradation process of nonfunctional ribosomes remained unknown. Using genetic screening, we further identified two ubiquitin-binding complexes, the Cdc48–Npl4–Ufd1 complex (Cdc48 complex) and the proteasome, as the factors involved in 25S NRD. We show that the nonfunctional 60S subunit is dissociated from the 40S subunit in a Cdc48 complex-dependent manner, before it is attacked by the proteasome. When we examined the nonfunctional 60S subunits that accumulated under proteasome-depleted conditions, the majority of mutant 25S rRNAs retained their full length at a single-nucleotide resolution. This indicates that the proteasome is an essential factor triggering rRNA degradation. We further showed that ribosomal ubiquitination can be stimulated solely by the suppression of the proteasome, suggesting that ubiquitin–proteasome-dependent RNA degradation occurs in broader situations, including in general rRNA turnover.

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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) Roles of the IL-7 receptor in late stages of T cell development in the thymus: S. TANI-ICHI, A. SHIMBA, K. WAGATSUMA, H. MIYACHI¹, S. KONAKA¹, K. IMAI, T. HARA and K. IKUTA (¹Reproductive Engineering Team, Institute for Virus Research, Kyoto University)**

IL-7 is an essential cytokine for T cell development and homeostasis. We previously reported that IL-7R α -deficient mice have severely reduced numbers of T cells in the thymus. However, the role of the IL-7R has not been precisely determined at late stages of T cell development, because IL-7R α -deficient mice have profound detrimental effects on early thymocytes. To address this question, we generated IL-7R α -floxed mice and crossed with CD4-Cre transgenic mice (IL-7RcKO). Total thymocyte numbers of IL-7RcKO mice were not different from littermate controls. Although CD4 T cells normally differentiated in the thymus, development of CD8 T cells, NKT cells and regulatory T cells was partially impaired. 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. Unexpectedly, CD4⁺CD8⁻ thymocytes were increased in IL-7RcKO mice. This was mainly attributed to increase of thymic B cells, especially IL-7R⁺ pre-B cells, while thymic $\gamma\delta$ T cells were also slightly increased. These results demonstrate that the IL-7R is required for development of CD8 T cells, NKT cells and regulatory T cells in the thymus. Moreover, our data suggest that the IL-7R transmits survival signal for positively selected CD8 T cells rather than influences the CD4/8 lineage choice. Finally, this study implies that suppression of B cell development in the thymus requires IL-7R expression on $\alpha\beta$ T cells.

2) Identification of Interleukin-7-producing cells in primary and secondary lymphoid organs using IL-7-GFP knock-in mice: T. HARA, S. SHITARA, K. IMAI, H. MIYACHI¹, S. KONAKA¹, H. YAO, S. TANI-ICHI and K. IKUTA (¹Reproductive Engineering Team, Institute for Virus Research, Kyoto University)

IL-7 is a cytokine crucial for development and maintenance of lymphocytes and other hematopoietic cells. However, how IL-7-expressing cells are distributed in lymphoid organs is not well known. To address this question, we established and analyzed IL-7-GFP knock-in mice. Thymic epithelial cells (TECs) expressed high GFP levels in the cortex and medulla, as detected with an anti-GFP antibody. Thymic mesenchymal cells also expressed GFP. Flow cytometry analysis suggested that cortical TECs expressed higher GFP levels than did medullary TECs. In bone marrow, immunohistochemistry indicated high levels of GFP in many VCAM-1⁺ mesenchymal stromal cells and in some VCAM-1⁻ cells. In addition, half of the VCAM-1⁺CD31⁻ stromal cells and some PDGFR α ⁺ stromal cells were GFP⁺, as detected by flow cytometry. Moreover, we detected GFP expression in fibroblastic reticular cells in the T-cell zone and cortical ridge of lymph nodes. Remarkably, lymphatic endothelial cells (LECs) expressed GFP at high levels within the lymph node medulla, skin epidermis and intestinal tissues. Additionally, we detected abundant IL-7 transcripts in isolated LECs, suggesting that LECs produce IL-7, a heretofore unknown finding. Furthermore, GFP is expressed in a subpopulation of intestinal epithelial cell, and that expression was markedly upregulated in a DSS-induced acute colitis model. Overall, IL-7-GFP knock-in mice serve as a unique and powerful tool to examine the identity and distribution of IL-7-expressing cells in vivo.

3) Role of hepatocyte-derived IL-7 in maintenance of intrahepatic NKT and T cells and development of B cells in fetal liver: B. LIANG, T. HARA, K. WAGATSUMA, J. ZHANG¹, K. MAKI, H. MIYACHI², S. KONAKA², C. YABE-NISHIMURA¹, S. TANI-ICHI and K. IKUTA (¹Department of Pharmacology, Kyoto Prefectural University of Medicine, ²Reproductive Engineering Team, Institute for Virus Research, Kyoto University)

The liver contains a variety of resident immune cells, such as NK cells, NKT cells, T cells, macrophages, and dendritic cells. However, little is known about how IL-7, which is produced by hepatocytes, functions locally in development and maintenance of liver immune cells. To address this question, we established IL-7-floxed mice and crossed them with albumin promoter-driven Cre (Alb-Cre) transgenic mice to establish conditional knockout of IL-7 in hepatocytes. The levels of IL-7 transcripts were reduced 10-fold in hepatocyte fraction. We found that the absolute numbers of NKT and T cells were significantly decreased in adult liver of IL-7^{f/f} Alb-Cre mice compared with

IL-7^{f/f} control mice. In contrast, NK cells, dendritic cells, and B cells were unchanged in the IL-7^{f/f} Alb-Cre liver. The number of V α 14⁺ invariant NKT cells was significantly reduced in liver but not in thymus and spleen of IL-7^{f/f} Alb-Cre mice. Furthermore, B cell development was impaired in perinatal liver of IL-7^{f/f} Alb-Cre mice. This study demonstrates that hepatocyte-derived IL-7 plays an indispensable role in maintenance of NKT and T cells in adult liver and development of B cells in fetal liver and suggests that hepatocytes provide a unique IL-7 niche for intrahepatic lymphocytes.

- 4) **Monoclonal antibodies 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. CRT exhibited three polypeptide forms; normal spliced form, alternative spliced forms and non-spliced full-length form. The amount of full-length form of CRT correlates to urogenital cancers (Kageyama et al. Clin Chem 2004). For diagnosis of urogenital cancers, we prepared monoclonal antibodies against CRT using dot blot method with PVDF membrane, but the detection sensitivity of CRT was poor for usual clinical detection systems. Then, we tried to prepare new mAbs with BSA-conjugated polypeptide unique to human full-length form CRT (NH₂-EDKDEDEDEED-COOH). Three independent monoclonal antibodies were produced, and the sensitivity for CRT detection system is under investigation.

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- 梁冰霏：肝臓におけるインターロイキン 7 の機能、平成 24 年度京都大学ウイルス研究所学術交流会、京都、2012 年 12 月 17 日

DEPARTMENT OF BIOLOGICAL RESPONSES
LABORATORY OF INFECTION AND PREVENTION

I. First Group

The research projects carried out in this group are aiming to uncover the molecular mechanisms of the regulation of inflammation in innate immunity. Since inflammation is mediated by the production of proinflammatory cytokines, we are studying the cytokine gene expression at the transcriptional and posttranscriptional levels.

1) Regulatory mechanism for the Regnase-1-mediated mRNA decay in innate immunity and its post-transcriptional regulation: T. MINO, A. FUKAO¹, T. IMAMURA, T. FUJIWARA¹ and O. TAKEUCHI (¹Laboratory of Disease Biology, Institute of Microbial Chemistry)

Post-transcriptional regulation that modifies mRNA stability and translation provides rapid and flexible control of gene expression. Control of mRNA stability is important for coordinating the initiation and resolution of inflammation. The RNase Regnase-1 (also known as Zc3h12a, Mcpip1) plays a critical role in preventing autoimmunity by controlling the stability of mRNAs such as *Interleukin-6 (Il6)* and *Regnase-1* itself. We further showed that Regnase-1 protein expression is drastically regulated in the course of inflammation. In this study, we demonstrate that Regnase-1 destabilizes translationally active mRNAs by association with the ribosome. Regnase-1 localized to cytoplasm and endoplasmic reticulum (ER), but not to processing bodies, cytoplasmic foci now known to be involved in the posttranscriptional processing of mRNAs. Regnase-1 was physically associated with the ribosome and protein synthesis was required for the Regnase-1-mediated *Il6* mRNA destabilizing. Treatment with translation inhibitors, such as anisomycin and cycloheximide, suppressed Regnase-1-mediated degradation of *Il6* mRNA, suggesting that Regnase-1 destabilizes translationally active mRNA. We demonstrated that most endogenous *Il6* mRNA localized to ER, where the mRNA is translated to protein, suggesting that Regnase-1 might function in recognizing the translationally active mRNA and triggering mRNA destabilizing immediately. The translation-coupled mRNA degradation provides a mechanism for the rapid response to signals, which occur as a result of the immunoresponse.

2) Regulation of innate immune signaling emanated from Toll-like receptors: D. ORI, S. TARTEY, A. WAKABAYASHI and O. TAKEUCHI

The Toll-like receptor (TLR) signaling leads to the activation of a set of transcription factors including NF- κ B and AP-1 for inducing proinflammatory cytokine genes. The mechanisms of TLR

signaling and its regulation have not been fully understood. We are investigating the regulatory mechanisms of cytokine gene expression by focusing on a set of potential regulators. Among them, the K63-type polyubiquitin binding proteins TAB2 and TAB3 were implicated in the positive regulation of the NF- κ B signaling. However, we found that these proteins were critical for the activation of MAP kinases, but not NF- κ B in B cells. Proliferation as well as antigen-specific antibody responses were impaired in the absence of TAB2 and TAB3, indicating the critical roles of the K63-type polyubiquitin chain in the B cell activation.

II. Second Group

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). TBP-2 has attracted much attention as a multifunctional regulator in cancer suppression, metabolism, vascular stress, as well as immune response and inflammation. TBP-2 also acts as a negative regulator of thioredoxin. We have been investigating redox signaling and host defense mechanism against oxidative stress. Thioredoxin is a key component of redox regulation and plays a protective role in various diseases, associated with oxidative stress and inflammation. Thioredoxin has recently been reported to ameliorate influenza virus-induced acute lung injury in mice. Meanwhile, the members of the thioredoxin system play a regulatory role in each cellular compartment. A member of the system in endoplasmic reticulum, Transmembrane Thioredoxin-Related Protein (TMX) has been reported to play an important protective role against inflammatory liver injury.

1) Metabolic energy control by Thioredoxin binding protein (TBP)-2/Txnip: H. MASUTANI and S. MASAKI

We have shown that TBP-2 plays essential roles in metabolic energy control. TBP-2 is a critical molecule in fasting response. Disruption of TBP-2 in obese mice (ob/ob) dramatically improved hyperglycemia, without affecting obesity or adipocytokine concentration. TBP-2-deficiency augmented glucose-induced insulin secretion through the transcriptional regulation of the uncoupling protein-2 (UCP-2) gene, via the control of PPAR gamma coactivator (PGC)-1- α in beta cells. TBP-2-deficiency also exhibited enhanced insulin sensitivity with activated IRS-1/Akt signaling in skeletal muscle. However, the molecular mechanism has not been elucidated. We showed that TBP-2 expression is induced by high glucose in C2C12 muscle cells through the activation of carbohydrate responsive elements and CT rich element of the TBP-2 promoter. Overexpression of TBP-2 suppressed glucose uptake in C2C12 muscle cells. The mechanism of action of TBP-2 is under investigation by proteomics approaches. Elucidation of the molecular mechanisms of TBP-2 may provide a new pharmacological basis for developing

approaches against obesity and type 2 diabetes mellitus.

2) Deficiency of Thioredoxin binding protein (TBP)-2 enhances TGF- β signaling and promotes Epithelial to Mesenchymal Transition: S. MASAKI and H. MASUTANI

Transforming growth factor beta (TGF- β) has critical roles in regulating cell growth, differentiation, apoptosis, and epithelial-mesenchymal transition (EMT) of various cancer cells. TGF- β -induced EMT is an important step during carcinoma progression to invasion state. Thioredoxin binding protein-2 (TBP-2, also called as Txnip or VDUP1) is downregulated in various types of human cancer and its deficiency results in the early onset of cancer. However, it remains unclear how TBP-2 suppresses the invasion and metastasis of cancer. We demonstrated that TBP-2 deficiency increases the transcriptional activity in response to TGF- β and also enhances TGF- β -induced Smad2 phosphorylation levels. Knockdown of TBP-2 augmented the TGF- β -responsive expression of Snail and Slug, transcriptional factors related to TGF- β -mediated induction of EMT and promoted TGF- β -induced spindle-like morphological changes consistent with the depletion of E-Cadherin in A549 cells. These results indicate that TBP-2 deficiency enhances TGF- β signaling and promotes TGF- β -induced EMT. The control of TGF- β -induced EMT is critical for the inhibition of the invasion and metastasis. Thus, TBP-2, as a novel regulatory molecule of TGF- β signaling, is likely to be a prognostic indicator or a potential therapeutic target for preventing tumor progression.

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DEPARTMENT OF CELL BIOLOGY
LABORATORY OF SUBCELLULAR BIOGENESIS

- 1) **Spindle orientation machinery in adherent cells and in 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)

Directing the axis of cell division toward extrinsic and intrinsic cues plays a fundamental role in morphogenesis, asymmetric cell division, and stem cell self-renewal. Recent studies highlight the misorientation of the cell division axis as a cause of mammalian diseases, including polycystic kidney disease. Although the core regulators for oriented cell division have been identified in invertebrate model systems, we still have an imprecise picture of the relevant signaling networks in the mammalian system. The reasons for this include the lack of established approaches in mammalian cells to survey the molecules required for the spindle orientation. 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. These results were published in Nat. Commun.

- 2) **Roles of cholesterol metabolites in the control of centrosome: 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 pregnenolone, a steroid which is produced from cholesterol by the steroidogenic enzyme Cyp11a1, has an essential role in centriole cohesion during mitosis. During prometa-metaphase, pregnenolone localizes to the mitotic spindles. The RNAi-mediated knockdown of Cyp11a1 induced multipolar spindles in mitotic HeLa cells. Introduction of pregnenolone, but not progesterone or 17-hydrpxy-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 ana/telophase in normal condition, takes place in prometa/metaphase in the Cyp11a1-depleted cells. This precocious centriole disengagement is again suppressed by the introduction of pregnenolone into these cells. Premature centriole disengagement induced by loss of pregnenolone is suppressed by inhibition of Plk1, which promotes centriole disengagement in early mitosis, but not by inhibition of separase, a key factor for the centriole disengagement at anaphase onset. These results demonstrate that pregnenolone is required for the maintenance of centriole engagement during prometa/metaphase.

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DEPARTMENT OF CELL BIOLOGY

LABORATORY OF SUBCELLULAR BIOGENESIS

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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 *Hes7* regulates the periodic somite formation. Both sustained expression and loss of expression of *Hes7* lead to somite fusion, suggesting that oscillatory expression of *Hes7* is required for somite segmentation. We recently found that negative feedback with delayed timing regulates oscillatory expression of *Hes7*, and that particularly, an intronic delay is essential for *Hes7* oscillations. By making and evaluating mathematical modeling, we are studying how the dynamics of gene expression are controlled in these cells.

1) Accelerating the tempo of the segmentation clock by reducing the number of introns in the *Hes7* gene: Y. HARIMA, Y. TAKASHIMA, Y. UEDA, T. OHTSUKA and R. KAGEYAMA

Periodic somite segmentation is controlled by the cyclic gene *Hes7* whose oscillatory expression depends upon negative feedback with a delayed timing. The mechanism of regulation of the pace of segmentation remains to be determined, but mathematical modeling has predicted that negative feedback with shorter delays would give rise to damped but more rapid oscillations. Here, we show that reducing the number of introns within the *Hes7* gene shortens the delay and results in

a more rapid tempo of both *Hes7* oscillation and somite segmentation, increasing the number of somites and vertebrae in the cervical and upper thoracic region. These results suggest that the number of introns is important for the appropriate tempo of oscillatory expression, and that *Hes7* is a key regulator of the pace of the segmentation clock.

2) Essential roles of the histone methyltransferase ESET in the epigenetic control of neural progenitor cells during development: S.L. TAN, M. NISHI, T. OHTSUKA, T. MATSUI, K. TAKEMOTO, A. KAMIO-MIURA, H. ABURATANI, Y. SHINKAI and R. KAGEYAMA

In the developing brain, neural progenitor cells (NPCs) switch the differentiation competency via changing gene expression profiles that are governed partly by epigenetic control such as histone modification, although the precise mechanism is unknown. Here we found that ESET/Setdb1/KMT1E, a histone H3 Lys-9 (H3K9) methyltransferase, was highly expressed at early stages of brain development but down-regulated over time, and that ablation of *ESET* led to decreased H3K9 trimethylation and misregulation of genes, resulting in severe brain defects and early lethality. In the mutant brain, endogenous retrotransposons were derepressed, and non-neural gene expression was activated. Furthermore, early neurogenesis was most severely impaired, while astrocyte formation was enhanced. We conclude that there is an epigenetic role of ESET in temporal and tissue-specific gene expression resulting in the proper control of brain development.

3) The role of *Hes* genes in intestinal development, homeostasis and tumor formation: T. UEO, I. IMAYOSHI, T. KOBAYASHI, T. OHTSUKA, H. SENO, H. NAKASE, T. CHIBA and R. KAGEYAMA

Notch signaling regulates intestinal development, homeostasis and tumorigenesis, but its precise downstream mechanism remains largely unclear. Here we found that inactivation of the Notch effectors *Hes1*, *Hes3* and *Hes5*, but not *Hes1* alone, led to reduced cell proliferation, increased secretory cell formation, and altered intestinal structures in the adult. However, in *Apc* mutation-induced intestinal tumors, inactivation of *Hes1* alone was sufficient for reducing tumor cell proliferation and inducing differentiation of tumor cells into all types of intestinal epithelial cells, but without affecting the homeostasis of normal crypts due to the genetic redundancy. These results indicated that *Hes* genes cooperatively regulate intestinal development and homeostasis, and raised the possibility that *Hes1* is a good target to induce differentiation of tumor cells.

4) MicroRNA9 regulates neural stem cell differentiation by controlling *Hes1* expression

dynamics in the developing brain: S.L. TAN, T. OHTSUKA, A. GONZALEZ and R. KAGEYAMA

It was previously shown that *Hes1* expression is oscillatory in neural stem cells but sustained and high in the roof plate and the floor plate, and that such different dynamics of *Hes1* expression (oscillatory versus sustained) regulate different proliferation and differentiation characteristics of these cells (active in neural stem cells but rather dormant in roof/floor plate cells). The mechanism of how different dynamics of *Hes1* expression is controlled remains to be determined. Here, we found that the seed sequence of microRNA-9 (miR-9) is complementary to the 3'-UTR sequence of *Hes1* mRNA. MiR-9 is highly expressed in the ventricular zone of the developing brain, which contains neural stem cells, but it is not expressed in the roof plate or the floor plate. Overexpression of miR-9 negatively regulates the *Hes1* protein expression by interacting with the 3'-UTR of *Hes1* mRNA, thereby inducing cell cycle exit and neuronal differentiation. Conversely, knockdown of miR-9 inhibits neuronal differentiation. Furthermore, knockdown of miR-9 inhibits the oscillatory expression of *Hes1* mRNA in neural stem cells. These results indicate that miR-9 regulates the proliferation and differentiation of neural stem cells by controlling the dynamics of *Hes1* expression in the developing brain.

5) A mechanism for gene-environment interaction in the etiology of congenital scoliosis: D.B. SPARROW, G. CHAPMAN, A.J. SMITH, M.Z. MATTAR, J.A. MAJOR, V.C. O'REILLY, Y. SAGA, E.H. ZACKAI, J.P.DORMANS, B.A. ALMAN, L. MCGREGOR, R. KAGEYAMA, K. KUSUMI and S.L. DUNWOODIE

Congenital scoliosis, a lateral curvature of the spine caused by vertebral defects, occurs in approximately 1 in 1,000 live births. Here we demonstrate that haploinsufficiency of Notch signaling pathway genes in humans can cause this congenital abnormality. We also show that in a mouse model, the combination of this genetic risk factor with an environmental condition (short-term gestational hypoxia) significantly increases the penetrance and severity of vertebral defects. We demonstrate that hypoxia disrupts FGF signaling, leading to a temporary failure of embryonic somitogenesis. These results potentially provide a mechanism for the genesis of a host of common sporadic congenital abnormalities through gene-environment interaction.

6) Control of *Hes7* Expression by *Tbx6*, the Wnt Pathway and the Chemical Gsk3 Inhibitor LiCl in the Mouse Segmentation Clock: A. GONZALEZ, I. MANOSALVA, T. LIU and R. KAGEYAMA

The mouse segmentation is established from somites, which are iteratively induced every

two hours from the presomitic mesoderm (PSM) by a system known as the segmentation clock. A crucial component of the segmentation clock is the gene *Hes7*, which is regulated by the Notch and Fgf/Mapk pathways, but its relation to other pathways is unknown. In addition, chemical alteration of the Wnt pathway changes the segmentation clock period but the mechanism is unclear. To clarify these questions, we have carried out *Hes7* promoter analysis in transgenic mouse embryos and have identified an essential 400 bp region, which contains binding sites of Tbx6 and the Wnt signaling effector Lef1. We have found that the *Hes7* promoter is activated by Tbx6, and normal activity of the *Hes7* promoter in the mouse PSM requires Tbx6 binding sites. Our results demonstrate that Wnt pathway molecules activate the *Hes7* promoter cooperatively with Tbx6 in cell culture and are necessary for its proper expression in the mouse PSM. Furthermore, it is shown that the chemical Gsk3 inhibitor LiCl lengthens the oscillatory period of *Hes7* promoter activity. Our data suggest that Tbx6 and the Wnt pathway cooperatively regulate proper *Hes7* expression. Furthermore, proper *Hes7* promoter activity and expression is important for the normal pace of oscillation.

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DEPARTMENT OF CELL BIOLOGY
LABORATORY OF SIGNAL TRANSDUCTION

Our research objective is to understand the pathogenesis of animal retroviruses, functions of endogenous retroviruses and potential iatrogenic risks by infection of endogenous retroviruses in xenotransplantation and vaccination. We are currently studying simian retroviruses, bovine endogenous retroviruses, feline endogenous retroviruses, porcine endogenous retroviruses and koala retroviruses.

1) Early events in RD-114 virus infection in dogs: R. YOSHIKAWA, M. SHIMOJIMA, K. MAEDA, T. HAYASHI, M. MORIMOTO, Y. KISO, K. BABA, J. YASUDA and T. MIYAZAWA

The genomes of mammalian species contain enormous sequences derived from endogenous retroviruses (ERVs). Although many ERVs have lost their infectivity, several ERVs maintain their infectivity. All domestic cats have an infectious ERV, termed RD-114 virus and several feline cell lines (Crandell-Rees feline kidney cells: CRFK cells) produce infectious RD-114 virus (Yoshikawa *et al.*, (2010) *J. Clin. Microbiol.* 48: 3366-3369; Okada *et al.*, (2010) *Virus Res.* 155: 268-273). We previously found that several canine live attenuated vaccines were contaminated with an infectious RD-114 virus. We also reported that RD-114 virus efficiently infected and proliferated in canine fibroblast cells and canine ASCT1 and ASCT2, sodium-dependent neutral amino acid transporters, are functional receptors for RD-114 virus. By experimental infection using specific pathogen-free dogs, we confirmed that RD-114 virus infected canine white blood cells, testes, mesentery lymph nodes and spleen. However, RD-114 virus could not be re-isolated from peripheral blood mononuclear cells (PBMCs). When we investigated whether RD-114 virus replicated in canine PBMCs in vitro, RD-114 virus hardly proliferated in canine PBMCs. We also found that the replication of RD-114 virus was inhibited by antiviral factors such as canine APOBEC3H and tetherin/BST2 (BST2). The expression level of canine APOBEC3H and BST2 was relatively high in canine PBMCs. In addition, we detected anti-RD-114 Env and neutralizing antibodies in plasmas of several dogs inoculated with RD-114 virus. Therefore, we conclude that the infection and replication of RD-114 viruses are controlled by both antiviral factors and acquired immunity in dogs.

2) Bovine trophoblastic cell differentiation and binucleation involves enhanced endogenous retrovirus element expression: K. KOSHI, Y. SUZUKI, Y. NAKAYA, K. IMAI, M. HOSOE, T. TAKAHASHI, K. KIZAKI, T. MIYAZAWA and K. HASHIZUME

Endogenous retrovirus (ERV) envelope (*env*) genes are involved in the differentiation of trophoblastic cells in humans and mice. However, there is limited information about their roles in ruminant trophoblastic cells. Thus, we attempted to explore the possible roles of ERV elements in the binucleation of bovine trophoblastic cells using in vitro bovine trophoblastic (BT) cell lines. In this study, blastocysts and elongated embryos were obtained from Japanese Black cows, and endometrial and fetal membrane tissues were collected from day 17 to 37 of gestation. The gene expression levels of four ERV elements, *bERVE* (bovine endogenous retrovirus envelope element-like transcript) -A, *bERVE-B*, BERV (bovine endogenous retrovirus) -K1 *env*, and BERV-K2 *env*, were analyzed in the fetal and endometrial tissue and cultured BT cell lines using quantitative RT-PCR. On-Matrigel gel and on-collagen gel culturing were used to induce binucleate cell (BNC) formation in the BT cell lines. How the culture conditions affected the expression of BNC-specific genes and ERV elements was examined by quantitative RT-PCR and immunocytochemistry. *bERVE-A*, *bERVE-B*, BERV-K1 *env*, and BERV-K2 *env* were expressed in almost all BT cell lines; however, only *bERVE-A* and BERV-K1 *env* were detected in trophoblastic tissues during the peri-implantation period. In the on-Matrigel cultures, the expression levels of BNC-specific genes and molecules were enhanced in the BT cells. The expression levels of *bERVE-A* and BERV-K1 *env* were also increased in the BT cells during on-Matrigel culturing. The BT cell expression levels of these ERV elements were consistent with those of BNC-specific genes during on-Matrigel culturing ($P<0.01$). These results suggest that *bERVE-A* and BERV-K1 *env* are involved in the expression of BNC-specific genes and the progression of bovine trophoblastic cell binucleation, as their expression levels increased during periods of increased BNC-specific molecule expression, which is strongly suggestive of the development of BNC from mononucleate trophoblastic cells. The on-Matrigel culture system is a convenient in vitro tool for studying bovine trophoblastic cell lineages.

3) Dynamic evolution of endogenous retrovirus-derived genes expressed in bovine conceptuses during the period of placentation: S. NAKAGAWA, H. BAI, T. SAKURAI, Y. NAKAYA, T. KONNO, T. MIYAZAWA, T. GOJOBORI and K. IMAKAWA

In evolution of mammals, some of essential genes for placental development are known to be of retroviral origin, as syncytin-1 derived from an envelope (*env*) gene of an endogenous retrovirus (ERV) aids in the cell fusion of placenta in humans. Although the placenta serves the same function in all placental mammals, *env*-derived genes responsible for trophoblast cell fusion and maternal immune tolerance differ among species and remain largely unidentified in the bovine species. To examine *env*-derived genes playing a role in the bovine placental development comprehensively, we determined the transcriptomic profiles of bovine conceptuses during three

crucial windows of implantation periods using a high-throughput sequencer. The sequence reads were mapped into the bovine genome, in which ERV candidates were annotated using RetroTector[®] (7624 and 1542 for ERV-derived and *env*-derived genes, respectively). The mapped reads showed that about 18 percent (284 genes) of *env*-derived genes in the genome were expressed during placenta formation, and about four percent (63 genes) were detected for all days examined. We verified three *env*-derived genes that are expressed in trophoblast cells by polymerase chain reaction. Out of these three, the sequence of *env*-derived gene with the longest open reading frame (named BERV-P *env*) was found to show high expression levels in trophoblast cell lines, and to be similar to those of syncytin-Car1 genes found in dogs and cats, despite their disparate origins. These results suggest that placentation depends on various retrovirus-derived genes that could have replaced endogenous predecessors during evolution.

4) Identification of feline ASCT1 and ASCT2 as RD-114 virus receptors: S. SHIMODE, R. YOSHIKAWA and T. MIYAZAWA

All domestic cats have an infectious endogenous retrovirus, termed RD-114 virus. Several feline cell lines produce RD-114 virus. Recently, we found that several live attenuated vaccines produced using feline cells were contaminated with infectious RD-114 virus. RD-114 virus is known to infect non-feline cell lines, including human and canine cell lines. The human and canine receptors for RD-114 virus are two types of sodium-dependent neutral amino acid transporters, termed ASCT1 and ASCT2. RD-114 virus utilizes both ASCT1 and ASCT2, but the virus uses ASCT2 more efficiently than ASCT1. Although RD-114 virus infects feline cell lines, there has been no report on the virus receptors in cats. Here we identified feline ASCT1 (FeASCT1) and ASCT2 (FeASCT2) as RD-114 virus receptors. We found that both FeASCT1 and FeASCT2 function as RD-114 receptors. Amino acid sequences of FeASCT1 and FeASCT2 show 77-95% identities with other mammals' ASCT1 and ASCT2. Interestingly, RD-114 virus has an immunosuppressive domain in its transmembrane envelope protein. Therefore, RD-114 virus may induce immunosuppression, if it has adapted to grow efficiently in cats. We will determine whether RD-114 virus is pathogenic in cats in the future.

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**CENTER FOR HUMAN RETROVIRUS RESEARCH
LABORATORY OF VIRAL PATHOGENESIS**

Goal of our research group: To determine the molecular mechanism of viral replication and pathogenesis.

1) HIV Restriction Factor and Cellular Response:

P. GEE, Y. KANEMURA, N. KASAI, H. EBINA and Y. KOYANAGI

It has been shown that SAMHD1, a deoxynucleoside triphosphate (dNTP) triphosphohydrolase, prevents reverse transcription of human immunodeficiency virus type 1 (HIV-1) RNA in myeloid cells and resting CD4⁺ T cells. Both clearance of cellular nucleic acids and the inhibition of HIV-1 are assumed to result in inducing the persistent immune activation. To understand this mechanism we explored SAMHD1-associating molecules in human cells and found several candidates. SAMHD1 is likely to have a modulator function in cellular responses in addition to its enzymatic activity, suggesting that this protein may be an important player in cells by regulating immune responses during viral infection.

2) HIV-1-Host Evolutionary Interaction: K. SATO, J. SHIBATA, T. KOBAYASHI, M. FUKUHARA, Y. KIMURA, N. MISAWA and Y. KOYANAGI

APOBEC3G is known as the first restriction factor that inhibits HIV-1 replication by inducing G-to-A hypermutation in the viral genome, while BST2 has been identified as another restriction factor that impairs the release of nascent virions by tethering them on the surface of infected cells. To counter these restriction factors, HIV-1 has equipped itself with its own weapons: Vif degrades APOBEC3G, while Vpu antagonizes BST2. These findings have allowed us to further our understanding of virus-host interactions. Interestingly, *vif*-deficient HIV-1 is incapable of replicating in APOBEC3G-expressing human cells both *in vitro* and *in vivo*, indicating that APOBEC3G is an authentic restriction factor and provides intrinsic immunity against HIV-1. However, it remains unclear how APOBEC3G is associated with intrinsic immunity. Using humanized mice, we have a series of projects aimed at elucidating how APOBEC3G attacks HIV-1 replication in human T cells and myeloid cells *in vivo*. In contrast, *vpu*-deficient HIV-1 is still able to replicate in BST2-expressing cells although BST2 clearly impairs the release of *vpu*-deficient HIV-1 virions. These observations indicate us that BST2-mediated anti-HIV-1 activity is vulnerable, and that Vpu is dispensable for HIV-1 infection. It has been shown that chimpanzee simian immunodeficiency virus (SIVcpz) is the ancestor of HIV-1 and SIVcpz Nef, not Vpu, possesses anti-chimpanzee BST2 activity. This finding has given rise to several questions. Why has HIV-1

Vpu acquired the counteracting potential against human BST2? Was it necessary or important for HIV-1 to become a successful pathogen? Or is the relationship between HIV-1 Vpu and human BST2 still “immature”? From comparative investigation of SIVcpz and HIV-1, we are focusing on the evolutionary interplay between human and chimpanzee Vpu and BST2.

3) HIV-1 Alternative Integration: H. EBINA, Y. KANEMURA and Y. KOYANAGI

Because retroviral cDNA integration is crucial for its replication, several anti-integrase (IN) compounds have been generated and shown to provide a significant clinical outcome and by rapidly reducing viral load in HIV-1-infected individuals from taking a daily anti-IN compound in combination with other retroviral drugs. However, it is known that exogenous DNA has the potential to integrate into the host chromosome through the DNA repair system of non-homologous end joining and/or homologous recombination pathway independent of IN. Therefore, we assessed the ability of HIV-1 to establish infection in the presence of IN inhibitors. We observed a low, yet clear infection of IN inhibitor-treated cells that had a very similar integration level to that of IN-deficient HIV-1, D64A. More importantly, the IN-independent integration could be significantly enhanced by the pretreatment of cells with DNA-damaging agents. These results suggest that retroviral cDNA is inserted into the host chromosome through host DNA repair machinery aside from the IN-dependent pathway. We also found that the HIV-1 provirus generated through the IN-independent pathway has the potential to produce progeny viruses. (Ebina et al. *Virology*, 2012)

4) Experimental-Mathematical Investigation of Enterovirus 71: M. FUKUHARA, K. SATO, S. IWAMI and Y. KOYANAGI

Enterovirus 71 (EV71) is the causative agent of hand-foot-and-mouth disease and can trigger viral encephalitis. EV71 outbreaks are a major public health concerns in Asia-Pacific countries. We created a novel mathematical model of viral replication that is incorporated from EV71 experimental data. Subsequently, we found that EV71 productivity and transmissibility but not the cytotoxicity are drastically different among EV71 strains and can be associated with their epidemiological backgrounds. The synergistic experimental-mathematical strategy is a powerful tool and will be used to quantitatively investigate the dynamics of EV71 infections not normally accessible by conventional experimental strategies. (Fukuhara et al. *J. Virol.*, 2013)

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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, Y. MITOBE, Y. MITAGAMI, H. KINOSADA, M. TANABE and M. MATSUOKA

Human T-cell leukemia virus type 1 (HTLV-1) causes not only a neoplastic disease, adult T-cell leukemia (ATL), but also inflammatory diseases including HTLV-1 associated myelopathy/tropical spastic paraparesis and uveitis. HTLV-1 encodes regulatory genes (*tax* and *rex*) and several accessory genes, such as *p30*, *p12*, *p13* and *HTLV-1 bZIP factor (HBZ)*. Tax and HBZ are thought to play important roles in HTLV-1-induced pathogenesis. Tax expression is frequently silenced in ATL cells while the *HBZ* gene transcription is detected in all of the ATL cell lines and primary ATL cases, indicating that HBZ is critical for ATL leukemogenesis. Recently, we have reported that HBZ-transgenic (Tg) mice develop T-cell lymphomas and systemic inflammatory diseases, such as dermatitis and alveolitis. This data suggests that HBZ is closely linked with both T-cell lymphoma and inflammatory diseases. Immunological analyses showed that regulatory T cells (Tregs) were increased in HBZ-Tg. 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 chronic inflammation and malignant transformation *in vivo*. Our observations imply that *HBZ* has a crucial role in HTLV-1-associated pathogenesis. We are now investigating about the association between inflammation and oncogenesis induced by HBZ using our mouse model.

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

HBZ has contradictory effects on various signaling pathways compared with Tax. Tax activates both the classical and alternative NF- κ B pathways, whereas HBZ specifically suppresses the classical NF- κ B pathway by targeting p65. Tax suppresses TGF- β signaling through inhibition of Smad proteins, although HBZ can form a complex with Smad2/3 and p300 to activate the transcription of TGF- β -responsive genes, such as *Foxp3*. Recently we have reported that HBZ interacts with several Wnt pathway-related proteins, such as DAPLE, LEF-1, and TCF-1, and inhibits the canonical Wnt pathway, whereas Tax activates it. Interestingly, HBZ up-regulates a noncanonical Wnt ligand, Wnt5a, and supports proliferation and migration of ATL cells, suggesting

one of the mechanisms of HBZ-mediated oncogenesis. We identified other cellular targets of HBZ by yeast two-hybrid screening and the transcriptional profiling. We are analyzing their significances in ATL leukemogenesis.

3) Development of new therapeutic strategies for HTLV-1 infection using the nonhuman primate model: J. YASUNAGA, P. MIYAZATO, J. TANABE, K. SUGATA, G. MA, M. MIURA and M. MATSUOKA

Both HTLV-1 and simian T-cell leukemia virus type 1 (STLV-1) belong to the Delta type retrovirus, and the structure of these viruses is very close. We noticed that approximately 60% of Japanese macaques are naturally infected with STLV-1, and found that the dynamics of STLV-1-infected cells in the monkeys are quite similar to that of HTLV-1-infected cells in the human carriers. STLV-1 infected mainly CD4⁺ T-cells, and induced the clonal proliferation of infected cells. In a STLV-1 infected Japanese macaque, T-cell lymphoma developed. STLV-1 Tax and STLV-1 bZIP factor (SBZ) have the same molecular functions of HTLV-1 Tax and HBZ, respectively; Tax of HTLV-1 and STLV-1 activate NF- κ B, AP-1, NFAT, and Wnt pathways, whereas HBZ and SBZ inhibit them. In addition, HBZ and SBZ activate TGF- β signaling and Tax of both viruses suppress it. These observations indicate that STLV-1-infected Japanese macaque is a highly valuable animal model to study the association between viral pathogenesis and immune response in HTLV-1 carriers, and to develop new antiviral treatments.

4) Characterization of DNA repair proteins involved in retroviral integration: M. MIURA 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. Murine leukemia virus (MLV) prefers the regions near transcriptional start sites, CpG islands and DNase hyper-sensitive sites for its integration although 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.

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

Current anti-retroviral therapy (ART) potently suppresses HIV-1 replication, and dramatically improves prognosis of HIV-1-infected individuals. However, long-term antiviral therapies induce the emergence of drug-related adverse effects as well as drug-resistant viruses, which are a major obstacle of efficient therapies. In order to develop new small-molecule-based anti-HIV drugs, we have screened tens of thousands of compounds and several with anti-HIV activity were identified. Among them, some compounds seemed to inhibit HIV replication by a novel mode of action. From the results of antiviral spectrum, these compounds showed inhibitory activity against not only HIV but also some other enveloped viruses. Now, we are going to identify the mechanism of action and their efficacy *in vivo* using a mouse model.

6) Comparison of the potency of antiviral agents in cell-free infection versus cell-to-cell transmission of HIV: K. SHIMURA and M. MATSUOKA

HIV infects target cells by cell-free infection as well as cell-to-cell transmission. The latter mechanism involves the efficient spread of HIV through the virological synapses formed between HIV-infected and uninfected cells. To date, evaluation of the anti-HIV activity of different compounds has been performed mainly using the cell-free infection model. However, some reports published recently described that the inhibitory potency of some anti-HIV drugs was affected by different HIV infection pathways. In order to precisely reveal this phenomenon, we are studying the antiviral activity of several kinds of anti-HIV drugs under both cell-free and cell-to-cell transmission using multicolor flow cytometric analysis.

7) Identification of potential inhibitors for simian retrovirus type 4: H. TOGAMI, K. SHIMURA, and M. MATSUOKA

Recently, Japanese monkeys housed in the Primate Research Institute, Kyoto University, died of a hemorrhagic syndrome. Simian retrovirus type 4 (SRV-4) was identified as the causative virus of this disease. It is important to study the prevention/treatment strategy for controlling SRV-4 infection among this population. We extensively evaluated anti-SRV-4 activity using a series of anti-HIV drugs. Among them, zidovudine (AZT), tenofovir disoproxil fumarate (TDF), and raltegravir (RAL) efficiently inhibited SRV-4 infection. To our surprise, AZT and TDF showed only

minimal inhibitory effect under cell-to-cell SRV-4 transmission, while RAL was still active even in this condition. These results indicate that RAL is a key drug for controlling SRV-4 infection.

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- 川月章弘、安永純一郎、松岡雅雄：HTLV-1 bZIP Factor(HBZ) Interacts with Enhancer of
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1 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) Posttranscriptional Regulation of Histone Methyltransferase GLP in Embryonic Mouse Germ cells. K. DEGUCHI, M. TACHIBANA, Y. SHINKAI

The epigenetic status of germ cells change dynamically during development. In this study, we analyzed the dynamics of histone H3 lysine 9 dimethylation (H3K9me2), a highly conserved mark of epigenetic silencing, and the expression of 2 lysine methyltransferases, G9a/Ehmt2/KMT1C and GLP/Ehmt1/KMT1D, in murine male embryonic germ cells after sex determination. Our previous studies have established that G9a and GLP are the primary enzymes for H3K9me2, and predominantly exist as a G9a-GLP heteromeric complex that appears to be a functional H3K9 methyltransferase *in vivo*. During the embryonic developmental stages E13.5 to E18.5 in mice, gonadal H3K9me2 levels were substantially lower in germ cells than in cells of non-germ lineage. Immunohistochemical analysis showed that during this phase in development, GLP but not G9a level was also significantly lower in male germ cells. However, *GLP* mRNA was present in E13, E16 and postnatal day 0 (P0) male germ cells, with levels similar to those in cells of non-germ lineage. Interestingly, GLP is upregulated in embryonic male germ cells deficient for *Nanos2*, which encodes a germ cell-specific RNA-binding protein. Our data suggest that GLP protein expression is posttranscriptionally regulated in murine embryonic male germ cells after sex determination and that low H3K9me2 level results from the absence of GLP (severe reduction of the G9a-GLP heteromeric complex).

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/SFI-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. Using this method, we checked epigenetic status of Sry locus in gonadal somatic cells. We revealed histone H3 lysine 4 is dimethylated in these cells at E11.5, suggesting possible involvement of histone methylation in Sry activation.

LIST OF PUBLICATIONS

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Makoto Tachibana The histone demethylase Jmjd1a controls sex determination via activating Sry expression The 6th International symposium on vertebrate sex determination, Kona, Hawaii, USA (April 23~27, 2012)

EXPERIMENTAL RESEARCH CENTER FOR INFECTIOUS DISEASES
LABORATORY OF PRIMATE MODEL

- 1) **Lymph nodes harbor viral reservoirs that cause rebound of plasma viremia in SIV-infected macaques upon cessation of combined antiretroviral therapy: M. HORIIKE, S. IWAMI, M. KODAMA, A. SATO, Y. WATANABE, M. YASUI, Y. ISHIDA, T. KOBAYASHI, T. MIURA, and T. IGARASHI.**

Because of the existence of viral reservoir, current combined anti-retroviral therapy (cART) is unable to eradicate virus from infected individuals. Toward functional cure for AIDS, we set out to identify viral reservoir during cART, employing SIV/macaque model for AIDS. Long-term cART was achieved by devising administration regimen. Systemic analysis at the completion of therapy revealed that viral RNA was expressed in lymphatic tissues, although plasma viral burdens were under detection limit. Failure to histochemically detect virus-infected cells prompted us to an alternative approach, an analysis during viral rebound upon cessation of cART. We detected viral protein-expressing T-lymphocytes in the follicles of mesenteric lymph nodes of an animal experiencing viral rebound.

It is implicated that lymph node harbor virus producing reservoirs once drug is withdrawn from treated individuals. The tissue could serve as a major reservoir even during cART. (This study was published in *Virology*.)

- 2) **Synthesis and biological evaluation of deoxy-hematoxylin derivatives as a novel class of anti-HIV-1 agents: H. ISHII, H. KOYAMA, K. HAGIWARA, T. MIURA, G. XUE, Y. HASHIMOTO, G. KITAHARA, Y. AIDA, and M. SUZUKI**

Development of combination anti-retroviral therapy (cART) has transformed acquired immune deficiency syndrome (AIDS) a manageable disease. Currently, there are 30 anti-human immunodeficiency virus (HIV) drugs, belonging to several classes of action, available to HIV-infected patients. However, individual underlying condition limits applicable drugs and eventual emergence of drug resistant mutant virus urges continuous development of new anti-HIV drugs.

As an attempt to generate new anti-HIV-1 drug, we systematically deoxygenated hematoxylin, which has been used not only as mordant dye but also as herbal medicine for digestive diseases, and examined the derivatives for anti-HIV activities. One of the compounds exhibited favorable profiles, such as lower cytotoxicity, greater inhibition of nuclear import of preintegration

complex and viral replication compared to the starting material.

This study would provide an example of structure-activity relationship-guided development and elaboration of a new class of anti-HIV-1 agents. (This study was published in *Bioorganic & Medicinal Chemistry Letters*)

3) Identifying viral parameters from in vitro cell cultures: S. IWAMI, P.D. HOLDER, C. A.BEAUCHEMIN, S. MORITA, T. TADA, K. SATO, R.J.DE BOER, K. AIHARA, Y. KOYANAGI, T. IGARASHI, and T. MIURA

Virus replication assay in cell culture is a vital tool to assess biological properties of the infectious agent. Although it is a sum of complex interplay of parameters such as target cell infection, virus production and cell death, we only take face value and do not know these underlying processes. For better understanding of virus replication, we employed mathematical approach to quantitatively describe these processes.

Fitting a mathematical model with experimental data from SHIV KS661 replication in macaque HSC-F cells, we successfully estimated an infected cell half-life of 14.1 hours, a half-life of SHIV KS661 infectiousness of 17.9 hours, a virus burst size of 22.1 thousand RNA copies and a basic reproductive number of 62.8. Furthermore, computation revealed that one out of 350 progeny virus particles of SHIV KS661 was infectious. These parameters would assist our understanding of several aspects in virus replication, such as “eclipse phase” and division of virus particles into infectious and non-infectious. The methodology and parameters would also guide to better understand the pathogenesis of SHIV and HIV infection.

(This study was published in *Frontiers in Microbiology and Retrovirology*)

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EXPERIMENTAL RESEARCH CENTER FOR INFECTIOUS DISEASES

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- 仲屋友喜、越勝男、中川草、木崎景一郎、小林剛、橋爪一善、宮沢孝幸：ウシ科動物の進化とウシ内在性レトロウイルス-K1 の関係性 第 154 回日本獣医学会学術集会 2012 年 9 月 14-16 日
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- 加藤文博、川岸崇裕、日向亮輔、大石真也、藤井信孝、三浦智行、小林剛、五十嵐樹彦：抗デングウイルス活性を有するかご物の探索および作用機序の解析 第 60 回日本ウイルス学会学術集会、大阪、2012 年 11 月 13 日
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- 大附寛幸、一瀬裕太郎、小林剛、原田恵嘉、吉村和久、鳴海哲夫、玉村啓和、松下修三、

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米田舞、大附寛幸、一瀬裕太郎、松田健太、松下修三、五十嵐樹彦、三浦智行：新規 CCR5 指向性 SHIV のサルへの順化と中和抵抗性の解析 第 155 回日本獣医学会、東京、2013 年 3 月 28-30 日

CENTER FOR EMERGING VIRUS RESEARCH

I . First Group

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. Bone marrow stromal antigen 2 (BST2; also known as tetherin) was identified as an inhibitory cellular protein of human immunodeficiency virus type 1 (HIV-1) replication, which tethers virions to the cell surface to prevent their release. On the other hand, the HIV-1 accessory protein, viral protein U (Vpu), has the ability to downregulate and counteract BST2. Vpu also possesses the ability to downmodulate cellular CD4 and SLAMF6 molecules expressed on infected cells. However, the role of Vpu in HIV-1 infection *in vivo* remains unclear.

1) **Role of viral accessory proteins for HIV-1 infection *in vivo*: K. SATO, N. MISAWA, T. KOBAYASHI, J. SHIBATA, and Y. KOYANAGI**

Using a human hematopoietic stem cell-transplanted humanized mouse model, we demonstrate that Vpu contributes to the efficient spread of HIV-1 *in vivo* during the acute phase of infection. Although Vpu did not affect viral cytopathicity, target cell preference, and 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 HIV-1-infected mice. We provide a novel insight suggesting that Vpu concomitantly downregulates BST2 and CD4, but not SLAMF6, from the surface of infected cells. Furthermore, we show evidence suggesting that BST2 and CD4 impair the production of cell-free infectious virions but do not associate with the efficiency of cell-to-cell HIV-1 transmission. Taken together, our findings suggest that Vpu downmodulates BST2 and CD4 in infected cells and augments the initial burst of HIV-1 replication *in vivo*. This is the first report demonstrating the role of Vpu in HIV-1 infection in an *in vivo* model.

II. Second Group

The aim of research in this group is to clarify the survival strategies of gram-negative bacteria. Various bacterial species in this phylum have been identified as causative agents of many infectious diseases. Therefore, it is of great importance to understand their survival strategy to cope with emerging infectious diseases. The cell structure of gram-negative bacteria is characterized by 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 cytoplasm. Because the outer membrane is essential for the growth of gram-negative bacteria, knowledge of the biosynthesis and quality control systems of the outer membrane would contribute to develop new drugs against

gram-negative pathogenic bacteria. We study these systems using *Escherichia coli*, the most extensively studied model organism to date.

- 1) **Characterization of a novel protease involved in biogenesis of the *Escherichia coli* outer membrane proteins.** S. NARITA, T. SUZUKI¹, N. DOHMAE¹ and Y. AKIYAMA² (¹Biomolecular Characterization Team, RIKEN Advanced Science Institute, ²Department of Viral Oncology, IVR)

To maintain the function of the outer membrane as a permeability barrier with selectivity, gram-negative bacteria are equipped with quality control systems that sense and combat against defects of outer-membrane constituents. When misfolding of outer membrane proteins occurs, RseA, the anti- σ^E factor, is sequentially cleaved by a periplasmic protease DegS and an inner-membrane protease RseP, which results in the activation of σ^E and the transcription of multiple genes involved in the extracytoplasmic stress responses. Although many genes have been identified as constituents of the σ^E regulon, not a few of them remain uncharacterized such as *yfgC*, which encodes a putative periplasmic protease. Here, we characterized this protein. The *Escherichia coli* $\Delta yfgC$ mutant showed increased sensitivity to detergents and antibiotics. Accordingly, proper folding of LptD, which is involved in the transport and assembly of lipopolysaccharide to the outer membrane, was also affected. These defects were suppressed by overexpression of LptE, an outer membrane lipoprotein involved in the assembly of LptD. Defects in outer membrane permeability of the $\Delta yfgC$ mutant were exacerbated by simultaneous disruption of genes encoding periplasmic chaperones or subunits of the BAM complex. These results indicate that YfgC may contribute to maintain the quality of the outer membrane by governing the assembly of outer membrane proteins.

LIST OF PUBLICATIONS

CENTER FOR EMERGING VIRUS RESEARCH

I . First Group

- Watanabe, T., Urano, E., Miyauchi, K., Ichikawa, R., Hamatake, M., Misawa, N., Sato, K., Ebina, H., Koyanagi, Y., and Komano, J. The hematopoietic cell-specific Rho GTPase inhibitor ARHGDIB/D4GDI limits HIV-1 replication. *AIDS Res. Hum. Retroviruses*, 28(8): 913-922, 2012.
- Sato, K., Misawa, N., Fukuhara, M., Iwami, S., An, D.S., Ito, M., and Koyanagi, Y. Vpu augments the initial burst phase of HIV-1 propagation and downregulates BST2 and CD4 in humanized mice. *J. Virol.*, 86(9): 5000-5013, 2012.

- Iwami, S., Holder, P.B., Beauchemin, A.A.C., Morita, S., Tada, T., Sato, K., Igarashi, T., and Miura, T. Quantification system for the viral dynamics of a highly pathogenic simian/human immunodeficiency virus based on an in vitro experiment and a mathematical model. *Retrovirology*, 9:18, 2012.
- Sato, K., Gee, P., and Koyanagi, Y. Vpu and BST2: still not there yet? *Front. Microbiol.*, 3:131, 2012.
- Iwami, S., Sato, K., de Boer, J.R., Aihara, K., Miura, T., and Koyanagi, Y. Identifying viral parameters from in vitro cell cultures. *Front. Microbiol.*, 3:319, 2012.
- 岩見真吾, 佐藤佳, 小柳義夫. ヒト化マウスを用いたヒト特異的疾患研究のイノベーション: 応用数理と実験医学の融合. *応用数理 (日本応用数理学会誌)*, 22巻2号, pp.7-16, 2012.

- Sato K., Misawa N., Satou Y., Matsuoka M., Ito M. and Koyanagi Y. Induction of immune activation by the depletion of regulatory CD4+ T cell during acute HIV-1 infection in humanized mouse model. 19th Conference on Retroviruses and Opportunistic Infections (CROI), poster, Seattle, 2012 年 3 月 5 日
- Sato K. HIV modeling in humanized mice I. 1st Samsung Humanized Mice Symposium, Seoul, 2012 年 4 月 14 日
- Sato K., Misawa N., Fukuhara M., Iwami S., An D.S., Ito M. and Koyanagi Y. Positive contribution of HIV-1 Vpu for viral propagation *in vivo*. Retroviruses Meeting Cold Spring Harbor, poster, New York, USA, 2012 年 5 月 24 日
- 佐藤佳. 感染急性期の HIV-1 増殖における制御性 T 細胞の動態と Vpr の寄与、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」、札幌. 2012 年 6 月 19 日
- 佐藤佳. ヒト化マウスを用いた、EBウイルス関連血球貪食リンパ組織球症の新規動物モデル、第8回麒麟塾、東京. 2012年7月7日
- 佐藤佳. ヒト化マウスモデルを用いたHIV複製定量系とその応用、第22回日本数理生物学会大会、岡山. 2012年9月10日
- 佐藤佳、三沢尚子、佐藤賢文、松岡雅雄、伊藤守、小柳義夫. Vprの制御性T細胞特異的な消耗促進作用による生体内HIV-1増殖亢進、第60回日本ウイルス学会学術集会、大阪. 2012年11月14日
- 福原充子、佐藤佳、吉川禄助、宮沢孝幸、小柳義夫. ベータレトロウイルスの新規抗BST2活性、第60回日本ウイルス学会学術集会、大阪. 2012年11月15日
- 佐藤佳、三沢尚子、福原充子、岩見真吾、Dong Sung An、伊藤守、小柳義夫. 生体内HIV-1複製におけるVpuの機能解析、第26回日本エイズ学会学術集会、日吉. 2012年11月24日

福原充子、岩見真吾、佐藤佳、小柳義夫. 数理モデルを用いたエンテロウイルス71複製ダイナミクスの解析、第35回日本分子生物学会、福岡. 2012年12月11日
佐藤佳、三沢尚子、福原充子、岩見真吾、伊藤守、小柳義夫. ウイルス性膜タンパク質Vpuによる生体内HIV-1増殖促進作用、第35回日本分子生物学会、福岡. 2012年12月12日

II. Second Group

Tao, K., Narita, S., Tokuda, H. Defective lipoprotein sorting induces *lolA* expression through the Rcs stress response phosphorelay system. J. Bacteriol., 194: 3643-3650, 2012.

大門康志、成田新一郎、秋山芳展：大腸菌における σ^E 依存性表層ストレス応答と toxin-antitoxin system の関わり. 第9回21世紀大腸菌研究会、長浜、2012 年6月21日-22日

Shin-ichiro Narita and Yoshinori Akiyama.: Characterization of YfgC, a protease homolog involved in assembly of the *Escherichia coli* outer membrane proteins. Gordon Research Conference on Bacterial Cell Surfaces, West Dover, VT, USA, June 24-29, 2012.

成田新一郎、秋山芳展：大腸菌外膜タンパク質の品質管理にかかわる新規プロテアーゼホモログBepAの解析. 2012年度国立遺伝学研究所研究会「代謝、増殖、分裂研究会」、三島、2012年12月8日-9日

大門康志、成田新一郎、志波 優、吉川博文、秋山芳展：大腸菌における σ^E の必須性と toxin-antitoxin システムの関わり. 2012年度国立遺伝学研究所研究会「代謝、増殖、分裂研究会」、三島、2012年12月8日-9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 Konaka and Miyachi. Results of last three years are as follows.

1) Freezing embryos

2010	101 strains	18,620 embryos
2011	117 strains	25,130 embryos
2012	140 strains	32,836 embryos

2) Transgenic mouse production with cloned DNAs

	No of constructs	No of embryos injected	No of transgenic pups obtained
2010	90	32,875	124(0.3%)
2011	81	29,031	227(0.8%)
2012	77	31,452	176(0.6%)

3) Production of chimeric mouse

	No of ES clones	No of embryos injected	No of coatcolor chimera obtained
2010	106	7,106	394(5.5%)
2011	107	5,828	324(5.5%)
2012	63	5,145	192(3.7%)

LIST OF PUBLICATIONS

Reproductive engineering team

Hara, T., S. Shitara, K. Imai, H. Miyachi, S. Konaka, H. Yao, S. Tani-Ichi, and K. Ikuta. 2012.

- Identification of IL-7-producing cells in primary and secondary lymphoid organs using IL-7-GFP knock-in mice. *Journal of Immunology* (Baltimore, Md.: 1950) 189 (4) (Aug 15): 1577-84.
- Imayoshi, I., K. Hirano, S. Konaka, H. Miyachi, and R. Kageyama. 2012. In vivo evaluation of PhiC31 recombinase activity in transgenic mice. *Neuroscience Research* 73 (2) (Jun): 106-14.
- Imayoshi, I., K. Hirano, M. Sakamoto, G. Miyoshi, T. Imura, S. Konaka, H. Miyachi, and R. Kageyama. 2012. A multifunctional teal-fluorescent Rosa26 reporter mouse line for cre- and flp-mediated recombination. *Neuroscience Research* 73 (1) (May): 85-91.
- Imayoshi, I., S. Tabuchi, K. Hirano, M. Sakamoto, S. Konaka, H. Miyachi, A. Yamanaka, and R. Kageyama. 2012. Light-induced silencing of neural activity in Rosa26 knock-in mice conditionally expressing the microbial halorhodopsin eNpHR2.0. *Neuroscience Research* (Mar 23).
- Liang, B., T. Hara, K. Wagatsuma, J. Zhang, K. Maki, H. Miyachi, S. Konaka, C. Yabe-Nishimura, S. Tani-Ichi, and K. Ikuta. 2012. Role of hepatocyte-derived IL-7 in maintenance of intrahepatic NKT cells and T cells and development of B cells in fetal liver. *Journal of Immunology* (Baltimore, Md.: 1950) 189 (9) (Nov 1): 4444-50.

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 Assistant Prof. 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, online reservation of seminar rooms, SSH and all Outgoing TCP services except for P2P. Main services are working on Sun Sparc platform with Solaris 10 and DELL with Linux.

This year we replaced a file server which had been used to backup user's directories, web pages and institute's local news regularly. Moreover, to prevent any unknown wifi routers or devices from being connected, we set up the MAC address filtering for our network. All IVR-LAN users are asked to make registration of their computers before connecting them on the net.

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 sequencing to find epigenetic regulations which might involved in cell differentiation.

LIST OF PUBLICATIONS

COMPUTER NETWORK

Tan S., Nishi M., Ohtsuka T., Matsui T., Takemoto K., Kamio-Miura A., Aburatani H., Shinkai Y. and Kageyama R. Essential roles of the histone methyltransferase ESET in the epigenetic control of neural progenitor cells during development. *Development* vol. 139:3806-3816, 2012

Tan S., Takemoto K., Matsui T., Kageyama R. and Shinkai Y. ESET-mediated endogenous retrovirus silencing. 63rd Fujihara Seminar, 京都, 2012年7月31日

STAFF CHANGES OF THE INSTITUTE

Appointments

During the period of January to December 2012, the following new staffs were appointed; Dr. Osamu Takeuchi as a Professor of Department of Biological Responses, Dr. Hiroaki Mitsuya as a Visiting Professor of Experimental Emerging Virus Research, Dr. Takao Masuda as a Visiting Associate Professor of Department of Biological Responses, Dr. McCloskey Asako as an Assistant Professor of Department of Genetics and Molecular Biology, Dr. Takashi Mino as an Assistant Professor of Department of Biological Responses, Dr. Kei Sato as an Assistant Professor of Center for Human Retrovirus Research, Dr. Yousuke Yamaoka and Dr. Akiko Makino as an Assistant Professor of Center for Emerging Virus Research, Drs. Kenji Inaba, Toru Minamino, Naoya Oohara, Naoto Ito, Kouji Moriishi, Yoshiharu Matsuura and Kouichi Watashi as a Lecture (part time) of Department of Viral Oncology, Dr. Gen Yamada as a Lecture (part time) of Department of Genetics and Molecular Biology, Dr. Fumitoshi Ishino as a Department of Cell Biology, Drs. Akio Adachi, Eiji Morita, Toshiki Watanabe and Shinichi Oka as a Lecture (part time) of Center for Human Retrovirus Research.

Departure

Dr. Youichi Shinkai moved to RIKEN, Dr. Isamu Matsunaga moved to Okatani Hospital, Dr. Hirotaka Kuwata moved to Showa university, Dr. Takeshi Kobayashi moved to Research Institute for Microbial Diseases, Osaka University, Dr. Toshiaki Tsubota moved to Graduate school of Medicine Kyoto University, Dr. Ayano Satsuka moved to Northwestern University, USA, Dr. Haruo Ohmori retired from the Institute in March, Drs. Masafumi Takiguchi, Yasushi Kawaguchi, Kenji Nakahigashi, Yasuhiko Horiguchi, Sho Yamasaki, Yasuhito Tanaka, Kazufumi Matsushita, Yutaro Kumagai, Yukihiro Nishiyama, Yutaro Kumagai, Yukihiro Nishiyama, Yoichiro Iwakura, Yoshiyuki Suzuki Tatsuo Shioda, Hirofumi Akari, Tatsuya Tsurumi, Tsuneo Morishima, Ikuo Wada and Koki Taniguchi left the Institute. 2012.

THE SCIENTIFIC LECTURES OF THE INSTITUTE FOR VIRUS RESEARCH

The annual scientific lecture of this Institute was held on July 26, 2012 at the Kyoto University Shirankaikan Inamori Hall.

Program

Opening Remarks: Masao Matsuoka

1. Molecular mechanism of inflammation control by innate immunity, Osamu Takeuchi, this Institute
2. What is osteoimmunology and where is it going? , Hiroshi Takayanagi, The University of Tokyo
3. RNA classification by the length and functionality, Mutsuhito Ohno, this Institute
4. Functional exploration of enigmatic noncoding RNAs as the genomic dark matter, Tetsurou Hirose, National Institute of Advanced Industrial Science and Technology

SEMINARS OF THE INSTITUTE FOR VIRUS RESEARCH

Thirty-three seminars were held at the Institute for Virus Research under the auspices of the Institute in 2012. Nineteen lectures were from abroad and fourteen others were from Japan.

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| January 11 | Dr. Sadayuki Ohkura, MRC, UK. "Mechanism of TRIM5/Fv1 recognition of retroviral capsid; what pattern do they recognise?" |
| February 21 | Dr. Shinji Masui, Kyoto University, Japan. "Molecular mechanisms for the maintenance of cellular pluripotency". |
| March 9 | Dr. Ikuo Wada, Fukushima medical university, Japan. "Molecular mechanisms for the maintenance of cellular pluripotency". |
| March 13 | Dr. Tetsuro Matsuzawa, Kyoto University, Japan. "Imagining ability: "kokoro" learned from Chimpanzee". |
| March 14 | Dr. François Guillemot, MRC National Institute for Medical Research, Mill Hill, UK. "Transcriptional regulation of stem cell divisions in the embryonic and adult brain". |
| March 15 | Dr. Yasuhiro Ikeda, Mayo Clinic, USA. "A novel gamma retroviral cis-acting element controls nuclear export of viral transcripts". |
| March 15 | Dr. Masafumi Takiguchi, Japan. "Selection and recognition of escape HIV-1 mutants". |
| March 22 | Dr. Sho Yamasaki, Kyusyu University, Japan. "C-type lectin-mediated pathways for host defense against Mycobacterium tuberculosis". |
| April 4 | Dr. Naoya Tsurushita, JN Biosciences LLC, Japan. "Current state of biotechnology in USA and Japan". |

- May 28 Dr. Hitoshi Sakano, The University of Tokyo, Japan. “Developmental Regulation of Neural Circuit Formation in the Mouse Olfactory System”.
- June 13 Dr. Toshiaki Nakashiba, RIKEN-MIT Center, USA. “The role of dentate gyrus granule cells in pattern separation and pattern completion”.
- June 19 Dr. Anish Sen Majumdar, Stempeutics Research, India. “A Phase I/II Clinical Trial to Assess the Safety and Efficacy of Bone Marrow Derived Allogeneic “Human Mesenchymal Stem Cells” in Patients with Critical Limb Ischemia”.
- July 5 Dr. Mitsuru Matsumoto, Tokushima University, Japan. “Roles of thymic epithelial cells in the medulla for the establishment of self-tolerance”.
- July 7 Dr. Taekjip Ha, University of Illinois at Urbana-Champaign, USA. “Towards single molecule systems biology”.
- July 12 Dr. Takeshi Yoshida, NIAID, USA. “Contribution of HIV-1 Vpu for virus release”.
- July 13 Dr. Benjamin David Simons, Cambridge University, UK. “ Strategies of stem cell self-renewal: from maintenance to cancer”.
- July 20 Dr. Tohru Minamino, Osaka University, Japan. “Molecular Mechanism of Bacterial Flagellar Type III Protein export”.
- July 27 Dr. Pierre P. Vanderhaeghen, University of Brussels, Belgium. “Mechanisms of specification of cortical neurons, from mouse to man”.
- July 31 Dr. Takashi Irie, Hiroshima University, Japan. “The functions of accessory proteins implicate the life cycle of Sendai virus”.

- September 14 Dr. Kenji Inaba, Kyusyu University, Japan. “Disulfide bond formation and cleavage systems involved in protein quality control”.
- September 28 Dr. Yoshinori Fukazawa, Oregon Health & Science University, USA. “Lymph node T cell responses predict the efficacy of live attenuated SIV vaccines”.
- October 4 Dr. Hisashi Akiyama, Boston University, USA. “CD169 is the dendritic cell receptor essential for HIV-1 *trans*-infection”.
- October 22 Dr. Didier Trono, École Polytechnique Fédérale de Lausanne, Switzerland. “KRAB’n’KAP: From controlling endogenous retroelements to supporting the replication dynamics of human viral pathogens”.
- October 29 Dr. Nicolas Chevrier, Harvard University, USA. “System-level analysis of the toll-like receptor network delineates viral and inflammatory sensing circuits”.
- October 29 Dr. Victor Appay, INSERM, France. “Deconvoluting CD8⁺ T-cell efficacy against HIV”.
- November 1 Dr. Charles R M Bangham, Imperial College London, UK. “Dynamics of HTLV-1 replication in vivo”.
- November 9 Dr. Benjamin Pfeuty, Lille University, France. “Modeling study of the coordination between cell proliferation and differentiation during embryonic development”.
- November 16 Dr. Naoya Tsurushita, The University of Tokyo, Japan, “Current state of biotechnology in USA and Japan”.
- November 22 Dr. Kenneth James Campbell, Children’s Hospital Research Foundation, USA. “Genetic control of neural diversity and circuit formation in the developing mouse telencephalon”.

- November 26 Dr. Goichi Miyoshi, New York University, USA. “Prox1 regulates the development of interneuron subtypes in the cerebral cortex”.
- December 4 Dr. François Clavel, INSERM, France. “HIV-1 susceptibility to human TRIM5 α : an unforeseen driving force in HIV disease pathogenesis”.
- December 7 Dr. Hiroshi Hanafusa, Nagoya University, Japan. “ROCO family kinase LRRK1 regulates endosomal trafficking of the EGF receptor” .
- December 27 Dr. Nobuhiko Kayagaki, Genentech Inc, USA. “Canonical and noncanonical inflammation pathways; identification of a novel inflammasome pathway”.