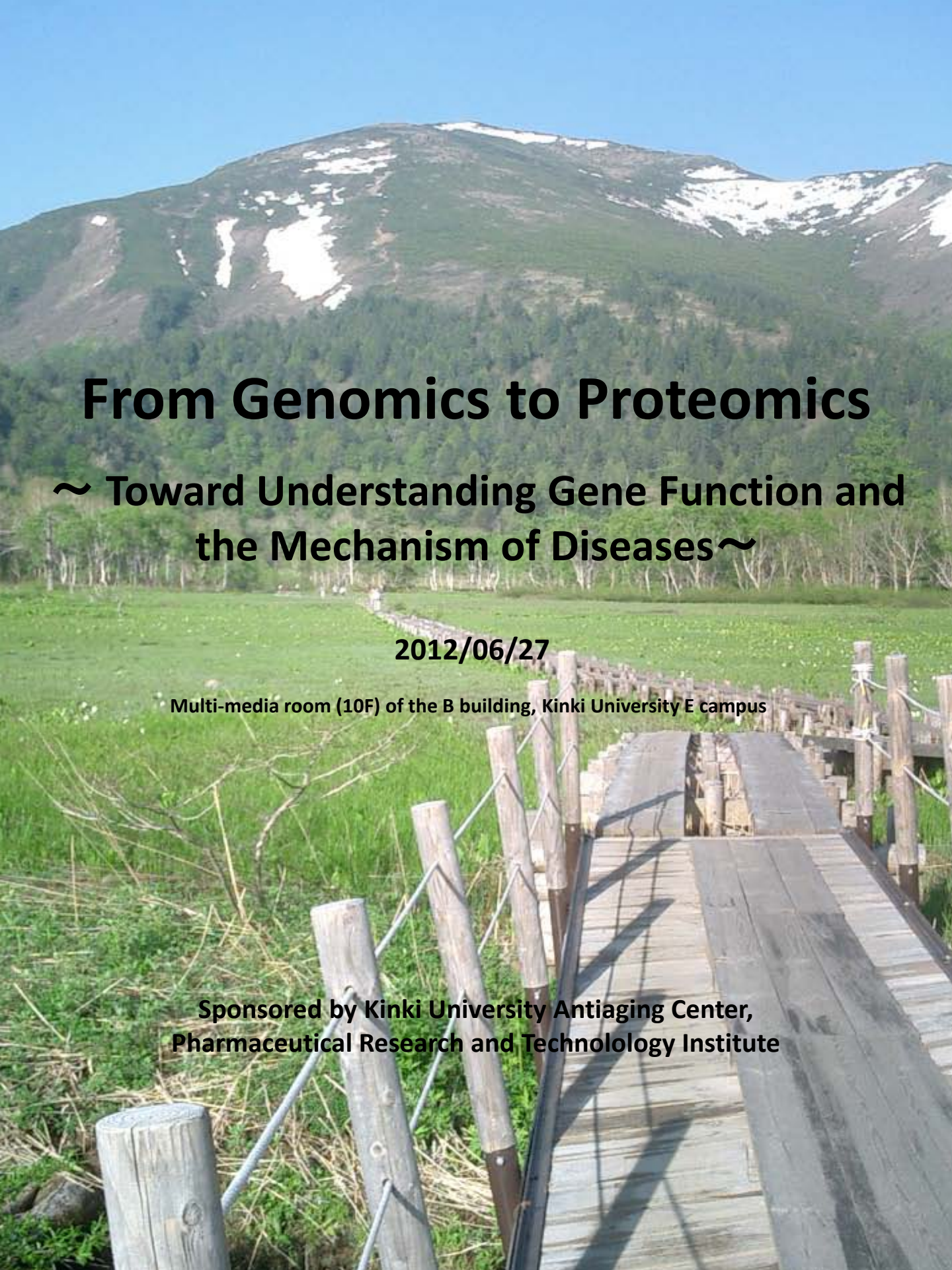

From Genomics to Proteomics

～ Toward Understanding Gene Function and the Mechanism of Diseases～

2012/06/27

Multi-media room (10F) of the B building, Kinki University E campus

**Sponsored by Kinki University Antiaging Center,
Pharmaceutical Research and Technolology Institute**

14:00-14:10 **Opening Remark** (Dr. Kakehi)

14:10-15:00 **Keynote Lecture: Dr. Osamu Takeuchi**
Chaired by Dr. Sugiura
Laboratory of Infection and Prevention,
Institute for Virus Research, Kyoto University
Posttranscriptional control of inflammation by an RNase, Regnase-1.

15:00-15:10 Tea break

15:10-15:50 **Dr. Kazuaki Kakehi** Chaired by Dr. Suzuki
Laboratory of Biopharmaco Informatics
Faculty of Pharmacy, Kinki University
Glycoproteomics for finding disease markers and its application to regulation studies on developmental biology

15:50-16:30 **Dr. Reiko Sugiura** Chaired by Dr. Fujiwara
Laboratory of Molecular Pharmacogenomics,
Faculty of Pharmacy, Kinki University
Functional genomics of calcineurin in model organism

16:30-16:40 Tea break

16:40-17:20 **Dr. Masaaki Miyazawa** Chaired by Dr. Yamada
Department of Immunology,
Kinki University Faculty of Medicine
Evolution of genetically determined resistance mechanisms to retroviral infections in humans and mice

17:20-17:25 **Concluding Remark** (Dr. Sugiura)

Dr. Osamu Takeuchi

Professor,
Laboratory of Infection and Prevention,
Institute for Virus Research,
Kyoto University
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Experience:

1997-2001 Ph.D. Graduate School of Medicine, Osaka University, Osaka, Japan
2002-2004 Post-Doctoral Fellow at the Dana-Farber Cancer Institute, Harvard University (Dr. Korsmeyer's laboratory), USA
2004-2007 Assistant Professor, Research Institute for Microbial Diseases (RIMD), Osaka University
2007-2012 Associate Professor, RIMD, Osaka University
2008-2012 Associate Professor, IFReC, Osaka University
2012- Professor, Institute for Virus Research, Kyoto University

Research Interests:

Innate immunity mediates inflammation in response to pathogen infection. However, the inflammation is tightly controlled to prevent autoimmune diseases. I am interested in the molecular mechanisms regulating inflammation appropriate, and I am focusing on posttranscriptional control of cytokine production by an RNase Regnase-1 critical for inhibiting autoimmune inflammatory disease in mice.

Selected Publications:

1. Miyake T, Satoh T, Kato H, Matsushita K, Kumagai Y, Vandenbon A, Tani T, Muta T, Akira S, Takeuchi O. (2010) IκBζ is essential for natural killer cell activation in response to IL-12 and IL-18. **Proc Natl Acad Sci U S A.** 107, 17680-5.
2. Satoh T*, Takeuchi O*, Vandenbon A, Yasuda K, Tanaka Y, Kumagai Y, Miyake T, Matsushita K, Okazaki T, Saitoh T, Honma K, Matsuyama T, Yui K, Tsujimura T, Standley DM, Nakanishi K, Nakai K, Akira S. (*equal contribution) (2010) The Jmjd3-Irf4 axis regulates M2 macrophage polarization and host responses against helminth infection. **Nat Immunol.** 11, 936-44.
3. Takeuchi O, Akira S. (2010) Pattern Recognition Receptors and Inflammation. **Cell**, 140, 805-820.
4. Satoh T, Kato H, Kumagai Y, Yoneyama M, Sato S, Matsushita K, Tsujimura T, Fujita T, Akira S, Takeuchi O. (2010) LGP2 is a positive regulator of RIG-I- and MDA5-mediated antiviral responses. **Proc Natl Acad Sci U S A**., 107, 1512-1517.
5. Kawagoe T*, Takeuchi O*, Takabatake Y, Kato H, Isaka Y, Tsujimura T, Akira S. (*equal contribution) (2009) TANK is a negative regulator of Toll-like receptor signaling and is critical for the prevention of autoimmune nephritis. **Nat Immunol.**, 10, 965-972.
6. Matsushita K*, Takeuchi O*, Standley DM, Kumagai Y, Kawagoe T, Miyake T, Satoh T, Kato H, Tsujimura T, Nakamura H & Akira S (*equal contribution) (2009) Zc3h12a is an RNase essential for controlling immune responses by regulating mRNA decay. **Nature**, 458, 1185-1190.
7. Kato H*, Takeuchi O*, Sato S, Yoneyama M, Yamamoto M, Matsui K, Uematsu S, Jung A, Kawai T, Ishii KJ, Yamaguchi O, Otsu K, Tsujimura T, Koh CS, Reis E Sousa C, Matsuura Y, Fujita T, Akira S (*equal contribution). (2006) Differential roles of MDA5 and RIG-I helicases in the recognition of RNA virusses. **Nature**., 441, 101-105.

Posttranscriptional control of inflammation by an RNase, Regnase-1.

Osamu Takeuchi

Laboratory of Infection and Prevention, Institute for Virus Research, Kyoto University, Japan

The innate immune system initially recognizes invasion of pathogens and evoke inflammation by producing a set of proinflammatory cytokines. Innate immunity utilizes receptors such as Toll-like receptors (TLRs) and RIG-I-like receptors (RLRs) for sensing pathogenic components, triggering intracellular signaling pathways which leads to transcription of cytokine genes. Although these responses are vital for clearing microbial infection, excess inflammation can be the cause of septic shock or autoimmunity. Therefore, inflammation is tightly controlled by a transcriptional and posttranscriptional mechanisms. We identified a novel RNase named Regnase-1 (or Zc3h12a) expressed in immune cells. Regnase-1 harbors an RNase domain and a CCCH-type zinc finger domain for Regnase-1 destabilizes interleukin-6 (IL-6) mRNA through its 3’ untranslated region in a manner dependent on the RNase activity. Reciprocally, deficiency in Regnase-1 in mice resulted in enhanced IL-6 mRNA expression in response to TLR stimulation, and Regnase-1-deficient mice spontaneously develop severe autoimmune inflammatory disease. These results indicate that Regnase-1 is essential for preventing unwanted inflammation by cleaving a set of mRNAs including Il6. We further found that the Regnase-1 protein expression is drastically changed in the course of inflammation. In response to TLR stimulation, Regnase-1 is rapidly phosphorylated by IkB kinase complex, and degraded in a ubiquitin-proteasome-dependent fashion in a kinetics similar to that of IkBa. These results demonstrate that the TLR signaling not only activates transcriptional expression of IL-6 mRNA, but also stabilizes IL-6 mRNA by degrading Regnase-1. In this symposium, I would like to discuss how posttranscriptional regulation controls inflammation and prevents autoimmune diseases.

MEMO

Glycoproteomics for finding disease markers and its application to regulation studies on developmental biology

Kazuaki Kakehi

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A number of clinical biomarkers for tumors are often glycoproteins such as carcinoembryonic antigen as a marker of colorectal cancer, cancer antigen 125 for diagnosis of ovarian cancer and prostate-specific antigen. In addition, aberrant glycosylation has been known to be associated with various human diseases.

Biosynthesis of the glycans in glycoproteins is regulated by combination of a number of factors such as (a) expression of related glycosyltransferases and/or glycosidases, (b) proper locations, and (c) the functional machinery of sugar nucleotides. Thus, microenvironmental changes in the synthesis of glycans greatly affect their synthetic efficiency and also their structures.

In this presentation, I will show newly developed methods for the analysis of glycans and its application to comprehensive analysis of the glycans derived from cancer cells and iPS cells. The methods described here will lead to finding biomarkers for various diseases as well as new evaluation/regulation tools for regenerative medicine and cell therapy.

Methods for the analysis of N-/O-glycans

In the series of our previous papers, we proposed methods for the analysis of glycans in biological samples. The methods involve several steps (i.e. releasing of glycans, separation, and structural analysis). Initially, total glycan pool obtained from the cell membrane fractions by enzymatic/chemical methods was fluorescently labeled with 2-aminobenzoic acid (2AA), and separated based on the number of sialic acid residues attached to the glycans using a serotonin-immobilized stationary phase. This step allows quantitative determination of total amount of glycans and also those of each category of glycans (asialo/high-mannose type glycans, mono-, di-, tri- and tetrasialoglycans). Then, the fractions are analyzed by LC/MS_n technique. Capillary affinity electrophoresis and digestion with specific exoglycosidases for linkage analysis are also employed.

Profiling studies of N-/O-glycans in cancer cells

We performed comparative studies on the distribution of N-/O-glycans in cell membrane fractions of some leukemia and epithelial cancer cells, and found specific distribution of the glycans. As an example, we found that MKN45 cells specifically express large amount of N-glycans having multi fucose residues. In the analysis of Oglycans, we found that leukemia cells generally showed simple glycan profiles and commonly contained sialyl-T (NeuAc ▪ 2-3Gal ▪ 1-3Gal*N*Ac) and disialyl-T (NeuAc ▪ 2-3Gal ▪ 1-3(NeuAc ▪ 2-6)Gal*N*Ac) antigens as major O-glycans. In contrast, epithelial cancer cell lines usually showed extremely complex profiles. The information will be a starting point for biological/clinical studies on the new aspect of cancer biology.

Glycoproteins expressing specific glycans in cancer cells

To confirm the proteins which express specific glycans in cancer cells, combination of lectin capturing and peptide/glycan analysis was employed. When using *Datura Staramonium* agglutinin (DSA; specific to polylactosamine-type glycans), we found that some glycoproteins such as CD107a and CD107b commonly contained polylactosamine-type glycans in all the cancer cells but others such as integrin and CD66b in specific cancer cells contained these glycans.

Application to regulation/evaluation of iPS cells

Regenerative medicine is a new branch of medicine that attempts to change the course of chronic disease and in many instances will regenerate tired and failing organ systems lost due to age, disease, damage, or congenital defects. So far, the transplantation of induced pluripotent stem (iPS)-derived cells has shown promising therapeutic effects in a few animal disease models, for example, in a rat model for Parkinson disease and a mouse model for acute myocardial infarction. Indeed, although considerable progress has been made toward understanding embryonic stem cells, we cannot yet claim that they can be used safely in clinical applications. At the same time, a number of challenges remain in the design of materials that are non-immunogenic, scalable, mechanically tunable, and bioactive in their presentation of key regulatory signals to cells. To validate/assure the quality of the stem cells for practical clinical use, a number of methods have been developed. Cell surface antigens consisting of glycans, namely stage-specific embryonic antigen (SSEA-1, -3, and -4) as well as the tumorrejection antigens (TRA-1-60, and 1-81) have been considered to be specific and good markers of pluripotent stem cells.

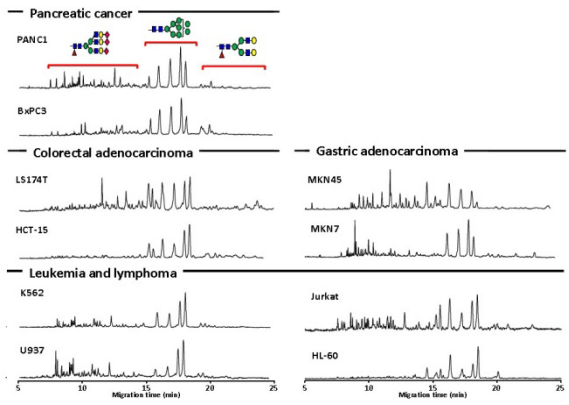

Fig. 1. N-glycan profiles from some cancer cells.

Glycans cover all cellular surfaces, and are involved in many facets of stem cell biology and technology. As shown in Fig. 1, cancer cells employed as model show characteristic glycan profiles, and we can confirm more than 80 glycans in each cell line. We would like to emphasize that glycan profiles obtained by separation techniques with structural information are useful guide for validation/assurance of stem cells and induced pluripotent stem cells for their clinical use.

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MEMO

Functional genomics of calcineurin in model organism

Reiko Sugiura

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Kinki University, Osaka, Japan

Calcineurin (CN) is a highly conserved Ca^{2+}/calmodulin-dependent Ser/Thr protein phosphatase that plays a critical role in Ca^{2+} signaling, including T-cell activation, cardiac development, and hypertrophy, through the activation of NF-AT, a family of transcription factors. We have been studying CN-mediated signaling pathways using fission yeast *Schizosaccharomyces pombe* as a model organism. We have previously demonstrated that CN plays an important role in maintaining the Cl^--homeostasis of and demonstrated that the Pmk1 MAPK signaling and CN play antagonistic roles in the Cl^- homeostasis (Sugiura *et al*., ***Nature*** 1999, 2003). Our genetic screen based on the functional interaction between calcineurin and Pmk1 MAPK has efficiently isolated negative regulators of the Pmk1 MAPK pathway. These include *pmp1*$^+$ encoding a dual-specificity MAPK phosphatase, *pek1*$^+$ encoding a MAPK kinase and *rnc1*$^+$ encoding a novel KH-type RNA-binding protein (Sugiura *et al*., EMBO J. 1998, ***Nature*** 1999, 2003, ***Mol. Bio. Cell***. 2006, 2007, 2009, 2010).
We also developed a molecular genetic screen for mutants which showed immunosuppressant-sensitive growth, in order to identify genes which share an essential function with calcineurin. Notably, our screen identified numerous genes encoding key players of Golgi/endosome membrane trafficking. These include *ypt3*$^+$ and *ryh1*$^+$, both encoding the Golgi-regulating small GTPase, *gdi1*$^+$ encoding the Rab GDP-dissociation inhibitor (GDI), *rho3*$^+$ encoding the Rho small GTPase, *apm1*$^+$, *aps1*+, *apl1*$^+$, and *apl4*$^+$, encoding each of the subunit of the clathrin adaptor complex AP-1, and most recently, *sip1*$^+$ encoding the adaptor protein for AP-1 complex (Sugiura *et al*., ***Mol. Bio. Cell.*** **2002, 2004, 2008,** ***J. Biol. Chem.*** **2000, 2001, 2005, 2008, 2012 Genetics 2000, 2006,** ***PLoS ONE*** **2011**).
Given the high degree of conservation of these genes and CN in higher eukaryotes, these results strongly suggested that calcineurin greatly impact Golgi/endosome trafficking by functionally interacting with regulators of membrane transport.
We further identified and characterized the *cbp1*$^+$ (calcineurin binding protein), which is highly similar to a new class of endogenous calcineurin inhibitor *RCAN1/MCIP1/DSCR1/Adapt78*. Although *RCAN1* has been associated with successful adaptation to oxidative stress and calcium stress and with devastating diseases such as Alzheimer's and Down syndrome, no rationale for these findings has been tested. Notably, we found that Cbp1 inhibits the Spc1 SAPK signaling and regulates oxidative responses. To our knowledge, this is the first demonstration that *RCAN* homologue acts as a repressor of p38 signaling pathway. These findings may shed light on the molecular basis of pathogenesis of diseases such as Alzheimer's and Down syndrome.

MEMO

Evolution of genetically determined resistance mechanisms to retroviral infections in humans and mice

Masaaki Miyazawa, Yoshiyuki Hakata, Jun Li, and Sachiyo Tsuji-Kawahara

Department of Immunology, Kinki University Faculty of Medicine, Osaka-Sayama, Osaka, Japan

Retroviruses contain reverse transcriptase in their viral particles and convert their RNA genomes into double-stranded DNA upon fusion of their envelopes with target cell membranes. The resultant viral DNA is actively transported into cellular nuclei and integrated into chromosomal DNA as proviruses. The above integration of proviral genomes inevitably disrupts the integrity of cellular genomes, and thus retroviruses are one of the greatest threats to the genetic integrity of higher organisms.

To prevent random integration of proviral DNA, mammals have acquired intracellular resistance mechanisms that interfere with the above processes of reverse transcription, viral DNA transport, and proviral integration[1)]. One such resistance factor is a cellular cytidine deaminase, apolipoprotein B mRNA editing enzyme catalytic polypeptide-like 3 (APOBEC3), which coverts cytidines within the nascent negative strand DNA to uridines during the process of reverse transcription, resultantly causing degradation of the uracil-containing DNA strand and G-to-A hypermutation in the complementary positive strand of the proviral DNA. However, retroviruses have evolved to overcome the above powerful mutator, which they encounter in the cells of their natural hosts. Thus, primate lentiviruses, including human and simian immunodeficiency viruses, have acquired the *vif* gene, and its product, Vif protein, binds to the APOBEC3 proteins of their natural hosts to cause rapid degradation of the deaminase through polyubiquitination, ultimately preventing the incorporation of APOBEC3 proteins into budding virions. As the binding of viral Vif protein with cellular APOBEC3 is species-specific, mouse APOBEC3 can interfere with the replication of wild-type human immunodeficiency virus (HIV) in the presence of its Vif, while human APOBEC3 proteins only restrict Vif-deficient, but not wild-type, HIV. Similarly, human APOBEC3 proteins strongly restrict mouse retroviruses, such as murine leukemia viruses (MuLVs), while MuLVs were believed to have acquired mechanisms that exclude mouse APOBEC3 from their virions. Thus, it was postulated that APOBEC3 enzymes function as restriction factors against retroviruses of non-cognate origins.

During the process of identifying the host gene, *Rfv3*, that controls the production of MuLV-neutralizing antibodies in infected mice[1, 2, 3)], we performed extensive microarray analyses on the expression levels of host genes that are harbored within a candidate region of chromosome 15 narrowed down through genetic backcross analyses[2)], and found that the *Apobec3* gene was expressed at high levels in MuLV-resistant strains of mice, while the expression of the same gene was much lower in susceptible strains[1)]. Subsequent analyses[4)] revealed that laboratory strains of mice can be divided into two groups: one group represented by the prototypic MuLV-resistant strain C57BL/6 (B6) and C57BL/10 express higher levels of APOBEC3 mRNA and protein in hematopoietic tissues, while the other

group represented by MuLV-susceptible BALB/c and A strains express lower levels of APOBEC3. These allelic differences are also associated with several amino acid substitutions in the primary structure of APOBEC3 protein and different patterns of mRNA splicing between the two groups of laboratory mice[4]. In fact, in the B6 groups of mice, the major transcript of the *Apobec3* gene lacks exon 5, while the majority of APOBEC3 mRNA in the BALB/c group contains exon 5.

The polymorphic residues in the N-terminal portion of the APOBEC3 outside of the deaminase catalytic domain are responsible for the higher MuLV restrictive function of the B6 gene product and are exposed to the putative DNA-binding groove. The higher levels of APOBEC3 mRNA expression in B6 mice were associated with the insertion of a retroviral long terminal repeat (LTR) sequence as an enhancer into intron 2 of the B6 *Apobec3* allele. By manipulating genomic clones of the mouse *Apobec3* gene, we[5] have recently demonstrated that two genetic determinants, different numbers of TCCT repeats within the pre-mRNA lariat sequence of intron 4 and a single nucleotide polymorphism within exon 5, control the inclusion of exon 5 into mouse APOBEC3 mRNA. Interestingly, the presence of exon 5 interferes with the protein translation, and thus even when similar levels of mRNA are expressed *in vitro*, exon 5-lacking protein is produced much more efficiently than the exon 5-containing one.

The above results may indicate that MuLV-resistant mice, such as B6, have acquired the genetic changes in intron 4 (the loss of one TCCT) and exon 5 (the single nucleotide substitution) and these were positively selected due to resultant resistance to retroviral infections. However, phylogenetic analyses of *Mus* species[5] indicated the opposite conclusion: the ancient ancestor of current *Mus* species seems to have possessed the *Apobec3* genotype that directed preferential expression of the exon 5-lacking mRNA. During the process of their north-bound migration from the Indian subcontinent toward Russia and eastern Europe, ancestors of current *Mus musculus* acquired retroviral LTR in intron 2 and became able to express higher levels of the exon 5-lacking *Apobec3* mRNA. Current B6 mice have inherited this genetic trait. On the other hand, the other group of *Mus* ancestors migrated west toward southern Europe and northern Africa through the Mediterranean coasts, and they acquired the linked TCCT repeat in intron 4 and the exon 5 nucleotide substitution that directs the inclusion of exon 5 and thus less efficient translation of APOBEC3 protein. Current BALB/c and A strains of mice have inherited this genotype from ancestral *Mus domesticus*. Thus, the apparently susceptible genotype has evolved later in the process of diversification of *Mus* species and is distributed more widely than the resistant genotype throughout the wild mice species in the western Eurasian continent[5]. These unexpected results may indicate that the powerful mutator APOBEC3 might function as a “double-edged sword,” and although it is evolutionarily advantageous to express high levels of APOBEC3 when the host is facing repeated attacks by retroviruses, the expression of the highly functional cytidine deaminase may also cause some adverse effects, probably through the possible modification of transcriptomes or through the modification of the host genome itself.

In this talk, we will also present some data on the evolution of another resistance factor, the small GTPase Rac2, which functions to restrict HIV infection in humans[6].

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MEMO