Affiliated Facilities

Laboratory Animal Research Center
実験動物研究施設

Professor	Chieko Kai D.V.M., Ph.D	教　授　農学博士	甲　斐　知恵子
Associate Professor	Misako Yoneda D.V.M., Ph.D	准教授　農学博士	米　田　美佐子
Assistant Professor	Hiroki Sato Ph.D	助　教　理学博士	佐　藤　宏　樹
Assistant Professor	Tomoko Fujiyuki Ph.D	助　教　薬学博士	藤　幸　知　子

Our major research interests are to elucidate molecular mechanisms of pathogenicity and species specificity of negative and single strand RNA viruses (Mononegavirales), and to control viral diseases. For these purposes, we are studying virus replication and identifying viral and host factors important for the expression of pathogenicity using a novel reverse genetics technique. We are also developing new virus vaccines and virus vectors by genetic engineering. In the animal research center, more than 30,000 mice, mainly transgenic or knockout, are kept for research of IMSUT, and the technical staff support their breeding, frozen storage of eggs and microbiological cleaning

Phosphorylation of measles virus nucleoprotein affects viral growth by changing gene expression and genomic RNA stability.

Sugai A, Sato H, Yoneda M, Kai C.

The measles virus (MV) nucleoprotein associates with the viral RNA genome to form the nucleocapsid, providing a template for viral RNA synthesis. In our previous study, major phosphorylation sites of the nucleoprotein were identified as serine residues 479 and 510 (S479 and S510). However, the functions of these phosphorylation sites have not been clarified. In this study, we rescued recombinant MVs (rMVs) whose phosphorylation sites in the nucleoprotein were substituted (rMV-S479A, rMV-S510A, and rMV-S479A/S510A) by reverse genetics and used them in subsequent analyses. In a one-step growth experiment, rMVs showed rapid growth kinetics compared with wild-type MV, although the peak titer of the wild-type MV was the same as or slightly higher than those of the rMVs. Time course analysis of nucleoprotein accumulation also revealed that viral gene expression of rMV was enhanced during the early phase of infection. These findings suggest that nucleoprotein phosphorylation has an important role in controlling viral growth rate through the regulation of viral gene expression. Conversely, multistep growth curves revealed that nucleoprotein-phosphorylation intensity inversely correlated with viral titer at the plateau phase. Additionally, the phosphorylation intensity of the wild-type nucleoprotein in infected cells was significantly reduced through nucleoprotein-phosphoprotein binding. Excessive nucleoprotein-phosphorylation resulted in lower stability against RNase and faster turnover of viral genomic RNA. These results suggest that nucleoprotein-phosphorylation is also involved in viral genomic RNA stability.

Newly identified minor phosphorylation site threonine-279 of measles virus nucleoprotein is a prerequisite for nucleocapsid formation.

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versity of Tokyo, Tokyo, Japan

Minor phosphorylation residues in the MV nucleoprotein have yet to be identified, and their functions are poorly understood. In this study, we identified nine putative phosphorylation sites by mass spectrometry and demonstrated that threonine residue 279 (T279) is functionally significant. Minigenome expression assays revealed that a mutation at the T279 site caused a loss of activity. Limited proteolysis and electron microscopy suggested that a T279A mutant lacked the ability to encapsidate viral RNA but was not denatured. Furthermore, dephosphorylation of the T279 site by alkaline phosphatase treatment caused deficiencies in nucleocapsid formation. Taken together, these results indicate that phosphorylation at T279 is a prerequisite for successful nucleocapsid formation.

Experimental infection of macaques with a wild water bird-derived highly pathogenic avian influenza virus (H5N1).

Fujiyuki T, Yoneda M, Yasui F[1], Kuraishi T[2], Hattori S[2], Kwon HJ, Munekata K[1], Kiso Y, Kida H[3], Kohara M[1], Kai C.: [1]Department of Microbiology and Cell Biology, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan, [2]Amami Laboratory of Injurious Animals, Institute of Medical Science, The University of Tokyo, Kagoshima, Japan, [3]Graduate School of Veterinary Medicine, Hokkaido University, Hokkaido, Japan.

Highly pathogenic avian influenza virus (HPAIV) continues to threaten human health. Non-human primate infection models of human influenza are desired. To establish an animal infection model with more natural transmission and to determine the pathogenicity of HPAIV isolated from a wild water bird in primates, we administered a Japanese isolate of HPAIV (A/whooper swan/Hokkaido/1/2008, H5N1 clade 2.3.2.1) to rhesus and cynomolgus monkeys, in droplet form, via the intratracheal route. Infection of the lower and upper respiratory tracts and viral shedding were observed in both macaques. Inoculation of rhesus monkeys with higher doses of the isolate resulted in stronger clinical symptoms of influenza. Our results demonstrate that HPAIV isolated from a water bird in Japan is pathogenic in monkeys by experimental inoculation, and provide a new method for HPAIV infection of non-human primate hosts, a good animal model for investigation of HPAIV pathogenicity.

Publications

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Affiliated Facilities

Amami Laboratory of Injurious Animals
奄美病害動物研究施設

Professor	Chieko Kai, D.V.M., Ph.D.	教　授　農学博士	甲　斐　知恵子
Associate Professor	Shosaku Hattori, D.V.M., Ph.D.	准教授　農学博士	服　部　正　策

The Amami Laboratory of Injurious Animals was established in 1965 at Setouchi-cho in Amami-oshima Island in order to study on endemic diseases involving parasite, arthropods, and venomous snakes in the tropics or subtropics.
The Amami-oshima Island belongs to the Nansei (Southwest) Islands and the fauna is quite different from that in other islands of Japan. Since establishment of the laboratory, trials have been carried out to utilize small mammals found unique in the Amami islands as experimental animals in addition to studies on prevention of Habu bites. As well known, successful eradication of filariasis from this island is one of the monumental works of the laboratory. Our present works are as follows:

1. Research on the Habu control

Shosaku Hattori, Motonori Ohno[1], Naoko Oda-Ueda[2], Takahito Chijiwa[1], Aichi Yoshida[3], Yoshihiro Hayashi[4], Michihisa Toriba[5] and Tomohisa Ogawa[6]: [1]Department of Applied Life Science, Faculty of Bioscience, Sojo University, [2]Department of Biochemistry, Faculty of Pharmaceutical Science, Sojo University, [3]School of Health Science, Faculty of Medicine, Kagoshima University, [4]National Museum of Nature and Science, Tokyo, [5]The Japan Snake Institute, [6]Faculty of Agriculture, Tohoku University

Snake bites by the venomous snake Habu, *Protobothrops flavoviridis*, have been reported annually about 60 cases in the population of 100,000 in the Amami Islands. Moreover, there is no indication that the population of the Habu itself has decreased, despite a campaign for capture of snakes by the Kagoshima Prefectural Government. Rat-baited box traps have been introduced to catch the snakes and found to be quite effective. However, maintenance of live rats requires man power and its cost is expensive. Therefore, our effort has been focused on the development of attractant for Habu. The attractant extracted from rats seems ineffective if compared with use of live rats.

It was known that the Habu survived the injection of the Habu venom since early times, because some proteins in the serum of the Habu blood combine to the elements of the Habu venom. The research of these binding proteins has been initiated with an objective of clinical trials. Phospholipase A_2 and its isozymes isolated from Habu venom have myonecrotic activity and hemorrhagic activity, and metal protease has hemorahagic activity. The binding proteins isolated from serum of Habu inhibit myonecrotic activity of phospholipase A_2 and its isozymes. We found that protein-HSF and peptide-AHP isolated from the Habu serum effectively control the hemorrhage caused by venom of the Habu, *Ovophis okinavensis*, *Agkistrodon blomhoffi brevicaudus*, *Calloselasma rhodostoma*, *Bitis arietans*, *Bothrops asper*, and, *Trimeresurus stejnegeri*.

Further, a statistics analysis and the simulation were done with the snakes captured by the Government, and the analysis of population dynamics of Habu was attempted. As a result of investigating the individual measurement data of the captured Habu over 9 years, we were able to obtain the generous age composition of the Habu. From analyzing

of the age pyramid of the Habu and the result of questionnaire surveys for the inhabitant in the Amami-oshima Island, the total population of the Habu which lives in this island was estimated at about 80,000. By the analysis of the measured data of last nine years, the snake sizes were miniaturized, and the population of young snakes decreased. According to these investigations, the population of the Habu is expected to decrease in the near future.

These studies are supported by grants from the Ministry of Land, Infrastructure and Transport and the Kagoshima Prefectural Government.

2. Reproduction of squirrel monkeys and owl monekys.

Shosaku Hattori, Takeshi Kuraishi, Kumiko Ikeda, Hazuki Yoshimura and Chieko Kai

The squirrel monkey (*Saimiri boliviensis*) and the owl monkey (*Aotus lemurinus griseimenbra*) were widely distributed in the tropical rainforest in Central and South America. The advantage of using both species for medical researches resides in its small size and gentle behavior. In this laboratory, squirrel monkeys have a reproductive season between winter and early spring. They are polygamy. Their puberty is 3-4 years old in females and 4-5 years old in males. Their gestation period is about 150 days. In contrast, the owl monkey is annual breeding animals. They are monogamy. Their puberty is 3 years old for both sex. Their gestation period is about 130 days. Five newborns were given in reproductive groups of squirrel monkeys in 2013. Two of 5 newborns were nursed by laboratory staffs because of neglect of their mothers. On the other hand, 3 female owl monkeys were pregnant on December in 2013. Those mothers will have children on March or April in 2014.

3. Experimental infectious study of Cryptosporidium using cynomolgus monkeys.

Koichi Masuno[7], Yasuhiro Fukuda[8], Masahito Kubo[9], Ryo Ikarashi[8], Takeshi Kuraishi, Shosaku Hattori, Junpei Kimura[10], Chieko Kai, Tokuma Yanai[11] and Yutaka Nakai[8]: [7]Drug Developmental Research Laboratories, Shionogi & Co., Ltd, [8]Laboratory of Sustainable Environmental Biology, Tohoku University, [9]Laboratory of Veterinary Pathology, Yamaguchi University, [10]College of Veterinary Medicine, Seoul National University, [11]Veterinary Medicine, Gifu University

Infection by *Cryptosporidium andersoni* and *Cryptosporidium muris* have been reported in mice and cattle. Although the detection of those oocysts in human feces have been occasionally reported, the infectious characteristic have been unknown. To understand the infectious features of *C. andersoni* and *C. muris* in human, we used cynomolgus monkeys. The monkeys were orally inoculated with oocysts of *C. andersoni* and *C. muris* under normal and immunosuppressive conditions. The detection of oocysts in monkey feces was performed in the flotation method for about 40 days after inoculation, but no oocysts were found under both conditions. The result of gross and histopathological study showed no evidence of cryptosporidium infections. Therefore, Infectivity of C. andersoni and C. muris for human is considered to be very low.

4. Histopathological study in the Amami rabbit (*Pentalagus furnessi*).

Masahito Kubo[9], Takeshi Kuraishi and Shosaku Hattori

The Amami rabbit, one of the most primitive members of the family Leporidae, occurs only on the Amami-Oshima and Tokuno-Shima islands in the central part of the Nansei Archipelago, southern Japan. The species has been given Special Natural Monument of Japan status by the government, and they are protected from hunting and capturing. However, collisions with car and predation by invasive mammals (feral dogs, cats and mongoose) have caused a decline in the population. In order to contribute to the conservation and management of this endangered species, it is important to investigate diseases and the cause of death. There is, however, little information related to the disease of this rabbit. Here we report histopathological studies of formaline fixed tissue samples gathered from 131 carcases of Amami rabbit between August 2003 and March 2012. Protozoal infection, purulent bronchopneumonia, fibrinous pericarditis, endogenous lipid pneumonia, topical fungal pneumonia, topical pyogranulomatous pneumonia, pulmonary abscess and renal abscess were observed. In 43 of 113 carcases, accumulations of foamy macrophages were found. We disclosed that free-ranging amami rabbits have different kinds of lesions in this study. However, further accumulation of pathological information will be required on the conservation and management of this endangered rabbit.

5. Study of fibroblast like dedifferentiated fat cells (DFAT cells) derived from ceiling culture of mature adipocyte of a squirrel monkey

Takeshi Kuraishi, Shosaku Hattori and Chieko Kai

Recently, it has been reported that human mature adipocytes which are subjected to ceiling culture dedifferentiated into fibroblastic cell populations,

named dedifferentiated fat cells (DFAT cells). DFAT cells are seemed to be a good candidate source of adult stem cells in regenerative medicine, because these cells exhibit multilineage potential as adipose tissue-derived stromal cells (ADSCs). We isolated squirrel monkey DFAT cells from a small amount of adipose tissue. DFAT cells obtained from adipose tissue of a squirrel monkey exhibited fibroblastic cell morphology. Flow cytometric analysis revealed that the cell-surface antigen profile (CD90, CD73, CD29, CD49d) of DFAT cells was very similar to that of ADSCs. Stem cell markers mRNA expression such as Oct4 and CD90 were detected in both DFAT cells and ADSCs of the squirrel monkey. In vitro differentiation analysis revealed that DFAT cells of the squirrel monkey could successfully differentiated into adipocytes under adipogenesis culture condition. In contrast adipogenesis, Osteogenic and chondrogenic differentiation of squirrel monkey DFAT cells rarely observed under osteogenesis and chondrogenesis culture conditions. These data indicate that squirrel monkey DFAT cells we isolated have weak multilineage potential.

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服部正策，倉石武．はぶウマ抗毒素および免疫ウマ血清の尾静脈投与による抗出血検定．平成24年度奄美ハブ毒免疫機序研究報告書．（鹿児島県）．pp. 37−46，2013.

倉石武，服部正策，朝沼榎．ハブ毒タンパク質に対するハブ，ヒメハブ咬傷者および毒取扱者血清中のIgE，IgG1およびIgG4抗体の間接ELISA法による測定．平成24年度奄美ハブ毒免疫機序研究報告書．（鹿児島県）．pp 44−51，2013.

Affiliated Facilities

Laboratory of Molecular Genetics
遺伝子解析施設

Professor	Izumu Saito, M.D., Ph.D.	教　授	医学博士	斎　藤　　泉
Assistant Professor	Yumi Kanegae, Ph.D.	助　教	医学博士	鐘ヶ江　裕　美
Assistant Professor	Saki Kondo, Ph.D.	助　教	医学博士	近　藤　小　貴

This laboratory has two main activities: development and supply of new adenovirus vectors enabling strictly-controlled gene expression useful for studies in various fields including gene therapy, and supporting the researchers by advising on recombinant DNA technology and on biohazards under the safety guidelines.

The purposes of our laboratory are concerned about not only research but also support for all researchers in this institute. Our supporting activity is involved in advising service on gene-manipulation experiments and on biohazards under the safety guidelines and laws. For the research part, we intend to develop novel methods or new experimental systems leading in the field of gene expression and its regulation. We are concentrating mainly on developing efficient adenovirus expression vectors useful for various fields including gene therapy. We are maintaining more than ten collaborations within and outside of this institute. In these collaborations, we can supply adenovirus vectors (AdVs) enabling strictly regulated gene expression and helper-dependent AdVs (HD AdVs) of high capacity up to 30 kilobases (kb). Previously we developed a system for construction of E1-deleted AdV, also called first-generation (FG) AdVs, using a full-length viral genome with intact viral termini (Fukuda. *et al*., Microbiol. Immunol. 50: 643-654, 2006). This cassette is available from Takara Bio and Nippon Gene. We have also developed a method for ON/OFF switching of gene expression in mammalian cells using a combination of adenovirus vector and Cre/*loxP* system (Kanegae *et al*., Nucleic Acids Res. 23: 3816-3821, 1995; Kanegae *et al*., Gene 181: 207-212, 1996) as well as FLP/frt system (Nakano *et al*., Nucleic Acids Res. 29: e40, 2001; Kondo *et al*., Nucleic Acids Res. 31: e76, 2003; Kondo *et al*., Microbiol. Immunol., 50: 831-843, 2006; Kondo et al., J. Molec. Biol., 2009). These methods continuously promote studies of various fields of molecular biology and medicine.

There are two remarkable advances from our laboratory. We succeeded in developing new-generation AdVs that may replace current FG AdVs. The most important problem of AdV is severe immune responses *in vivo*. Firstly, we have identified adenovirus pIX gene as a main cause of inflammation: pIX gene is abnormally activated in AdV. Then we developed AdVs that do not express pIX protein. Transgene expression was lasted for six months in this new AdV (Nakai *et al*., Hum. Gene Ther. 18: 925-936, 2007). The AdV is now called the "low-inflammatory AdVs". For example, Cre-expressing AdV, AxCANCre, is replaced by the low-inflammatory Cre-expressing AdVs, AxEFNCre (Chiyo *et al*., Virus Res. 160: 89-97, 2011). Secondly, we have established a method for efficient production of AdVs lacking the genes of virus-associated (VA) RNAs that disturb cellular RNAi machinery (Maekawa *et al*., Sci. Rep. 2013) using 293hde12 cell line producing a large amount of the codon-human-

ized FLPe (hFLPe) recombinase (Kondo *et al.*, J. Molec. Biol., 2009). The VA-deleted AdVs possibly substitute for current FG AdVs.

We have established a unique system producing HD AdVs using 293hde12 cells. Our system is probably superior to currently available system, because in the latter HD AdVs are produced using cell lines expressing Cre, which is slightly toxic to cells when expressed in a large amount. One example using our HD AdV system is described in the section 3.

The research activities in 2013 are shown below.

1. Dual-safe adenovirus vector lacking virus-associated RNA genes with low inflammation enhanced shRNA activity

Yumi Kanegae, Aya Maekawa, Zheng Pei, Mariko Suzuki, Saki Kondo and Izumu Saito

FG AdVs have been considered an attractive tool for gene therapy because of high titer and high transduction efficiency. However, since AdVs cause high immune responses, application of AdVs were limited. We have reported low-inflammatory AdVs using EF1α promoter, which does not induce aberrant expression of viral pIX protein (Nakai *et al.*, Hum. Gene Ther., 2007). Another important problem is that two different virus-associated RNAs (VA RNAs) transcribed by PolIII are always transcribed from the AdV genome, we here newly developed AdVs lacking genes of VA RNAs. Although VA RNAs are not essential for adenovirus replication, VA-deleted AdVs have been very difficult to construct or showed extremely low titer. We succeeded in establishing an efficient production method of VA-deleted AdVs using hFLPe recombinase. Notably, the titer was almost comparable with current AdVs. Actually, we have already constructed more than twenty VA-deleted AdVs of high titer. Moreover, we combined the VA-deleted vector with low-inflammatory vector (Dual-safe AdVs).

As one example, we used this vector system for aiming gene therapy of chronic hepatitis caused by hepatitis C virus (HCV). Because VA RNAs interfere maturation of miRNA and/or compete with short-hairpin RNA (shRNA), VA-deleted AdVs were, in fact, able to enhance shRNA suppression of HCV replication. So this Dual-safe ADV carrying both interferon gene and anti-HCV shRNA cassette may be an efficient and safe tool for gene therapy of chronic hepatitis C.

2. A concern of adenovirus vectors: continuously expressed virus-associated (VA) RNAs influence the expression of host genes

Saki Kondo, Aya Maekawa, Mariko Suzuki, Izumu Saito and Yumi Kanegae

Since FG AdVs lack E1A gene, which is essential for all other viral promoters driven by RNA polymerase II, they were usually considered that they do not express any viral gene product. However, FG AdVs, in fact, express VA RNAs that are transcribed by RNA polymerase III. VA RNAs are non-coding small RNAs and VAI has been known to inhibit protein kinase R activity. Recently, it has been reported that they are processed to miRNAs that disturb cellular gene expressions. This function has only been reported in the late phase of the virus life cycle where the replication reached 10.000 copies per cell. It has not been examined whether smaller amounts of VA RNAs expressed from AdVs, which do not replicate in target cells, also disturb cellular gene expression, because high titer of VA-deleted AdVs have hardly been generated.

We recently developed an efficient production method to generate high-titer VA-deleted AdVs (Maekawa *et al.*, Sci. Rep., 2013). Using this vector we identified the possible target genes of VA RNAs that influence cellular gene expression comparing current AdVs with VA-deleted AdVs by microarray analysis. TIA-1, which has been reported to be downregulated by abundant VA RNAs, was also decreased at mRNA levels compared with that of current AdVs. Moreover, we found novel VA RNA target genes including a transcription factor, which may influence cellular signaling pathway that cannot be ignored when using current AdVs infection. These results demonstrated that VA RNAs actually influence on cell functions and suggested that VA-deleted AdVs may be safer vectors than current AdVs and may substitute for current AdVs.

3. Structural determinants in GABARAP required for the selective binding and recruitment of ALFY to LC3B positive structures.

Alf Hakon Lystad[1], Yoshinobu Ichimura[2], Kenji Takagi[3], Yinjie Yang[2], Serhiy Pankiv[1], Yumi Kanegae, Shun Kageyama[2], Mariko Suzuki, Izumu Saito, Tsunehiro Mizushima[3], Masaaki Komatsu[2] and Anne Simonsen[1]: [1]Institute of Basic Medical Biosciences, University of Oslo, [2]Protein Metabolism Project, Tokyo Metropolitan Institute of Medical Science, [3]Picobiology Institute, Graduate School of Life Science, University of Hyogo.

Sequestration of cytoplasmic cargo for degradation by macroautophagy (hereafter autophagy) is facilitated by binding of cargo-interacting proteins, so called autophagy receptors, to Atg8-homolog proteins, which upon induction of autophagy becomes covalently linked to phosphatidylethanolamine (PE) in the autophagic membrane. Whereas yeast has a single Atg8 gene, mammals have seven

Atg8 homologs, which can be divided into two subfamilies; the LC3 family and the GABARAP family. The reason for such an expansion of this protein family in higher eukaryotes is unclear, but it coincides with the expansion of cargo-recognition proteins and is likely to provide specificity to cargo recruitment. The currently known autophagy receptors include receptors for recognition of bacteria, viral particles, mitochondria, peroxisomes, midbody remnants and protein aggregates. They generally interact with two hydrophobic pockets in the Atg8 proteins through a linear motif called an LC3-interacting region (LIR), having the consensus sequence [W/F/Y]-x-x-[I/L/V][1].

ALFY (Autophagy-linked FYVE protein, also called WDFY3) is a large phosphatidyl-inositol 3-phosphate-binding protein shown to be recruited to ubiquitin-positive structures during stress. ALFY interacts with the ubiquitin-binding autophagy receptors p62/SQSTM1 and NBR1 and contributes to autophagic clearance of aggregated proteins. Because ALFY is a very large protein: the molecular weight is 392kDa. To study the role in the autophagy clearance we constructed GFP-ALFY fusion protein of 420kDa. Since the length of its cDNA is 11.5kb, it is impossible to generate an FG AdV containing such large cDNA. Therefore, we constructed HD AdVs expressing GFP-fusion of wild-type ALFY and its mutant ALFY.

We showed that ALFY binds selectively to the GABARAP subfamily, and weakly to LC3C, through a conserved LIR motif in its WD40 region. We demonstrated that the interaction of ALFY with GABARAPs is indispensable for recruitment of LC3 B to ALFY-p62-positive structures. We further identified three conserved residues in the GABARAPs that confer selectivity to the interaction with ALFY and showed that introduction of these residues in the corresponding positions of LC3B is sufficient to enable interaction of ALFY with LC3B.

Publications

Pei, Z., Shi, G., Kondo, S., Ito, M., Maekawa, A., Suzuki, M., Saito, I., Suzuki, T., and Kanegae, Y. Adenovirus vectors lacking virus-associated RNA expression enhance shRNA activity to suppress hepatitis C virus replication. Sci. Rep., 20: 3575, 2013.

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Affiliated Facilities

Medical Proteomics Laboratory
疾患プロテオミクスラボラトリー

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The mission of our laboratory is to develop advanced technologies for antibody engineering, mass spectrometry, electron microscopy to perform an integrative protein analysis from a physicochemical, structural and systems biology point of view. Currently, we mainly focus on the researches on functional protein-protein interactions related to a variety of diseases such as cancer and infection.
We are also engaged in collaborative researches regarding mass spectrometry, electron microscopy, peptide synthesis, protein purification and the related functional analyses and have made a substantial contribution to many scientific achievements.

<Group I>

AIM: Life, as we understand it, requires of a concerted and complex set of interactions between different biological molecules, such as DNA, RNA, proteins, lipids, and carbohydrates. We sought to understand the nature of these interactions at the molecular and energetic level. Our dissecting tools are applied to study a broad range of biological phenomena, and to develop the next generation of therapeutic antibodies in the era of Bio-better and Bio-superior.

1. Dynamic elements govern the catalytic activity of CapE, a capsular polysaccharide-synthesizing enzyme from Staphylococcus aureus.

Miyafusa T, Caaveiro JM, Tanaka Y, Tsumoto K.

CapE is an essential enzyme for the synthesis of capsular polysaccharide (CP) of pathogenic strains of Staphylococcus aureus. Herein we demonstrate that CapE is a 5-inverting 4,6-dehydratase enzyme. However, in the absence of downstream enzymes, CapE catalyzes an additional reaction (5-back-epimerization) affording a by-product under thermodynamic control. Single-crystal X-ray crystallography was employed to identify the structure of the by-product. The structural analysis reveals a network of coordinated motions away from the active site governing the enzymatic activity of CapE. A second dynamic element (the latch) regulates the enzymatic chemoselectivity. The validity of these mechanisms was evaluated by site-directed mutagenesis.

2. Bidirectional binding property of high glycine-tyrosine keratin-associated protein contributes to the mechanical strength and shape of hair.

Matsunaga R, Abe R, Ishii D, Watanabe S, Kiyoshi M, Nöcker B, Tsuchiya M, Tsumoto K.

Since their first finding in wool 50years ago, keratin-associated proteins (KAPs), which are classified into three groups; high sulfur (HS) KAPs, ultra high sulfur (UHS) KAPs, and high glycine-tyrosine (HGT) KAPs, have been the target of curiosity for scientists due to their characteristic amino acid sequences. While HS and UHS KAPs are known to function in disulfide bond crosslinking, the function of HGT KAPs remains unknown. To clarify the function as well as the binding partners of HGT KAPs, we prepared KAP8.1 and other KAP family proteins, the trichocyte intermediate filament proteins (IFP) K85 and K35, the head domain of K85, and the C subdomain of desmoplakin C-terminus (DPCT-C) and investigated the interactions between them in vitro. Western blot analysis and isothermal titration calorimetry (ITC) indicate that KAP8.1 binds to the head domain of K85, which is helically aligned around the axis of the intermediate filament (IF). From these results and transmission electron microscopy (TEM) observations of bundled filament complex in vitro, we propose that the helical arrangement of IFs found in the orthocortex, which is uniquely distributed on the convex fiber side of the hair, is regulated by KAP8.1. Structure-dependent binding of DPCT-C to trichocyte IFP was confirmed by Western blotting, ITC, and circular dichroism. Moreover, DPCT-C also binds to some HGT KAPs. It is probable that such bidirectional binding property of HGT KAPs contribute to the mechanical robustness of hair.

3. Incorporation of rapid thermodynamic data in fragment-based drug discovery

Kobe A, Caaveiro JM, Tashiro S, Kajihara D, Kikkawa M, Mitani T, Tsumoto K.

Fragment-based drug discovery (FBDD) has enjoyed increasing popularity in recent years. We introduce SITE (single-injection thermal extinction), a novel thermodynamic methodology that selects high-quality hits early in FBDD. SITE is a fast calorimetric competitive assay suitable for automation that captures the essence of isothermal titration calorimetry but using significantly fewer resources. We describe the principles of SITE and identify a novel family of fragment inhibitors of the enzyme ketosteroid isomerase displaying high values of enthalpic efficiency.

4. Selective binding of antimicrobial porphyrins to the heme-receptor IsdH-NEAT3 of Staphylococcus aureus

Vu NT, Moriwaki Y, Caaveiro JM, Terada T, Tsutsumi H, Hamachi I, Shimizu K, Tsumoto K.

The Isd (iron-regulated surface determinant) system of the human pathogen Staphylococcus aureus is responsible for the acquisition of heme from the host organism. We recently reported that the extracellular heme receptor IsdH-NEAT3 captures and transfers noniron antimicrobial porphyrins containing metals in oxidation state (III). However, it is unclear if geometric factors such as the size of the metal (ionic radius) affect binding and transfer of metalloporphyrins. We carried out an ample structural, functional, and thermodynamic analysis of the binding properties of antimicrobial indium(III)-porphyrin, which bears a much larger metal ion than the iron(III) of the natural ligand heme. The results demonstrate that the NEAT3 receptor recognizes the In(III)-containing PPIX in a manner very similar to that of heme. Site-directed mutagenesis identifies Tyr642 as the central element in the recognition mechanism as suggested from the crystal structures. Importantly, the NEAT3 receptor possesses the remarkable ability to capture dimers of metalloporphyrin. Molecular dynamics simulations reveal that IsdH-NEAT3 does not require conformational changes, or large rearrangements of the residues within its binding site, to accommodate the much larger (heme)2 ligand. We discuss the implications of these findings for the design of potent inhibitors against this family of key receptors of S. aureus.

5. Hyperthin nanochains composed of self-polymerizing protein shackles

Ryo Matsunaga, Saeko Yanaka, Satoru Nagatoishi, and Kouhei Tsumoto

Protein fibrils are expected to have applications as functional nanomaterials because of their sophisticated structures; however, nanoscale ordering of the functional units of protein fibrils remains challenging. Here we design a series of self-polymerizing protein monomers, referred to as protein shackles, derived from modified recombinant subunits of pili from Streptococcus pyogenes. The monomers polymerize into nanochains through spontaneous irreversible covalent bond formation. We design the protein shackles so that their reactions can be controlled by altering redox conditions, which affect disulphide bond formation between engineered cysteine residues. The interaction between the monomers improves their polymerization reactivity and determines morphologies of the polymers. In addition, green fluorescent protein-tagged protein shackles can polymerize, indicating proteins can be stably attached to the nanochains with its functionality preserved. Furthermore we demonstrate that a molecular-recognizable nanochain binds to its partner with an enhanced binding ability in solution. These characteristics are expected to be applied for novel protein nanomaterials.

6. Effects of subclass change on the structural stability of chimeric, humanized, and human antibodies under thermal stress

Takahiko Ito and Kouhei Tsumoto

To address how changes in the subclass of antibody molecules affect their thermodynamic stability, we prepared three types of four monoclonal antibody molecules (chimeric, humanized, and human) and analyzed their structural stability under thermal stress by using size-exclusion chromatography, differential scanning calorimetry (DSC), circular dichroism (CD), and differential scanning fluoroscopy (DSF) with SYPRO Orange as a dye probe. All four molecules showed the same trend in change of structural stability; the order of the total amount of aggregates was IgG1＜IgG2＜IgG4. We thus successfully cross-validated the effects of subclass change on the structural stability of antibodies under thermal stress by using four methods. The T (h) values obtained with DSF were well correlated with the onset temperatures obtained with DSC and CD, suggesting that structural perturbation of the CH2 region could be monitored by using DSF. Our results suggested that variable domains dominated changes in structural stability and that the physicochemical properties of the constant regions of IgG were not altered, regardless of the variable regions fused.

7. Improving the affinity of an antibody for its antigen via long-range electrostatic interactions.

Fukunaga A, Tsumoto K.

To address how long-range electrostatic force can affect antibody-antigen binding, we focused on the interactions between human cardiac troponin I and its specific single-chain antibodies (scFvs). We first isolated two scFvs against two linear epitopes with distinct isoelectric points. For the scFv against the acidic epitope (A1scFv), we mutated five residues of framework region 3 of the light chain to Lys or Arg, designated as the K- or R-mutant, respectively. For the scFv against the basic epitope (A2scFv), we mutated four or three residues in framework region 3 of the light or heavy chain to Asp, to generate the VL- and VH-mutant, respectively. Surface plasmon resonance analyses showed that the kon values of all of the mutants were greater than that of wild type, even though framework region 3 does not make direct contact with the epitope. The affinity of the K-mutant was pM range, and that of the R-mutant improved further by more than two orders of magnitude due to a decrease in the dissociation rate constant. For the A2scFv mutants, the affinity of the VL-mutant for its target improved through an increase in the kon value without a decrease in the koff value. The stability slightly decreased in all mutants. These results suggest that introducing electrostatic interaction can improve the affinity of an antibody for its target, even if the mutation reduces stability of the antibody.

8. Identification of small-molecule inhibitors of the human S100B-p53 interaction and evaluation of their activity in human melanoma cells

Yoshimura C, Miyafusa T, Tsumoto K.

The interaction between human S100 calcium-binding protein B (S100B) and the tumor suppressor protein p53 is considered to be a possible therapeutic target for malignant melanoma. To identify potent inhibitors of this interaction, we screened a fragment library of compounds by means of a fluorescence-based competition assay involving the S 100B-binding C-terminal peptide of p53. Using active compounds from the fragment library as query compounds, we constructed a focused library by means of two-dimensional similarity searching of a large database. This simple, unbiased method allowed us to identify several inhibitors of the S100B-p53 interaction, and we elucidated preliminary structure-activity relationships. One of the identified compounds had the potential to inhibit the S 100B-p53 interaction in melanoma cells.

9. Mutations for decreasing the immunogenicity and maintaining the function of core streptavidin

Yumura K, Ui M, Doi H, Hamakubo T, Kodama T, Tsumoto K, Sugiyama A.

The defining property of core streptavidin (cSA) is not only its high binding affinity for biotin but also its pronounced thermal and chemical stability. Although potential applications of these properties including therapeutic methods have prompted much biological research, the high immunogenicity of this bacterial protein is a key obstacle to its clinical use. To this end, we have successfully constructed hypoimmunogenic cSA muteins in a previous report. However, the effects of these mutations on the physicochemical properties of muteins were still unclear. These mutations retained the similar electrostatic charges to those of wild-type (WT) cSA, and functional moieties with similar hydrogen bond pattern. Herein, we performed isothermal titration calorimetry, differential scanning calorimetry, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis to gain insight into the physicochemical properties and functions of these modified versions of cSA. The results indicated that the hy-

poimmunogenic muteins retained the biotin-binding function and the tetramer structure of WT cSA. In addition, we discuss the potential mechanisms underlying the success of these mutations in achieving both immune evasion and retention of function; these mechanisms might be incorporated into a new strategy for constructing hypoimmunogenic proteins.

10. Effects of chain length of an amphipathic polypeptide carrying the repeated amino acid sequence (LETLAKA)(n) on a-helix and fibrous assembly formation.

Takei T, Hasegawa K, Imada K, Namba K, Tsumoto K, Kuriki Y, Yoshino M, Yazaki K, Kojima S, Takei T, Ueda T, Miura K.

Polypeptide α3 (21 residues), with three repeats of a seven-amino-acid sequence (LETLAKA)(3), forms an amphipathic α-helix and a long fibrous assembly. Here, we investigated the ability of α3-series polypeptides (with 14-42 residues) of various chain lengths to form α-helices and fibrous assemblies. Polypeptide α2 (14 residues), with two same-sequence repeats, did not form an α-helix, but polypeptide α2L (15 residues; α2 with one additional leucine residue on its carboxyl terminal) did form an α-helix and fibrous assembly. Fibrous assembly formation was associated with polypeptides at least as long as polypeptide α2L and with five leucine residues, indicating that the C-terminal leucine has a critical element for stabilization of α-helix and fibril formation. In contrast, polypeptides α5 (35 residues) and α6 (42 residues) aggregated easily, although they formed α-helices. A 15-35-residue chain was required for fibrous assembly formation. Electron microscopy and X-ray fiber diffraction showed that the thinnest fibrous assemblies of polypeptides were about 20 Å and had periodicities coincident with the length of the α-helix in a longitudinal direction. These results indicated that the α-helix structures were orientated along the fibrous axis and assembled into a bundle. Furthermore, the width and length of fibrous assemblies changed with changes in the pH value, resulting in variations in the charged states of the residues. Our results suggest that the formation of fibrous assemblies of amphipathic α-helices is due to the assembly of bundles via the hydrophobic faces of the helices and extension with hydrophobic noncovalent bonds containing a leucine.

<Group II>

1. Global characterization of the proteome and phosphoproteome in human glioblastoma initiating cells by high-resolution mass spectrometry

Hiroko Kozuka-Hata, Ryo Koyama-Nasu[1], Yumi Goto, Yukiko Nasu-Nishimura[1], Hiroko Ao-Kondo, Kouhei Tsumoto, Tetsu Akiyama[1] and Masaaki Oyama: [1]Laboratory of Molecular and Genetic Information, Institute of Molecular and Cellular Biosciences, University of Tokyo

Glioblastoma is one of the most common and aggressive brain tumors with the median survival of twelve months after diagnosis. Despite extensive studies of this malignant tumor, the outcomes of the treatment have not significantly improved over the past decade. To elucidate the underling mechanisms of its tumorigenicity, we performed parallel analyses of the comprehensive proteome and phosphoproteome in glioblastoma initiating cells that are widely recognized as key players in showing resistance to chemotherapy and radiation. Using high-resolution nanoflow LC-MS/MS (LTQ Orbitrap Velos) in combination with GELFREE™ 8100 fractionation system, we identified a total of 8,856 proteins and 6,073 phosphopeptides, respectively. Global protein network analysis revealed that the molecules belonging to ribosome, spliceosome and proteasome machineries were highly enriched at the proteome level. Our in-depth phosphoproteome analysis based on two fragmentation methodologies of CID and HCD detected various phosphorylation sites on neural stem cell markers such as nestin and vimentin, leading to identification of thirty-six phosphorylation sites including eleven novel sites of nestin protein. The SILAC-based quantitative analysis showed that 516 up-regulated and 275 down-regulated phosphorylation sites upon epidermal growth factor stimulation. Interestingly, the phosphorylation status of the molecules related to mTOR signaling pathway was dynamically changed upon EGF stimulation. More intriguingly, we also identified some novel phosphopeptides encoded by the undefined sequence regions of the human transcripts, which could be regulated upon external stimulation in glioblastoma initiating cells. Our result unveils an expanded diversity of the regulatory phophoproteome defined by the human transcriptome.

2. Integrative analysis of phosphoproteome and transcriptome dynamics defines drug-resistance properties of breast cancer

Masaaki Oyama, Takeshi Nagashima[2], Hiroko Kozuka-Hata, Noriko Yumoto[2], Yuichi Shiraishi[2], Kazuhiro Ikeda[3], Yoko Kuroki[2], Noriko Gotoh[4], Satoshi Inoue[3], Hiroaki Kitano[5] and Mariko Okada-Hatakeyama[2]: [2]RIKEN, [3]Research Center for Genomic Medicine, Saitama Medical University, [4]Division of Systems Biomedical Technology, IMSUT, [5]Sony Computer Science Laboratories, Inc.

Signal transduction system, in orchestration with subsequent transcriptional regulation, widely regulates complex biological events such as cell proliferation and differentiation. Therefore, a comprehensive and fine description of their dynamic behavior provides a fundamental platform for systematically analyzing the regulatory mechanisms that result in each biological effect. Here we developed an integrated framework for time-resolved description of phosphoproteome and transcriptome dynamics based on the SILAC-nanoLC-MS and GeneChip system. In this study, we analyzed cellular information networks mediated by estrogen receptor/ErbB2 pathways, which have long been implicated in drug response of breast cancer. Through shotgun identification and quantification of phosphorylated molecules in breast cancer MCF-7 cells, we obtained a global view of the dynamics regarding breast cancer-related signaling networks upon estrogen (E2) or heregulin (HRG) stimulation. Comparative analysis of wild-type and tamoxifen-resistant MCF-7 cells revealed altered behaviors of signaling hub dynamics, indicating distinct signaling network properties between these two cell types. Pathway and motif activity analyses using the transcriptome data suggested that deregulated activation of GSK3β and MAPK1/3 signaling might be associated with altered activation of CREB and AP-1 transcription factors in tamoxifen-resistant MCF-7 cells. Thus, our integrative analysis of phosphoproteome and transcriptome in human breast cancer cells revealed distinct signal-transcription programs in tamoxifen-sensitive and insensitive tumor cells, which potentially defines drug-resistance properties against tamoxifen.

3. System-level analysis of CagA-dependent signaling network dynamics by Helicobacter pylori infection

Hiroko Kozuka-Hata, Masato Suzuki[6], Kotaro Kiga[6], Shinya Tasaki, Jun-ichiro Inoue, Tadashi Yamamoto[7], Chihiro Sasakawa[6] and Masaaki Oyama: [6]Division of Bacterial Infection, Department of Microbiology and Immunology, IMSUT, [7]Division of Oncology, Department of Cancer Biology, IMSUT

The signal transduction system within a cell regulates complex biological events in response to bacterial infection. The previous analyses of cell signaling in Helicobacter pylori-infected gastric epithelial cells have revealed that CagA, a major virulence factor of Helicobacter pylori, is delivered into cells via the type IV secretion system and perturbs signaling networks through the interaction with the key signaling molecules such as SHP-2, Grb2, Crk/Crk-L, Csk, Met, and ZO-1. Although the biological activity of tyrosine-phosphorylated CagA has intensively been studied, system-wide effects of the virulence factor on cellular signaling have yet to be analyzed. Here we performed time-resolved analyses of phosphoproteome and CagA-interactome in human gastric AGS cells by CagA-positive/negative Helicobacter pylori infection. Our highly sensitive nanoLC-MS/MS analyses in combination with the Stable Isotope Labeling by Amino acids in Cell culture (SILAC) technology defined CagA-dependent perturbation of signaling dynamics along with a subset of CagA-associated possible modulators on a network-wide scale. Our result indicated that the activation level of the phosphotyrosine-related signaling molecules in AGS cells was suppressed overall in the presence of CagA during Helicobacter pylori infection. As Helicobacter pylori infection plays pivotal roles in the progression of gastric diseases including carcinogenesis, a comprehensive and fine description of the signaling dynamics would serve as a fundamental platform to theoretically explore for the potential drug targets through analyzing the regulatory mechanisms at the system-level.

4. SILAC-based quantitative phosphoproteome analysis of glioblastoma stem cell differentiation by high-resolution nanoLC-MS/MS

Yuta Narushima, Hiroko Kozuka-Hata, Ryo Koyama-Nasu[1], Yumi Goto, Tomoko Hiroki, Kouhei Tsumoto, Tetsu Akiyama[1] and Masaaki Oyama

Glioblastoma is one of the most malignant brain tumors with the median survival of twelve months after diagnosis. Glioblastoma stem cells (GSCs) have been considered as a cause of glioblastoma's aggressive malignancy and possess the properties of cancer stemness such as self-renewal, pluripotency, high tumorigenicity and resistance to chemotherapy. In order to develop new therapies against GSCs, their differentiation-inducing mechanisms are intensively studied because the characteristics of tumorigenicity and drug resistance are known to be lost in differentiated states. Several cell signaling pathways including Notch, Sonic hedgehog and Wnt have been found to maintain stemness of GSCs, in which post-translational modifications, especially phosphorylation, have critical roles. In this study, we applied a combination of Stable Isotope Labeling by Amino acids in Cell culture (SILAC), TiO_2 phosphopeptide enrichment and nanoLC-MS/MS to quantitative phosphoproteome analysis of serum-induced differentiation in GSCs.

Here, we tried to perform SILAC-based comparative quantification of the phosphoproteome in the differentiation of GSCs isolated from the human glioblastoma patients. First, we observed the alteration of cellular morphology and confirmed the change of stemness and differentiation markers be-

tween serum-containing and serum-free conditions. Next, we recently obtained the phosphoproteome data by high-resolution nanoLC-MS/MS and analyzed the signaling network status with some pathway analysis software. We will report system-wide characterization of phosphorylation-dependent networks related to stemness conservation and differentiation in GSCs.

5. Photo-crosslinking-based proteomics elucidates direct protein-protein interactions involving a defined binding domain

Nobumasa Hino[2], Masaaki Oyama, Aya Sato[2], Takahito Mukai[2], Hiroko Kozuka-Hata, Tadashi Yamamoto[7], Kensaku Sakamoto[2] and Shigeyuki Yokoyama[2]

Signal transduction pathways are essentially organized through the distribution of various binding domains in signaling proteins, with each domain binding to its target molecules. To identify the targets of these domains, we developed a novel proteomic approach, based on photo-cross-linking and mass spectrometry. Through the use of an expanded genetic code, a photoreactive amino acid, *p*trifluoromethyl - diazirinyl - L - phenylalanine, was site-specifically incorporated into the SH2 domain of the adaptor protein GRB2 in human embryonic kidney cells. By exposing the cells to 365-nm light after an EGF stimulus, the SH2 of GRB2 was cross-linked with the endogenous proteins directly interacting with it. These targets were identified by a comparative mass-spectrometric strategy. Thus, we discovered that GRB2-SH2 directly binds to the GIT 1 scaffold protein and the AF6 protein, a putative effector of the RAS protein. Furthermore, heterogeneous nuclear ribonucleoproteins F, H1, and H2 were found to be direct targets of GRB2-SH2.

6. Mass spectrometry-based annotation of the human short ORFeome

Masaaki Oyama, Hiroko Kozuka-Hata, Sumio Sugano[8], Tadashi Yamamoto[7] and Jun-ichiro Inoue: [8]Laboratory of Functional Genomics, Department of Medical Genome Sciences, Graduate School of Frontier Sciences, The University of Tokyo

In parallel with the human genome projects, human full-length cDNA data has also been intensively accumulated. Large-scale analysis of their 5'-UTRs revealed that about half of these had a short ORF upstream of the coding region. Experimental verification as to whether such upstream ORFs are translated is essential to reconsider the generality of the classical scanning mechanism for initiation of translation and define the real outline of the human proteome. Our previous proteomics analysis of small proteins expressed in human K562 cells provided the first direct evidence of translation of upstream ORFs in human full-length cDNAs (Oyama et al., Genome Res, 14: 2048-2052, 2004). In order to grasp an expanded landscape of the human short ORFeome, we have performed an in-depth proteomics analysis of human K562 and HEK293 cells using a two-dimensional nanoLC-MS/MS system. The results led to the identification of eight protein-coding regions besides 197 small proteins with a theoretical mass less than 20 kDa that were already annotated coding sequences in the curated mRNA database. In addition to the upstream ORFs in the presumed 5'-untranslated regions of mRNAs, bioinformatics analysis based on accumulated 5'-end cDNA sequence data provided evidence of novel short coding regions that were likely to be translated from the upstream non-AUG start site or from the new short transcript variants generated by utilization of downstream alternative promoters. Protein expression analysis of the *GRINL1A* gene revealed that translation from the most upstream start site occurred on the minor alternative splicing transcript, whereas this initiation site was not utilized on the major mRNA, resulting in translation of the downstream ORF from the second initiation codon. These findings reveal a novel post-transcriptional system that can augment the human proteome via the alternative use of diverse translation start sites coupled with transcriptional regulation through alternative promoters or splicing, leading to increased complexity of short protein-coding regions defined by the human transcriptome (Oyama et al., Mol Cell Proteomics, 6: 1000-1006, 2007).

<Group III>

1. Development of new methods for analyzing the neural circuits in the retina

Neural circuits in the central nervous system are the basis of various high-order brain functions. It is also true in case of retina. In the retina, six main classes of cells connect each other systematically to make up complex neural circuits. Characteristics of the retinal functions have been examined precisely by the electrophysiological methods and models of cell connectivity have been proposed. But morphological studies of the actual neural connection, which constitute the physiological properties of higher order neurons, are not enough. We have been trying to reveal the actual neural circuit morphologically by using electron microscopic computed tomography (CT) and X-ray microscopy for years. But the results obtained are still insufficient especially for thick specimens. To date the inner structure of the cells can only been observed by transmission electron microscope (TEM). Recent

progress in scanning electron microscope (SEM) equipment lead us to develop a new method to observe TEM section in SEM. Samples were specifically treated to enhance electron contrast and more than 500 serial thin TEM sections were collected on the smooth conductive matrix. Using this method it became possible to observe whole structure of more than 500 μm square and more than 30 μm depth area with the resolution comparable to TEM. The whole cell data obtained by this method were used to simulate the intracellular signal transduction process in real cell space by Dr. Ichikawa[1] *et al* in [1]Division of Mathematical Oncology. Using similar methods we are now going to collect the information to decipher the wiring diagram of the retina and part of the data were used to analyze the function of Fezf2 transcription factor in developing mouse retina. (Collabolation with Dr. Watanabe[2] *et al* in [2]Division of Molecular and Developmental Biology)

2. Collaborative and supportive works as electron microscope core-laboratory

This group is also engaged in collaborative researches using electron microscope. We offer supports for the research projects those need electron microscopic analysis. The services available in this group are the conventional thin section transmission electron microscopy, immuno-electron microscopy, negative staining techniques and scanning electron microscopy. By using these individual technique or combination of some of these we can offer direct visual evidence that cannot be acquired by other methods. This year, 24 projects in 11 laboratories were performed as core-laboratory works.

a. Thin section transmission electron microscopy

Thin section transmission electron microscopy is the most widely used technique to observe the inner structure of cells and tissues. In this method, samples are fixed and embedded in epoxy resin, thin sections with about 70nm thickness are cut and observed in the electron microscope. In case of immuno-electron microscopy, thin sections are obtained by similar procedure, and the antigen epitopes exposed on the surface of the sections are marked by sequentially reacted with appropriate primary antibodies and colloidal gold labeled secondary antibodies. This year, thin section electron microscopy combined with immuno-electron microscopy were used in many collaborative works.

a-1. Ultrastructural analysis of entry and assembly of Herpes Simplex Virus

We have been performing several studies with research groups in Dr. Kawaguchi[3]'s laboratory: [3]Department of Infectious Disease Control, International Research Center for Infectious Diseases, regarding the infection/replication processes of herpes simplex virus (HSV). This year, thin section electron microscopy was used to analyze the function of viral proteins in trans-nuclear membrane processes of the newly formed viruses. By analyzing the virus forming processes in some mutant host cells, we could analyze viral proteins as well as candidate host molecules those may be involved in the trans-nuclear process of the HSV (ref. Liu[3] *et al*).

a-2. Improvement of antigen expression in rice-based oral colera vaccine (MucoRice-CTB). An Morphological and immune-electron microscopic analysis of the rice protein expression.

We have been performing several studies also with research groups in Dr. Kiyono[4]'s laboratory: [4]Division of Mucosal Immunology, Department of Microbiology and Immunology. In these studies, we analyzed the expression and localization of rice proteins and induced colera toxin B-subunit (CTB) under the RNAi-mediated suppression of some endogenous proteins by thin section transmission electron microscopy and immune-electron microscopy to increase CTB expresion. We found that suppression of some endogenous protein could increase CTB expression six times more than wild type. This study may contribute to the practical realization of rice-based oral colera vaccine (ref. Tokuhara[4] *et al* and Kurokawa[4] *et al*).

Some other collaborative research works using thin section electron microscopy and/or immuno-electron microscopy were performed with Dr. Noda[5] *et al* in [5]Division of Virology, Department of Microbiology, regarding the structure of the influenza viruses and ebola virus (ref. Sugita[5] *et al*), Dr. Sanada[6]'s group, in [6]Department of Gerontological Nursing/Wound Care Management, Graduate School of Medicine, The University of Tokyo, Dr. Hoshina[7] in [7]Division of Oncology, regarding the structure of the synapses (ref. Hoshina[7] *et al*), Dr. Kunieda[8] in [8]Laboratory of Physiological Chemistry, Department of Biological Sciences, Graduate school of Science, regarding the morphology of the Tardigrades, and so on.

b. Negative staining techniques

Negative staining techniques are simple and quick method to observe the morphology of the macro molecules. In the collaborative work with Dr. Noda[5] *et al*., this technique combined with thin section electron microscopy was used to analyze the morphology of the influenza virus ribonucleoprotein complex (ref. Sugita[5] *et al*). The negative

staining techniques were also used in some works to analyze the structure of the purified proteins and the proteins integrated in the plasma membrane.

c. Conventional scanning electron microscopy

Conventional scanning electron microscopy is a technique used to examine the surface structure of the cells, tissues or other non-biological materials. The collaborative works using scanning electron microscopy were done with Dr. Shibasaki[9] *et al*., [9]Laboratory of Developmental Genetics, Center for Experimental Medicine and Systems Biology, regarding the morphology of the cilia on the surface of the cells covering the inner wall of the ventricles (ref. Shibasaki *et al*). Other works are in progress with Dr. Sanada[6] *et al*, to analyze the effects of diabetes or bacterial infection during wound repair. Scanning electron microscopy was also used to analyze the morphological changes of cultured macrophages and non-biological materials as a collaborative work with Dr. Cheng[10] in [10]Olympus Co.

Publications

<Group I>

Moriwaki Y, Terada T, Caaveiro JM, Takaoka Y, Hamachi I, Tsumoto K, and Shimizu K. Heme-binding mechanism of structurally similar Isd NEAT domains of Staphylococcus aureus exhibiting different affinities for heme. Biochemistry, 52: 8866-8877, 2013.

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