Laboratory Animal Research Center
実験動物研究施設

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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.

A promoter level mammalian expression atlas.

The FANTOM Consortium (Kai, C., Nakamura, T., Sato, H., Sugiyama, T., Yoneda, M. et al) and the RIKEN PMI and CLST

Regulated transcription controls the diversity, developmental pathways and spatial organization of the hundreds of cell types that make up a mammal. Using single-molecule cDNA sequencing, we mapped transcription start sites (TSSs) and their usage in human and mouse primary cells, cell lines and tissues to produce a comprehensive overview of mammalian gene expression across the human body. We found that few genes are truly 'housekeeping', whereas many mammalian promoters are composite entities composed of several closely separated TSSs, with independent cell-type-specific expression profiles. TSSs specific to different cell types evolve at different rates, whereas promoters of broadly expressed genes are the most conserved. Promoter-based expression analysis revealed key transcription factors defining cell states and linked them to binding-site motifs. The functions of identified novel transcripts can be predicted by coexpression and sample ontology enrichment analyses. The functional annotation of the mammalian genome 5 (FANTOM5) project provides comprehensive expression profiles and functional annotation of mammalian cell-type-specific transcriptomes with wide applications in biomedical research.

An atlas of active enhancers across human cell types and tissues.

Andersson, R., The FANTOM Consortium

Enhancers control the correct temporal and cell-type-specific activation of gene expression in multicellular eukaryotes. Knowing their properties, regulatory activity and targets is crucial to understand the regulation of differentiation and homeostasis. We used the FANTOM5 panel of samples, covering the majority of human tissues and cell types, to produce an atlas of active, in vivo-transcribed enhancers. We showed that enhancers share properties with CpG-poor messenger RNA promoters but produce bidirectional, exosome-sensitive, relatively short unspliced RNAs, the generation of which is strongly related to enhancer activity. The atlas is used to compare regulatory programs between different cells at unprecedented depth, to identify disease-associated regulatory single nucleotide polymorphisms, and to classify cell-type-specific and

ubiquitous enhancers. We further explored the utility of enhancer redundancy, which explains gene expression strength rather than expression patterns. The online FANTOM5 enhancer atlas represents a unique resource for studies on cell-type-specific enhancers and gene regulation.

Characterization of two recent Japanese field isolates of canine distemper virus and examination of the avirulent strain utility as an attenuated vaccine

Takenaka, A., Yoneda, M., Seki, T., Uema, M., Kooriyama, T., Nishi, T., Fujita, K., Miura, R., Tsukiyama-Kohara, K., Sato, H., Kai, C.

Recently, several new strains of canine distemper virus (CDV) have been isolated in Japan. To investigate their pathogenesis in dogs, the Yanaka and Bunkyo-K strains were investigated by infecting dogs and determining clinical signs, amount of virus, and antibody responses. The Yanaka strain is avirulent and induced an antibody response. The Bunkyo-K strain induced typical CDV clinical signs in infected dogs and virulence was enhanced by brain passage. Molecular and phylogenetic analyses of H genes demonstrated the Bunkyo-K strains were of a different lineage from Asia-1 group including the Yanaka strain and Asia-2 group that contain recent Japanese isolates, which were recently identified as major prevalent strains worldwide but distinct from old vaccine strains. Based on these data, we tested the ability of the Yanaka strain for vaccination. Inoculation with the Yanaka strain efficiently induced CDV neutralizing antibodies with no clinical signs, and the protection effects against challenge with either old virulent strain or Bunkyo-K strain were equal or greater when compared with vaccination by an original vaccine strain. Thus, the Yanaka strain is a potential vaccine candidate against recent prevalent CDV strains.

Publications

Sugai, A., Sato, H., Hagiwara, K., Kozuka-Hata, H., Oyama, M., Yoneda, M., Kai, C. Newly identified minor phosphorylation site threonine-279 of measles virus nucleoprotein is a prerequisite for nucleocapsid formation. *J Virol.* 88(2): 1140-9, 2014.

Imamura, K., Imamachi, N., Akizuki, G., Kumakura, M., Kawaguchi, A., Nagata, K., Kato, A., Kawaguchi, Y., Sato, H., Yoneda, M., Kai, C., Yada, T., Suzuki, Y., Yamada, T., Ozawa, T., Kaneki, K., Inoue, T., Kobayashi, M., Kodama, T., Wada, Y., Sekimizu, K., Akimitsu, N. Long Noncoding RNA NEAT1-Dependent SFPQ Relocation from Promoter Region to Paraspeckle Mediates IL8 Expression upon Immune Stimuli. *Mol Cell*. 6; 53(3): 393-406, 2014.

Yasui, F., Kohara, M., Kitabatake, M., Nishiwaki, T., Fujii, H., Tateno, C., Yoneda, M., Morita, K., Matsushima, K., Koyasu, S., Kai, C. Phagocytic cells contribute to the antibody-mediated elimination of pulmonary-infected SARS-associated coronavirus. *Virology* 454-455: 157-68, 2014.

The FANTOM Consortium and the RIKEN PMI and CLST (Kai, C., Nakamura, T., Sato, H., Sugiyama, T., Yoneda, M. in 101 authors). A promoter level mammalian expression atlas. *Nature*, 50 (7493): 462-70, 2014.

Andersson, R., The FANTOM Consortium et al. (Kai, C., Nakamura, T., Sato, H., Sugiyama, T., Yoneda, M. in 261 authors). An atlas of active enhancers across human cell types and tissues. *Nature*, 507(7493): 455-61, 2014.

Liu, Y., Sato, H., Hamana, M., Moonan, N.A., Yoneda, M. and Kai, C. Construction of an expression system for bioactive IL-18 and generation of recombinant canine distemper virus expressing IL-18. *J Vet Med Sci*, 76(9): 1241-48, 2014.

Murakami, T., Ibi, K., Kuraishi, T., Hattori, S., Kai, C., Ishiguro, N. and Yanai, T. Failure of heterogeneous amyloid-enhancing factor in geriatric squirrel monkeys (Saimiri boliviensis). *J Med Primatol*. 43(6): 488-91, 2014.

Kai, C., Sugiyama, T., Fujiyuki, T. and Yoneda, M. A Novel strategy of oncotherapy using a recombinant oncolytic measles virus. *Int. J. Mol. Med*., 34, 2014. supplement 1, S131.

Takenaka, A., Yoneda, M., Seki, T., Uema, M., Kooriyama, T., Nishi, T., Fujita, K., Miura, R., Tsukiyama-Kohara, K., Sato, H., Kai, C. Characterization of two recent Japanese field isolates of canine distemper virus and examination of the avirulent strain utility as an attenuated vaccine. *Vet Microbiol.* 174(3-4): 372-81, 2014.

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
Project Assistant Professor	Takeshi Kuraishi, Ph.D.		特任助教	農学博士	倉　石　　武

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

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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. The analysis of specific transcription factors of Habu snake venom gland

Hitomi Nakamura[6], Tatsuo Murakami[7], Shosaku Hattori, Yoshiyuki Sakaki[8], Takatoshi Ohkuri[6], Takahito Chijiwa, Motonori Ohno and Naoko Oda-Ueda: [6]Department of Biochemistry, Faculty of Pharmaceutical science, [7]Department of Applied Life Science, Faculty of Bioscience, Sojo University, [8]RIKEN Genomic Sciences center

Protobothrops flavoviridis (Habu) is a Japanese venomous snake. The phospholipase A_2 (PLA_2) secreted from habu venom gland exert various toxic effects. It is thought that activation of PLA_2 genes specifically expressed in the venom gland is regulated by venom gland-specific transcription factors. Therefore, we search the transcription factors by using the full-length cDNA library for habu venom gland after milking of the venom. As a result, we identified three cDNAs encoding epithelium-specific ETS transcription factors (ESE)-1, 2, 3. ESE-3 showed specific expression in the venom gland and activation for the proximal promoters of venom PLA_2 genes. The binding specificity of ESE-3 to the ETS binding motif is correlated with transcriptional activities for the venom PLA_2 genes. This work showed that ESE-3 function as an activator on the promoters of the PLA_2 genes.

3. Reproduction of squirrel monkeys and owl monkeys.

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 breeding 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. Six newborns were given in reproductive groups of squirrel monkeys in 2014. Two of 6 newborns were nursed by laboratory staffs because of neglect of their mothers. On the other hand, 3 newborns were given in 3 female owl monkeys in 2014.

4. Cross-species transmission of AA amyloidosis between primates and other animal

Tomoaki Murakami[9], Kanata Ibi[10], Takeshi Kuraishi, Shosaku Hattori, Chieko Kai, Naotaka Ishiguro[11] and Tokuma Yanai[10]: [9]Laboratory of Veterinary Toxicology, Tokyo University of Agriculture and Technology, [10]Laboratory of Veterinary Pathology, Department of Veterinary Medicine, Gifu University, [11]Laboratory of Food and Environmental Hygiene, Department of Veterinary Medicine, Gifu University

We administered chimpanzee, bovine, chicken amyloid fibrils as amyloid-enhancing factor (AEF) into squirrel monkeys to investigate the possibility for cross-species AA amyloidisis transmission. AA amyloid depositions were not observed in all squirrel monkeys inoculated. The results in this work imply that cross-species transmission of AA amyloidosis may be very difficult in primates.

5. A case of dermatitis caused by trombiculid mite in an Amami rabbit (*Pentalagus furnessi*).

Masahito Kubo[12], Hiroshi Sato[13], Shosaku Hattori and Takeshi Kuraishi: [12]Laboratory of Veterinary Pathology, [13]Laboratory of Veterinary Parasitology, Joint faculty of veterinary medicine, Yamaguchi University.

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 a case of severe dermatitis produced by trombiculid mite attack in an Amami rabbit (*Pentalagus furnessi)*. A young-adult male rabbit was discovered in a carcass on the road. Notable dermatitis was observed on the ventral abdomen, axillary, and inguinal regions. Tissue samples of skin were fixed in 10% neutral-buffered formalin for histopathological and parasitological study. Fixed tissue samples were treated with routine histological methods and sections were stained with

hematoxylin-eosin. Ectoparasites collected from the skin samples from the abdominal and inguinal regions were mounted in gum-chloral media and examined under the microscope. Histologically, ectoparasites were observed on the skin surface. Tubular structures with hyalinized walls (stylostomes of trombiculid mite) were discovered in the covering crust and epidermis. According to the morphologic criteria for species differentiation, all mites examined were identified as *Leptotrombidium miyajimai*.

In conclusion, remarkable dermatitis caused by trombiculid mites (*L. miyajimai)* was found in a dead body of amami rabbit. This is the first report of skin lesions due to trombiculid mite attack in the Amami rabbit.

6. Isolation of bone-marrow derived mesenchymal stem cells (BM-MSC) from a carcass of squirrel monkey infant

Takeshi Kuraishi, Shosaku Hattori and Chieko Kai

Before clinical application and transplantation of MSC-derived cells, the safety and efficiency must be preclinically tested. This should be preferably carried out in a non-human primate animal model, because mouse stem cells have proven to show great differences from the human. We isolated MSC from bone marrow of a squirrel monkey infant. Under specific extracellular nutrient conditions, BM-MSC differentiated into osteogenic, chondrogenic, adipogenic lineage. Our results demonstrate that squirrel moneky bone marrow is a viable source of MSC capable of multilineage differentiation in vitro.

Publications

Nakamura, H., Murakami, T., Hattori, S., Sakaki, Y., Ohkuri, T., Chijiwa, T., Ohno, M. and Oda-Ueda, N. Epithelium specific ETS transcription factor, ESE-3, of *Protobothrops flavoviridis* snake venom gland transactivates the promoters of venom phospholipase A_2 isozyme genes. Toxicon. 92: 133-139, 2014.

Yamaguchi, K., Chijiwa, T., Ikeda, N., Shibata, H., Fukumaki, Y., Oda-Ueda, N., Hattori, S. and Ohno, M. The finding of a group IIE phospholipase A_2 gene in a specified segment of *protobothrops flavoviridis* genome and Its possible evolutionary relationship to group IIA phospholipase A_2 genes. Toxins. 6: 3471-3487, 2014.

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Kubo, M., Sato, H., Hattori, S. and Kuraishi, T. Dermatitis associated with infestation of a Trombiculid Mite, *Leptotrombidium miyajimai*, in an Amami rabbit (*Pentalagus furnessi*). J. Wildl. Dis. 50(2): 416-418, 2014.

服部正策，倉石武．ハブ粗毒筋注後の周辺の病理組織学的検索と免疫ウマ血清（№1469）の効果．平成25年度奄美ハブ毒免疫機序応用研究報告書．（鹿児島県）．pp. 42−51，2014.

倉石武，服部正策，朝沼榎．２回目の咬傷でアレルギー様症状を起こした咬傷者（１例）の追加調査（咬傷から５年後の血清中ハブ毒特異的IgE抗体の測定）．平成25年度奄美ハブ毒免疫機序応用研究報告書．（鹿児島県）．pp. 52−53，2014.

Laboratory of Molecular Genetics
遺伝子解析施設

Professor	Izumu Saito, M.D., D.M.Sc.	教 授	医学博士	斎 藤 泉
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Assistant Professor	Saki Kondo, D.M.Sc.	助 教	医学博士	近 藤 小 貴

This laboratory has three main activities: development and supply of novel adenovirus vectors useful for studies in various fields including gene therapy, study about hepatitis B virus 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). We have established a unique system producing HD AdVs using 293 hde12 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.

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; Kondo *et al*., Nucleic Acids Res. 31: e76, 2003) as well as FLP/frt system (Nakano *et al*., Nucleic Acids Res. 29: e 40, 2001; 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 293-hde12 cell line producing a large amount of the codon-hu-

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

The research activities in 2014 are shown below. They include in the three different fields: the virology of adenovirus, the application of AdVs in gene therapy and the study of hepatitis B virus.

1. VA RNAs suppress HDGF gene expression to support efficient viral replication: Identification of a target gene of VA RNAs

Saki Kondo, Kenji Yoshida[1], Mariko Suzuki, Izumu Saito and Yumi Kanegae: [1]Regenerative and Cellular Medicine Office, Sumitomo Dainippon Pharma Co., Ltd., Kobe

Non-coding small RNAs are involved in many physiological responses including viral life cycles. Adenovirus-encoding small RNAs, known as VA RNAs, are transcribed throughout the replication process in the host cells, and their transcript levels depend on the copy numbers of the viral genome. Therefore, VA RNAs are abundant in infected cells after genome replication, i.e. during the late phase of viral infection. Their function during the late phase is the inhibition of interferon-inducible protein kinase R (PKR) activity to prevent antiviral responses; recently, mivaRNAs, the microRNAs processed from VA RNAs, have been reported to inhibit cellular gene expression. Although VA RNA transcription starts during the early phase, little is known about its function. The reason may be because much smaller amount of VA RNAs are transcribed during the early phase than the late phase. In this study, we applied replication-deficient adenovirus vectors and novel AdVs lacking VA RNA genes to analyze the expression changes in cellular genes mediated by VA RNAs using microarray analysis. AdVs are suitable to examine the function of VA RNAs during the early phase, since they constitutively express VA RNAs but do not replicate except in 293 cells. We found that the expression level of hepatoma-derived growth factor (HDGF) significantly decreased in response to the VA RNAs under replication-deficient condition, and this suppression was also observed during the early phase under replication-competent conditions. The suppression was independent of mivaRNA-induced downregulation, suggesting that the function of VA RNAs during the early phase differs from that during the late phase. Notably, overexpression of HDGF inhibited AdV growth. This is the first report to show the function of VA RNAs during the early phase that may be contribute to efficient viral growth.

2. Preferable sites and orientations of transgene inserted in the adenovirus vector genome: the E3 site may be unfavorable for transgene position

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

The AdV can carry two transgenes in its genome, the therapeutic gene and a reporter gene, for example. The E3 insertion site has often been used for the expression of the second transgene. A transgene can be inserted at six different sites/orientations: E1, E3 and E4 sites, and right and left orientations. However, the best combination of the insertion sites and orientations as for the titers and the expression levels has not sufficiently been studied. We attempted to construct 18 AdVs producing GFP or LacZ gene driven by the EF1αpromoter and Cre gene driven by the α-fetoprotein promoter. The AdV containing GFP gene at E3 in the rightward orientation (GFP-E3R) was not available. The LacZ-E3R AdV showed 20-fold lower titer and 50-fold lower level of fiber mRNA than the control E1L AdV. Notably, we found four aberrantly spliced mRNAs in the LacZ-E3L/R AdVs, probably explaining their very low titers. Although the transgene expression levels in the E4R AdVs were about 3-fold lower than those in the E1L AdVs, their titers are comparable to E1L AdVs. We concluded that E1L and E4R sites/orientations are preferable for expressing the main target gene and a second gene, respectively.

3. Development of new methods to detect the replicating HBV genome of ccc and rc in the cells infected with adenovirus vectors expressing pregenome RNA

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The genome of hepatitis B virus (HBV) is incomplete double-stranded DNA (rc, relaxed-circular DNA), while the covalently closed circular DNA (ccc) genome is present in nuclei from which the pregenome RNA (pgRNA) is transcribed. However, there have been only a few methods for simultaneous detection of both rc and ccc. The conventional methods are not suited to distinguish the HBV DNA region in the plasmid from ccc genome newly produced by viral DNA replication. We have established two methods detecting only the replicating HBV genome by distinguishing from the transduced HBV DNA. For the first method, we used the plasmid containing the 1.2 copy-HBV genome for transfection into HuH-7 cells. Then, total DNA

extraction is treated with restriction enzyme that cleaves only plasmid DNA. For the second method, we used AdV expressing HBV pregenome. The AdV shows much higher transduction efficiency than the plasmid using transfection. Since AdV DNA does not remain on the cell surface (Pei *et al*., BBRC, 2012), we were able to detect the replicating HBV genomes by Southern blot analysis using AdVs. Moreover, the band of HBV genomes became undetectable when the cells treated by Lamivudine, which is an inhibitor of reverse transcriptase. In addition, the replicating HBV genome increased depending on the infection dose of AdV, indicating this new method using AdVs is suitable for quantitative analysis. In conclusion, we developed two methods useful for the study of the replication of HBV genome. Besides, these methods can probably be applied to screening of new anti-HBV drugs.

Publications

Lystad, A., Ichimura, Y., Takagi, K., Yang, Y., Pankiv, S., Kanegae, Y., Kageyama, S., Suzuki, M., Saito, I., Mizushima, T., Komatsu, M., and Simonsen, A. Structural determinants in GABARAP required for the selective binding and recruitment of ALFY to LC3B positive structures. EMBO Rep., 15: 557-565, 2014.

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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>

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

Hiroko Kozuka-Hata, Tomoko Hiroki, Ryo Koyama-Nasu[1], Yumi Goto, Kouhei Tsumoto, Junichiro Inoue, 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. 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, Kouhei Tsumoto, Junichiro Inoue, 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 glioblastoma initiating cells. Our shotgun phosphoproteomics-based strategy revealed that 731 phosphorylation sites on 415 proteins were significantly regulated in serum-induced differentiation of the glioblastoma initiating cells, in which Rho family GTPase signaling was strongly correlated with stem cell differentiation by statistical pathway analyses. Furthermore, some specific kinases, such as Fyn, ERK1/2 and TGFBR1/2 were also found to be potential regulators based on their phosphorylation profiles and upstream kinase prediction analyses. The experimental verification based on drug-dependent signaling perturbation should help us elucidate the principles of stemness-related network regulation and pave the way to develop novel targets for the treatment of signaling aberration from a systems perspective.

3. 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 GSK 3β 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.

4. 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.

5. Sophisticated proteomics technologies for identifying protein-protein interaction sites using cross-linking/high-resolution mass spectrometry

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

System-wide elucidation of protein-protein interaction (PPI) networks is very important to define the regulatory mechanisms in cellular machineries. As homogenous, stable and sufficient amounts of complexes are required for conventional structural analyses such as X-ray crystallography and NMR, an overview of network-wide PPI information in living systems remains mostly unclear.

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 II>

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. Identification and characterization of the X-dimer of human P-cadherin: implications for homophilic cell adhesion.

Kudo S, Caaveiro JM, Goda S, Nagatoishi S, Ishii K, Matsuura T, Sudou Y, Kodama T, Hamakubo T, Tsumoto K.

Cell adhesion mediated by cadherins depends critically on the homophilic trans-dimerization of cadherin monomers from apposing cells, generating the so-called strand-swap dimer (ss-dimer). Recent evidence indicates that the ss-dimer is preceded by an intermediate species known as the X-dimer. Until now, the stabilized form of the X-dimer had only been observed in E-cadherin among the classical type I cadherins. Herein, we report the isolation and characterization of the analogous X-dimer of human P-cadherin. Small-angle X-ray scattering (SAXS) and site-directed mutagenesis data indicates that the overall architecture of the X-dimer of human P-cadherin is similar to that of E-cadherin. The X-dimerization is triggered by Ca(2+) and governed by specific protein-protein interactions. The attachment of three molecules of Ca(2+) with high affinity (Kd = 9 μM) stabilizes the monomeric conformation of P-cadherin (ΔTm = 17 °C). The Ca (2+)-stabilized monomer subsequently dimerizes in the X-configuration by establishing protein-protein interactions that require the first two extracellular domains of the cadherin. The homophilic X-dimerization is very specific, as the presence of the highly homologous E-cadherin does not interfere with the self-recognition of P-cadherin. These data suggest that the X-dimer could play a key role in the specific cell-cell adhesion mediated by human P-cadherin.

2. Role of positions e and g in the fibrous assembly formation of an amphipathic α-helix-forming polypeptide.

Takei T, Tsumoto K, Yoshino M, Kojima S, Yazaki K, Ueda T, Takei T, Arisaka F, Miura K.

We previously characterized α3, a polypeptide that has a three times repeated sequence of seven amino acids (abcdefg: LETLAKA) and forms fibrous assemblies composed of amphipathic α-helices. Upon comparison of the amino acid sequences of α3 with other α-helix forming polypeptides, we proposed that the fibrous assemblies were formed due to the alanine (Ala) residues at positions e and g. Here, we characterized seven α3 analog polypeptides with serine (Ser), glycine (Gly), or charged residues substituted for Ala at positions e and g. The α-helix forming abilities of the substituted polypeptides were less than that of α3. The polypeptides with amino acid substitutions at position g and the polypeptide KEα3, in which Ala was substituted with charged amino acids, formed few fibrous assemblies. In contrast, polypeptides with Ala replaced by Ser at position e formed β-sheets under several conditions. These results show that Ala residues at position e and particularly at position g are involved in the formation of fibrous assemblies.

3. Thermodynamic and structural characterization of the specific binding of Zn(II) to human protein DJ-1.

Tashiro S, Caaveiro JM, Wu CX, Hoang QQ, Tsumoto K.

Mutations of DJ-1 cause familial Parkinson's disease (PD), although the role of DJ-1 in PD remains unresolved. Very recent reports have shown that DJ-1 interacts with copper ions. This evidence opens new avenues to understanding the function of DJ-1 and its role in PD. Herein, we report that Zn(II) binds to DJ-1 with great selectivity among the other metals examined: Mn(II), Fe(II), Co(II), Ni (II), and Cu(II). High-resolution X-ray crystallography (1.18 Å resolution) shows Zn(II) is coordinated to the protein by the key residues Cys106 and Glu 18. These results suggest that DJ-1 may be regulated and/or stabilized by Zn(II).

4. The staphylococcal elastin-binding protein regulates zinc-dependent growth/biofilm formation.

Nakakido M, Aikawa C, Nakagawa I, Tsumoto K.

Staphylococcus aureus is one of the most important human pathogens because it is a common cause of nosocomial infections. The elastin-binding protein of Staphylococcus aureus (EbpS) is an adhesin that is responsible for attachment to host cells via its binding to elastin. Despite its relatively weak contribution to adhesion, the ebpS gene is highly conserved among S. aureus isolates, suggesting that EbpS may have other crucial functions. Here, we

found that EbpS binds Zn(2+) with its N-terminal region, which leads to local conformational changes that result in the assembly of the EbpS protein. The growth rate of the EbpS-deficient strain was considerably decreased. Zn(2+) chelation decreased the growth rate of the wild-type strain but did not alter that of the EbpS-deficient strain. Furthermore, biofilm formation by the EbpS-deficient strain was abnormally enhanced in the Zn(2+) concentration-dependent manner. All the results suggest that ebpS deficiency led to a zinc concentration-dependent inability to modulate the growth/biofilm maturation phase appropriately. Given the high conservation of ebpS and that appropriate regulation of biofilm formation is thought to be essential for effective staphylococcal infection, inhibition of EbpS binding to Zn(2+) could lead to the development of novel therapeutic strategies for controlling S. aureus infections.

5. Alternative downstream processes for production of antibodies and antibody fragments.

Arakawa T, Tsumoto K, Ejima D.

Protein-A or Protein-L affinity chromatography and virus inactivation are key processes for the manufacturing of therapeutic antibodies and antibody fragments. These two processes often involve exposure of therapeutic proteins to denaturing low pH conditions. Antibodies have been shown to undergo conformational changes at low pH, which can lead to irreversible damages on the final product. Here, we review alternative downstream approaches that can reduce the degree of low pH exposure and consequently damaged product. We and others have been developing technologies that minimize or eliminate such low pH processes. We here cover facilitated elution of antibodies using arginine in Protein-A and Protein-G affinity chromatography, a more positively charged amidated Protein-A, two Protein-A mimetics (MEP and Mabsorbent), mixed-mode and steric exclusion chromatography, and finally enhanced virus inactivation by solvents containing arginine. This article is part of a Special Issue entitled: Recent advances in molecular engineering of antibody.

6. Next generation drying technologies for pharmaceutical applications.

Walters RH, Bhatnagar B, Tchessalov S, Izutsu K, Tsumoto K, Ohtake S.

Drying is a commonly used technique for improving the product stability of biotherapeutics. Typically, drying is accomplished through freeze-drying, as evidenced by the availability of several lyophilized products on the market. There are, however, a number of drawbacks to lyophilization, including the lengthy process time required for drying, low energy efficiency, high cost of purchasing and maintaining the equipment, and sensitivity of the product to freezing and various other processing-related stresses. These limitations have led to the search for next-generation drying methods that can be applied to biotherapeutics. Several alternative drying methods are reviewed herein, with particular emphasis on methods that are commonly employed outside of the biopharmaceutical industry including spray drying, convective drying, vacuum drying, microwave drying, and combinations thereof. Although some of the technologies have already been implemented for processing biotherapeutics, others are still at an early stage of feasibility assessment. An overview of each method is presented, detailing the comparison to lyophilization, examining the advantages and disadvantages of each technology, and evaluating the potential of each to be utilized for drying biotherapeutic products.

7. Peptide-dependent conformational fluctuation determines the stability of the human leukocyte antigen class I complex.

Yanaka S, Ueno T, Shi Y, Qi J, Gao GF, Tsumoto K, Sugase K.

In immune-mediated control of pathogens, human leukocyte antigen (HLA) class I presents various antigenic peptides to CD8(+) T-cells. Long-lived peptide presentation is important for efficient antigen-specific T-cell activation. Presentation time depends on the peptide sequence and the stability of the peptide-HLA complex (pHLA). However, the determinant of peptide-dependent pHLA stability remains elusive. Here, to reveal the pHLA stabilization mechanism, we examined the crystal structures of an HLA class I allomorph in complex with HIV-derived peptides and evaluated site-specific conformational fluctuations using NMR. Although the crystal structures of various pHLAs were almost identical independent of the peptides, fluctuation analyses identified a peptide-dependent minor state that would be more tightly packed toward the peptide. The minor population correlated well with the thermostability and cell surface presentation of pHLA, indicating that this newly identified minor state is important for stabilizing the pHLA and facilitating T-cell recognition.

8. Protective effect of the long pentraxin PTX3 against histone-mediated endothelial cell cytotoxicity in sepsis.

Daigo K, Nakakido M, Ohashi R, Fukuda R, Ma-

tsubara K, Minami T, Yamaguchi N, Inoue K, Jiang S, Naito M, Tsumoto K, Hamakubo T.

Pentraxin 3 (PTX3), a member of the long pentraxin subfamily within the family of pentraxins, is a soluble pattern recognition molecule that functions in the innate immune system. Innate immunity affords the infected host protection against sepsis, a potentially life-threatening inflammatory response to infection. Extracellular histones are considered to be the main cause of septic death because of their cytotoxic effect on endothelial cells, which makes them a potential therapeutic target. We found that PTX3 interacted with histones to form coaggregates, which depended on polyvalent interactions and disorder in the secondary structure of PTX3. PTX3 exerted a protective effect, both in vitro and in vivo, against histone-mediated cytotoxicity toward endothelial cells. Additionally, the intraperitoneal administration of PTX3 reduced mortality in mouse models of sepsis. The amino-terminal domain of PTX3, which was required for coaggregation with histones, was sufficient to protect against cytotoxicity. Our results suggest that the host-protective effects of PTX3 in sepsis are a result of its coaggregation with histones rather than its ability to mediate pattern recognition. This long pentraxin-specific effect provides a potential basis for the treatment of sepsis directed at protecting cells from the toxic effects of extracellular histones.

9. Effects of Syringe Material and Silicone Oil Lubrication on the Stability of Pharmaceutical Proteins.

Krayukhina E, Tsumoto K, Uchiyama S, Fukui K.

Currently, polymer-based prefillable syringes are being promoted to the pharmaceutical market because they provide an increased break resistance relative to traditionally used glass syringes. Despite this significant advantage, the possibility that barrel material can affect the oligomeric state of the protein drug exists. The present study was designed to compare the effect of different syringe materials and silicone oil lubrication on the protein aggregation. The stability of a recombinant fusion protein, abatacept (Orencia), and a fully human recombinant immunoglobulin G1, adalimumab (Humira), was assessed in silicone oil-free (SOF) and silicone oil-lubricated 1-mL glass syringes and polymer-based syringes in accelerated stress study. Samples were subjected to agitation stress, and soluble aggregate levels were evaluated by size-exclusion chromatography and verified with analytical ultracentrifugation. In accordance with current regulatory expectations, the amounts of subvisible particles resulting from agitation stress were estimated using resonant mass measurement and dynamic flow-imaging analyses. The amount of aggregated protein and particle counts were similar between unlubricated polymer-based and glass syringes. The most significant protein loss was observed for lubricated glass syringes. These results suggest that newly developed SOF polymer-based syringes are capable of providing biopharmaceuticals with enhanced physical stability upon shipping and handling.

10. Reversible ion transportation switch by a ligand-gated synthetic supramolecular ion channel.

Muraoka T, Endo T, Tabata KV, Noji H, Nagatoishi S, Tsumoto K, Li R, Kinbara K.

Inspired by the regulation of cellular activities found in the ion channel proteins, here we developed membrane-embedded synthetic chiral receptors 1 and 2 with different terminal structures, where receptor 1 has hydrophobic triisopropylsilyl (TIPS) groups and receptor 2 has hydrophilic hydroxy groups. The receptors have ligand-binding units that interact with cationic amphiphiles such as 2-phenethylamine (PA). Conductance study revealed that the receptors hardly show ion transportation at the ligand-free state. After ligand binding involving a conformational change, receptor 1 bearing TIPS termini displays a significant current enhancement due to ion transportation. The current substantially diminishes upon addition of β-cyclodextrin (βCD) that scavenges the ligand from the receptor. Importantly, the receptor again turns into the conductive state by the second addition of PA, and the activation/deactivation of the ion transportation can be repeated. In contrast, receptor 2 bearing the hydroxy terminal groups hardly exhibits ion transportation, suggesting the importance of terminal TIPS groups of 1 that likely anchor the receptor in the membrane.

11. The molecular interaction of a protein in highly concentrated solution investigated by Raman spectroscopy.

Ota C, Noguchi S, Tsumoto K.

We used Raman spectroscopy to investigate the structure and interactions of lysozyme molecules in solution over a wide range of concentrations (2.5-300 mg ml(-1)). No changes in the amide-I band were observed as the concentration was increased, but the width of the Trp band at 1555 cm(-1) and the ratios of the intensities of the Tyr bands at 856 and 837 cm(-1), the Trp bands at 870 and 877 cm(-1), and the bands at 2940 (CH stretching) and 3420 cm(-1) (OH stretching) changed as the concentration was changed. These results reveal that al-

though the distance between lysozyme molecules changed by more than an order of magnitude over the tested concentration range, the secondary structure of the protein did not change. The changes in the molecular interactions occurred in a stepwise process as the order of magnitude of the distance between molecules changed. These results suggest that Raman bands can be used as markers to investigate the behavior of high-concentration solutions of proteins and that the use of Raman spectroscopy will lead to progress in our understanding not only of the basic science of protein behavior under concentrated (i.e., crowded) conditions but also of practical processes involving proteins, such as in the field of biopharmaceuticals.

<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 neural 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. Until recently 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 ultrathin TEM sections 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. We analyzed serial thin sections of zebrafish retinal outer plexiform layer by this method and succeeded in tracing thin processes of bipolar cells of less than 100nm diameter into the photoreceptor terminal. We are now trying to collect the information to decipher the wiring diagram of the retina.

In another project, the whole cell data regarding the localization of intracellular organelles of a hepatic cell were obtained by this method and 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.

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, 23 projects in 16 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[2]'s laboratory: [2]Division of Molecular Virology, Department of Microbiology and Immunology, 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[2] *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[3]'s laboratory: [3]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 expression. 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. Kurokawa[3] *et al*).

Some other collaborative research works using thin section electron microscopy and/or immuno-electron microscopy were performed with Dr. Noda[4] *et al* in [4]Division of Virology, Department of Microbiology, regarding the structure of the influenza viruses and ebola virus, Dr. Sanada[5]'s group, in [5]Department of Gerontological Nursing/Wound Care Management, Graduate School of Medicine, Dr. Kunieda[6] in [6]Laboratory of Physiological Chemistry, Department of Biological Sciences, Graduate school of Science, regarding the morphology of the Tardigrades.

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[4] *et al*., this technique combined with thin section electron microscopy was used to analyze the morphology of the influenza virus ribonucleoprotein complex. 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. This method was used also to analyze the function of a protein during the in vitro formation of collagen fibers in collaboration with Dr. Tsumoto[7] *et al* in [7]Medical Proteomics Laboratory.

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. Sanada[5] *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[8] in [8]Olympus Co.

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<Group I>

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