Affiliated Facilities

Laboratory Animal Research Center
実験動物研究施設

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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 minus 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.

Measles virus selectively blind to signaling lymphocyte activation molecule as a novel oncolytic virus for breast cancer treatment.

Sugiyama T., Yoneda M., Kuraishi T., Hattori S., Inoue Y., Sato H. and Kai C.

Oncolytic viruses hold much promise as novel therapeutic agents that can be combined with conventional therapeutic modalities. Measles virus (MV) is known to enter cells using the signaling lymphocyte activation molecule (SLAM), which is expressed on cells of the immune system. Although human breast cancer cell lines do not express SLAM, we found that a wild-type MV (HL strain) efficiently infected various breast cancer cell lines, causing cell death. Based on this finding, we used reverse genetics to generate a recombinant MV selectively unable to use SLAM (rMV-SLAMblind). The rMV-SLAMblind lacked infectivity for SLAM-positive lymphoid cells, while retaining oncolytic activity against breast cancer cells. We showed that, unlike the MV vaccine strains, rMV-SLAMblind used PVRL4 (polio virus receptor-related 4) as a receptor to infect breast cancer cells and not the ubiquitously expressed CD46. Consistent with this, rMV-SLAMblind infected CD46-positive primary normal human cells at a much-reduced level, whereas a vaccine strain of the Edmonston lineage (rMV-Edmonston) efficiently infected and killed them. The rMV-SLAMblind showed antitumor activity against human breast cancer xenografts in immunodeficient mice. The oncolytic activity of rMV-SLAMblind was significantly greater than that of rMV-Edmonston. To assess the in vivo safety, three monkeys seronegative for MV were inoculated with rMV-SLAMblind, and no clinical symptoms were documented. On the basis of these results, rMV-SLAMblind could be a promising candidate as a novel oncolytic virus for breast cancer treatment.

The nucleocapsid protein of measles virus blocks host interferon response.

Takayama I., Sato H., Watanabe A., Omi-Furutani M., Kanki K., Yoneda M. and Kai C.

MV belongs to the genus Morbillivirus of the

family Paramyxoviridae. A number of paramyxoviruses inhibit host interferon (IFN) signaling pathways in host immune systems by various mechanisms. Inhibition mechanisms have been described for many paramyxoviruses. Although there are inconsistencies among previous reports concerning MV, it appears that P/V/C proteins interfere with the pathways. In this study, we confirmed the effects of MV P gene products of a wild MV strain on IFN pathways and examined that of other viral proteins on it. Interestingly, we found that N protein acts as an IFN-α/β and γ-antagonist as strong as P gene products. We further investigated the mechanisms of MV-N inhibition, and revealed that MV-N blocks the nuclear import of activated STAT without preventing STAT and Jak activation or STAT degradation, and that the nuclear translocation of MV-N is important for the inhibition. The inhibitory effect of the N protein was observed as a common feature of other morbilliviruses. The results presented in this study suggest that N protein of MV as well as P/V/C proteins is involved in the inhibition of host IFN signaling pathways.

Nipah virus N mRNA is downregulated through interaction between its 3'UTR and hnRNP D.

Hino K., Sato H., Sugai A., Kato M., Yoneda M. and Kai C.

Nipah virus (NiV) belongs to the Henipavirus within the family Paramyxoviridae. NiV causes acute encephalitis and respiratory disease in humans, has a high mortality rate, and is a threat in South Asia. The genome size of henipavirus is about 18,246 nucleotides, which is longer than other paramyxoviruses (whose genome size is about 15,384 nucleotides). The difference in genome size is due to the noncoding RNA region, in particular, the 3'UTR occupies over half of the noncoding RNA region. To determine the function of the NiV noncoding RNA region, we investigated the effect on the expression of reporter gene by NiV 3'UTRs. We found that the NiV N 3'UTR has strong repressor activity, and the 1 to 100 nucleotide region is the most responsible. Subsequently, we tried to found the proteins binding to that region, and identified hnRNP D. A mutation in the hnRNP D binding site or the knockdown of hnRNP D resulted in the increased expression of the NiV N 3'UTR reporter. Our findings indicated that NiV N expression would be repressed by hnRNP D through the NiV N 3'UTR, and revealed that the involvement of posttranscriptional regulation in the viral life cycle.

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Affiliated Facilities

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

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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]Department of Veterinary Anatomy, Faculty of Agriculture, University of 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.

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

The squirrel monkey, *Saimiri sciurea*, is widely distributed in the tropical rainforest in Central and South America between 10 degrees N and 17 degrees S of latitudes. The advantage of using this species for medical researches resides in its small size and gentle behavior. In this laboratory, about 3 ～7 newborns are given annually by 24 adult females.

The aim is to optimize the use of the non-human primate model in future the Amami Laboratory research activities. The laboratory newly established experimental infection systems which require or can be adapted to the squirrel monkey model, particularly the study of human falciparum malaria. Development of parasites, immune response to malaria parasites and pathological changes were investigated in in-vivo condition, further more, in vitro analysis of cell and molecular level was performed. It is also investigating the mechanisms of infection in immunology, vector development, a vaccine production program, and a clinical trials program.

3. Sleep profile in captive squirrel monkeys (*Saimiri boliviensis*) and owl monkeys (*Aotus lemurimus*).

Sachi Sri Kantha[7], Juri Suzuki[8], Shosaku Hattori, Takeshi Kuraishi and Chieko Kai: [7]Pharmaceutical English Section, Gifu Pharmaceutical University, [8]Center for Human Evolution Modeling Research, Kyoto University Primate Research Institute

Our objective in this study is to test the hypothesis that nocturnally-active owl monkeys and diurnally-active squirrel monkeys will exhibit differences in their sleep parameters namely, total sleep time (TST) and sleep episode length (SEL) because these primates have been exposed historically to different predation risks. Therefore, we are monitoring TST and SEL of～51 squirrel monkeys in breeding colony of Amami laboratory. TST and SEL of these monkeys were quantitated for 14～21 days (one experiment period) via actigraphy: by tagging an acclerometer-type miniature transmitter (Actiwatch-MINIMITTER) sensitive to omnidirectional movement, to the squirrel monkey's neck. Furthermore, we will compare TST and SEL data acquired from squirrel monkeys in this year with that of 16 owl monkeys in the last year.

4. Histopathological study in Amami rabbits (*Pentalagus furnessi*).

Masahito Kubo[9], Takeshi Kuraishi and Shosaku Hattori: [9]Laboratory of Veterinary Pathology, 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, deforestation and predation by invasive mammals, such as feral dogs, cats and mongoose, have caused a decline in the population. From the results of a pellet census carried out on Amami-Oshima Island 2002-2003, the population was estimated to be in the rage 2000-4800, which is 20 % lower than the estimate in 1994. 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 vestibular disorder due to chronic suppurative otitis media/interna in an Amami rabbit. An adult female Amami rabbit was rescued in March 2010 because of vestibular disorder, but this rabbit died six days after rescue. We found chronic suppurative otitis media/interna in the right ear by histopathological examination. Therefore, we considered to be a cause of vestibular disorder.

5. Isolation of fibroblastic cell populations by ceiling culture of mature adipocyte obtained from 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). These cells show the same multilineage potential as adipose tissue-derived stromal cells (ADSCs). It is

seemed to be a good useful source of mesenchymal stem cells (MSCs) in regenerative medicine. However, 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. The squirrel monkey is a preferable non-human primate animal model for this aim due to its genetic and physiological similarities to the human. We isolated squirrel monkey DFAT cells from a small amount of adipose tissue. DFAT cells obtained from adipose tissue 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 ASCs. Stem cell markers mRNA expression such as Oct4 and CD90 were detected in both DFAT cells and ASCs of the squirrel monkey. In further study, we must demonstrate that squirrel monkey DAFT cells are able to differentiate into adipocytes, osteoblasts, and chondrocytes under appropriate culture conditions.

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Affiliated Facilities

Laboratory of Molecular Genetics
遺伝子解析施設

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Assistant Professor	Yumi Kanegae, D.M.Sc.	助　教	医学博士	鐘ヶ江　裕　美
Assistant Professor	Saki Kondo, D.M.Sc.	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 20 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, will be replaced by the low-inflammatory Cre-expressing AdV, 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). The research activities in 2012 are shown below.

1. Efficient production of adenovirus vector lacking genes of virus-associated RNAs that disturb cellular RNAi machinery

Aya Maekawa, Zheng Pei, Mariko Suzuki, Hiromitsu Fukuda, Yohei Ono, Saki Kondo, Izumu Saito and Yumi Kanegae

FG AdVs were usually considered that they do not express any viral gene product. However, we have shown that, in fact, the expression of adenoviral protein IX (pIX) is induced by purpose gene promoter (Nakai *et al*., Hum. Gene Ther. 18: 925-936, 2007). pIX co-expression is one of the main causes of AdV-induced immune responses. Whereas CAG and SRα promoters considerably activated the pIX promoter through their enhancer effects, the EF1α promoter hardly did. Therefore, the EF1α promoter is valuable for the long-term expression of FG AdV. However, FG AdVs, in fact, also express VA RNAs that are transcribed by RNA polymerase III when using these vectors both in vitro and in vivo.

VA RNAs, VAI and VAII, located at about 30 map units on adenovirus 5 (Ad5), are non-coding RNAs consisting of 157-160 nucleotides (nt). These VA RNAs are extremely abundant during the late phase of infection and inhibit cellular RNA-interference pathways by saturating Exportin 5 and Dicer. VAI also inhibits protein kinase R (PKR) activity and, consequently, eliminates the block of the cellular translation machinery to allow the efficient production of viral proteins. Moreover, VA RNAs were processed and generate miRNAs, known as mivaRNAI and mivaRNAII, that disturb the expression of many cellular genes with the probable result of blocking cellular antiviral machinery. Maybe due to these functions, the titers of VA-deleted Ad5 are approximately 60-fold lower than that of wild-type Ad5 and development of efficient systems for producing E1-, E3- and VA-deleted AdVs are extremely difficult. However, it is no doubt that the AdVs lacking VA RNA genes (VA-deleted AdVs) are superior to current FG AdVs.

We succeeded in establishing an efficient method of producing VA-deleted AdVs. First, a VA-substituted "pre-vector" lacking the original VA RNA genes but alternatively possessing an intact VA RNA region was constructed. Then, this region was removed after a few passages in FLP-expressing 293 cells. The resulting transduction titers of VA-deleted AdVs were sufficient for practical use. It is notable that the titer of the Cre-expressing VA-deleted AdV was successfully obtained without trouble and was also quantitatively sufficient for general use, because Cre-expressing FG AdVs often show low titer and are sometimes difficult for expansion of the vector.

The VA-deleted AdV seems particularly useful for the field using adenovirus vector expressing siRNA, because VA RNAs expressed in the FG AdV may compete with siRNAs and reduce their effects. Therefore, VA-deleted AdVs lacking expression of VA RNAs are probably advantageous for basic studies, both *in vitro* and *in vivo*, and would offer safer gene therapy.

2. Adenovirus vectors lacking virus-associated RNA expression enhance shRNA downregulation of hepatitis C virus replication

Zheng Pei, Gudi Shi[1], Saki Kondo, Masahiko Ito[1], Tetsuro Suzuki[1], Izumu Saito and Yumi Kanegae: [1]Department of Virology and Parasitology, Hamamatsu Medical College, Hamamatsu

Strategies utilizing RNAi or functional RNAs have recently been applied for suppression of the purpose gene expression. AdVs have often been used for this purpose and recently more than one-hundred papers were published per year. Because viral E1a protein is essential for all early promoters by polymerase II, FG AdV is usually considered not to express any virus protein in target cells. However, two different VA RNAs are, in fact, transcribed by Pol III promoter from the FG AdV genome in the target cells. VA RNAs are processed to small RNAs (mivaRNAs) (Aparicio *et al*., Nucleic Acids Res., 2010) and suppress the expression of many cellular genes. Moreover, because VA RNAs acts as miRNAs and siRNAs in cells, VA RNAs may interfere the function of miRNA and shRNA through competition. Moreover, VA RNAs directly bind to Exportin 5 and Dicer and inhibit cellular RNA-interference machienary, it is anticipated that they might hamper the effect of miRNAs and shRNAs expressed for FG AdVs. However, this possibility was not able to be tested because VA-deleted AdVs had not been obtained.

Using the VA-deleted AdVs developed by Maekawa *et al*. described above, we directly examined using VA-deleted AdV and FG AdV whether VA RNAs inhibit the activity of various shRNA or not. We constructed the three sets of shRNA carrying VA-containing and VA-deleted AdVs and showed evidences for the first time that, in fact, VA RNAs do interfere shRNA activity. The VA-deleted AdVs expressing shRNA targeting at 5′-noncoding region of hepatitis C virus (HCV) more efficiency suppressed HCV replication than the VA-containing, current FG AdVs. Because shRNA technology is popularly used not only in basic studies but also as the strategy for gene therapy fields, VA-deleted AdV may be valuable tool in these fields. However, we also found that such effect was not observed for certain gene expressions. Further studies are needed to clarify these differences.

3. Comparison of expression levels of transgene inserted in E1, E3 and E4 positions with different orientations

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

First-generation adenovirus vector is widely used because of high transduction efficiency. Usually the inserted transgene is substituted for E1 genes located near the left terminal of the viral genome. In addition, E3 region and E4-adjacent position located at an internal position and near the right end of the genome, respectively, can be used as the insertion sites. Moreover, transgenes can be inserted in either rightward or leftward orientation. Therefore, six different AdVs in total can theoretically be constructed for one transgene. However, it has not been examined in detail to what extent these inserted positions and orientations influence on transgene expression levels. In order to obtain the maximum expression level of a transgene containing a strong, versatile promoter and, especially, a weak promoter such as tissue-specific promoters, the influences of different insertion sites and orientations of the transgene are important.

We tried to construct AdVs containing GFP and LacZ genes under the control of potent EF1α promoter, and Cre gene under the control of α-fetoprotein (AFP) promoter in different combinations of insertion positions and orientations. In total, we tried to obtain eighteen different AdVs and, of these, examined seventeen AdVs. Then we examined their titers, expression levels and mRNA structures. We observed that using the potent promoters AdVs of E1L (E1 insertion position and leftward orientation) showed the highest expression level and that both E4L AdVs and E4R AdVs exhibited lower expression levels. Interestingly, the results using versatile promoters and expressed proteins (GFP and LacZ) were similar to those that AFP promoter and Cre were used, suggesting that the surrounding circumstances gave a similar effect with a similar mechanism for potent promoters and tissue-specific promoter. Surprisingly, though E3L AdVs were obtained without any trouble, we were not able to obtain GFP-expressing E3R AdV in spite of repeated trials and the titer of E3R AdV expressing LacZ was low. We are currently examined mRNA structures adjacent of the E3 insertion position to elucidate the reason of this striking difference between E 3R AdVs and E3L AdVs.

Publications

Maekawa, A., Pei, Z., Suzuki, M., Fukuda, H., Ono, Y., Kondo, S., Saito, I. and Kanegae, Y. Efficient production of adenovirus vector lacking genes of virus-associated RNAs that disturb cellular RNAi machinery. Sci Rep. 3: 1136, 2013.

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Affiliated Facilities

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Assistant Professor	Hiroshi Sagara, Ph.D.	助　教	医学博士	相　良　　洋
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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. Mapping ultra-weak protein-protein interactions between heme transporters of Staphylococcus aureus.

Abe R, Caaveiro JM, Kozuka-Hata H, Oyama M, Tsumoto K.

Iron is an essential nutrient for the proliferation of Staphylococcus aureus during bacterial infections. The iron-regulated surface determinant (Isd) system of S. aureus transports and metabolizes iron porphyrin (heme) captured from the host organism. Transportation of heme across the thick cell wall of this bacterium requires multiple relay points. The mechanism by which heme is physically transferred between Isd transporters is largely unknown because of the transient nature of the interactions involved. Herein, we show that the IsdC transporter not only passes heme ligand to another class of Isd transporter, as previously known, but can also perform self-transfer reactions. IsdA shows a similar ability. A genetically encoded photoreactive probe was used to survey the regions of IsdC involved in self-dimerization. We propose an updated model that explicitly considers self-transfer reactions to explain heme delivery across the cell wall. An analogous photo-cross-linking strategy was employed to map transient interactions between IsdC and IsdE transporters. These experiments identified a key structural element involved in the rapid and specific transfer of heme from IsdC to IsdE. The result-

ing structural model was validated with a chimeric version of the homologous transporter IsdA. Overall, our results show that the ultra-weak interactions between Isd transporters are governed by bona fide protein structural motifs.

2. Crystal structure of the enzyme CapF of Staphylococcus aureus reveals a unique architecture composed of two functional domains.

Miyafusa T, Caaveiro JM, Tanaka Y, Tsumoto K

CP (capsular polysaccharide) is an important virulence factor during infections by the bacterium Staphylococcus aureus. The enzyme CapF is an attractive therapeutic candidate belonging to the biosynthetic route of CP of pathogenic strains of S. aureus. In the present study, we report two independent crystal structures of CapF in an open form of the apoenzyme. CapF is a homodimer displaying a characteristic dumb-bell-shaped architecture composed of two domains. The N-terminal domain (residues 1-252) adopts a Rossmann fold belonging to the short-chain dehydrogenase/reductase family of proteins. The C-terminal domain (residues 252-369) displays a standard cupin fold with a Zn2+ ion bound deep in the binding pocket of the β-barrel. Functional and thermodynamic analyses indicated that each domain catalyses separate enzymatic reactions. The cupin domain is necessary for the C3-epimerization of UDP-4-hexulose. Meanwhile, the N-terminal domain catalyses the NADPH-dependent reduction of the intermediate species generated by the cupin domain. Analysis by ITC (isothermal titration calorimetry) revealed a fascinating thermodynamic switch governing the attachment and release of the coenzyme NADPH during each catalytic cycle. These observations suggested that the binding of coenzyme to CapF facilitates a disorder-to-order transition in the catalytic loop of the reductase (N-terminal) domain. We anticipate that the present study will improve the general understanding of the synthesis of CP in S. aureus and will aid in the design of new therapeutic agents against this pathogenic bacterium.

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 ITC 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. 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.

5. 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 hypoimmunogenic muteins retained the biotin-bind-

ing 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.

6. Interleukin-11 links oxidative stress and compensatory proliferation.

Nishina T, Komazawa-Sakon S, Yanaka S, Piao X, Zheng DM, Piao JH, Kojima Y, Yamashina S, Sano E, Putoczki T, Doi T, Ueno T, Ezaki J, Ushio H, Ernst M, Tsumoto K, Okumura K, Nakano H.

Apoptotic cells can stimulate the compensatory proliferation of surrounding cells to maintain tissue homeostasis. Although oxidative stress is associated with apoptosis and necrosis, whether it contributes to compensatory proliferation is unknown. Here, we showed that interleukin-11 (IL-11), a member of the IL-6 family of proinflammatory cytokines, was produced by cells in an oxidative stress-dependent manner. IL-11 production depended on the activation in dying cells of extracellular signal-regulated kinase 2, which in turn caused the phosphorylation and accumulation of the transcription factor Fra-1 by preventing its proteasome-dependent degradation. Fra-1 was subsequently recruited to the Il11 promoter and activated gene transcription. Upon acute liver injury in mice, IL-11 was mainly produced by hepatocytes in response to reactive oxygen species that were presumably released from dying hepatocytes. IL-11 that was secreted by the dying cells then induced the phosphorylation of the transcription factor STAT3 in adjacent healthy hepatocytes, which resulted in their compensatory proliferation. Furthermore, an IL-11 receptor (IL-11 R) agonist enhanced the proliferation of hepatocytes and ameliorated oxidative stress upon acetaminophen-induced liver injury. Conversely, the effects of acetaminophen were exacerbated in mice deficient in the IL-11R α subunit. Together, these results suggest that IL-11 provides a functional link between oxidative stress and compensatory proliferation.

<Group II>

1. Post-translational modification of proteins during apoptotic cell death

Apoptotic cell death involves various biochemical reactions. Among them, post-translational modification of proteins is intensively investigated in this laboratory. First, intracellular proteolytic enzymes are activated prior to and during apoptosis. Caspases are now established as pivotal apoptosis-executing enzymes that cleave various substrates. Endogenous or viral proteins and synthetic substances inhibitory for caspases suppress the apoptotic cascade and rescue cells from cell death. On the other hand, proteasomes drive the cell cycle by degrading cyclins etc., and also play important parts in apoptosis, since proteasome inhibitors induce apoptotic cell death in growing cells but suppress apoptosis of some cells that is in quiescent state. Furthermore, in some specific cells such as polymorphonuclear leukocytes, other proteases might be involved in cell death.

a. Fas, a death receptor, is polymerized to high-molecular weight forms during Fas-mediated apoptosis in Jurkat T cells

Hidehiko Kikuchi, Fotoshi Kuribayashi and Shinobu Imajoh-Ohmi

An apoptotic receptor Fas mediates death signal from Fas ligand. A cell death-inducing monoclonal antibody CH11 mimics Fas ligand and triggers apoptotic signal mediated by Fas molecule. Plasma transglutaminases are found to involved in down-regulation of apoptosis induced by a cytotoxic anti-Fas monoclonal antibody in Jurkat cells. When cells were treated with the antibody in fetal calf serum-containing media, Fas was polymerized to higher-molecular-weight polypeptides as judged by immunoblotting. Under conditions where the transglutaminase activity was eliminated or supprressed, the polymerization of Fas was not observed, and concurrently cell death was hastened. Furthermore, an antibody against blood coagulation factor XIII strongly accelerated the Fas-mediated apoptosis, indicating that plasma transglutaminases catalyze polymerization of Fas and down-regulate apoptotic cell death.

b. Limited proteolysis of actin in apoptotic neutrophils

Junko Ohmoto and Shinobu Imajoh-Ohmi

Neutrophil actin is proteolyzed to a 40-kDa fragment during preparation/isolation from peripheral blood. The truncated actin lacks amino-terminal region of native protein and presumably cannot copolymerize to F-actin. The 40-kDa actin-derived fragment is apparently related to spontaneous apoptosis of neutrophils. To investigate the role of actin proteolysis, especially cause-and-effect relationship to neutrophil apoptosis, we have made a cleavage-site-directed antibody (#1090pAb) for the 40-kDa form of actin using synthetic peptides as haptens. The antibody reacted with the 40-kDa polypeptide but not with unproteolyzed native actin which remain abundant in the cell. Using this

antibody, we have found that (1) the truncated actin is generated during isolation of neutrophils from peripheral blood, (2) neutrophils without the truncated actin can be prepared in the presence of diisopropyl fluorophosphate, and (3) leukocyte elastase is possibly responsible for this limited proteolysis.

Herein we analyzed cellular localization of the truncated actin using #1090pAb. Confocal laser microscopic observation indicated that the plasma membrane of neutrophils were strongly stained with #1090pAb, but that intracellular regions near the membrane were sometimes stained weakly. We examined here whether or not the amino-teriminal region of the 40-kDa actin is on the cell surface of neutrophils using at the same time established antibodies for components of superoxide-generating system composed of transmembranous cytochrome and cytosolic activator proteins. Furthermore, flow-cytometric analysis revealed that #1090pAb stained the cell-surface antigen under the conditions that antibodies for cytosolic proteins did not. Our findings suggest that the truncated actin is, at least in its amino-terminal part, on the surface of neutrophils. However, another antibody against the amino-terminal region of native actin did not stain neutrophils from outside suggesting that the cleavage site is inaccessible to exogenous proteinases.

2. Identification of a cleavage product of BRCA2 in cancer cell lines and its formative mechanism

Akira Nakanishi[1,2], Yoshio Miki[2,3] and Shinobu Imajoh-Ohmi: [1]IMSUT, [2]Depertment of Molecular Genetics, Medical Research Institute, Tokyo Medical and Dental University, [3]Department of Genetic Diagnosis, The Cancer Institute, Japanese Foundation for Cancer Research

BRCA2 is a multifunctional tumor suppressor protein with critical roles in DNA repair, centrosome amplification, and cytokinesis. Loss of BRCA2 expression has been linked to several cancer types. We previously performed immunoblotting for BRCA2 in the MCF7 breast cancer cell line using two antibodies with different epitopes, i.e., recognizing the central region (res. 1651-1821) and C terminus (res. 2959-3418). The specific band for wild-type BRCA2 (molecular weight; 384,000) was detected at 239-kDa using the antibody that recognizes the central region. The 239-kDa band was not detected using the antibody that recognizes the C terminus. However, the mechanisms governing BRCA2 cleavage are not clearly understood. We hypothesized that cleavage of BRCA2 is achieved by proteolytic processing of wild-type BRCA2.

To identify the candidate protease, we immunoprecipitated whole cell extracts prepared from HeLa S3 cells using anti-BRCA2 antibody (central region). Captured BRCA2-associated protease was separated by gel electrophoresis and subjected to mass spectrometry. Mass spectrometry was also used to determine the mass of cleavage products and the localization of the scissile bond. To examine the role of the cleavage BRCA2 fragments, we generated cleavage-site-directed antibodies for immunoblotting, which specifically recognize the two fragments of BRCA2 but do not cross-react with the unproteolyzed wild-type BRCA2 (384-kDa).

Analysis of immunoprecipitated protein complexes and mass spectrometry revealed membrane type-1 matrix metalloproteinase (MT1-MMP) protein complexed with BRCA2. We also found that the sequence of the BRCA2 cleavage site was at ^{2135}N-L^{2136} by mass spectrometry. Furthermore, following transfection of BRCA2-FLAG into HeLa cells, we observed differences in intercellular localization of cleaved BRCA2 and BRCA2-FLAG under immunofluorescence microscopy using cleavage-site-directed antibodies and FLAG antibody.

We suggest that BRCA2 is a cleavage target of MT1-MMP and that MT1-MMP possesses an intercellular proteolytic function, which may be importantly involved in malignant transformation.

<Group III>

1. Global phosphoproteome analysis of glioblastoma initiating cells by high-resolution mass spectrometry

Hiroko Kozuka-Hata, Yukiko Nasu-Nishimura[1], Ryo Koyama-Nasu[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

Stem cells have been known to exist in each tissue of multicellular organisms and have the ability to differentiate into various cell types based on their self-renewal and differentiation potency. Although the existence of cancer stem cells had been postulated for decades, there had been no experimental evidence for their presence. Recent studies demonstrated the existence of cancer stem cells in glioblastoma, the most aggressive brain tumors with median survival of less than 12 months after diagnosis.

In order to systematically elucidate the aberrant signaling machinery activated in this malignant brain tumor, we investigated phosphoproteome dynamics of glioblastoma initiating cells using high-resolution nanoflow LC-MS/MS system in combination with SILAC technology. Through phosphopeptide enrichment by titanium dioxide beads, more than 6,000 phosphopeptides were identified based

on the two peptide fragmentation methodologies of collision induced dissociation (CID) and higher-energy C-trap dissociation (HCD). The SILAC-based quantification described 516 up-regulated and 275 down-regulated phosphorylation sites upon epidermal growth factor stimulation, including the comprehensive status of the phosphorylation sites on stem cell markers such as nestin. Very intriguingly, our in-depth phosphoproteome analysis led to identification of novel phosphorylated molecules encoded by the undefined sequence regions of the human transcripts, one of which was regulated upon external stimulation in human glioblastoma stem cells.

Our result unveiled an expanded diversity of the regulatory phosphoproteome 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. 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, Tetsu Akiyama[1] and Masaaki Oyama

Glioblastoma is one of the most malignant brain tumors with a mean survival time of 14 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 factors and mechanisms are intensively studied because the characteristics of tumorigenicity and drug resistance have been 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. However, the global change of phosphoproteome in GSC differentiation is still unclear in spite of the presence of some previous reports regarding the individual pathways. The technology for phosphoproteomics has increasingly become more comprehensive and sensitive due to the significant development in shotgun proteomics and phosphopeptide enrichment methodologies. 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 analysis of serum-induced differentiation in GSCs.

Here, we tried to perform SILAC-based comparative quantification of the phosphoproteome in differentiated and undifferentiated GB2 cells, which are GSCs isolated from the human glioblastoma tissue. First, we observed the alteration of cellular morphology and performed western blotting of GB 2 cell lysates to confirm the change of stemness and differentiation markers under the conditions between serum-containing and serum-free culture. After cultured in serum-containing medium, GB2 cells were changed into more squamous forms. Moreover, the protein expression of the stemness marker (Sox2) was dramatically decreased in serum-cultured GB2 cells, whereas that of the differentiation marker (glial fibrillary acidic protein) was increased. Next, we prepared phosphopeptide samples for mass spectrometry through TiO_2 phosphopeptide enrichment from the lysates of normal and differentiated GB2 cells after adaption to the SILAC medium. Now we're currently obtaining the phosphoproteome data by high-resolution nanoLC-MS/MS and analyzing the signaling network status with Ingenuity Pathways Analysis. We aim to determine which phosphorylation-related pathways are crucial 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 IV>

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 for analyzing thick specimens. Although the morphological connections of neurons have only been analyzed by using transmission electron microscope (TEM), recent progress in scanning electron microscope (SEM) equipment used in industrial area such as "Focused ion beem SEM (FIB-SEM)" and "Three dimensional SEM (3D-SEM)" lead us to apply these new technology for biological specimens. To evaluate these new equipments we tried to visualize whole cell inner structure and found it valuable for analyzing neural connection in the retina. In parallel, we developed new methods for observing inner structure of the cells by combining the TEM technology and SEM. By using these methods we successfully obtained the precise inner structure of the cells efficiently and with higher resolution. Using these methods we are now going to collect the information to decipher the wiring diagram of the retina. Part of this work has been performed as collaboration with Dr. Ichikawa[1]'s laboratory: [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, 26 projects in 16 laboratories were performed as core-laboratory works.

a. Thin section electron microscopy

Thin section 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]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 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.

a-2. Morphological and immuno-electron microscopic analysis of the mucosal M-cells

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 ultrastructure of the M-cells in the airway and intestinal epithelium by thin section transmission electron microscopy and scanning electron microscopy. Molecular characteristics of the M-cells were also analyzed using Immuno-electron microscopy. In another study, several species of proteins were induced to express in rice and examined the localization within the cell with immuno-electron microscopy. We revealed that the expressed epitopes were accumulated in the different compartments depending on the kind of the expressed protein.

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's group[5], in [5]Department of Gerontological Nursing/Wound Care Management, Graduate School of Medicine, The University of Tokyo (ref. Ibuki *et al*), Dr. Hoshina[6] in [6]Division of Oncology, regarding the structure of the synapses, Dr. Kunieda[7] in [7]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[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.

c. Scanning electron microscopy

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[8] *et al.,* [8]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. Other works are in progress with Dr. Sanada[5] *et al,* to analyze the effects of diabetes or bacterial infection during wound repair (ref. Ibuki *et al*). Scanning electron microscopy was also used to analyze the non-biological materials as a collaborative work with Dr. Cheng[9] in [9]Olympus Co.

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<Group I>

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