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, virus vectors, and oncolytic virus 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.

Development of new therapy for canine mammary cancer with recombinant measles virus.

Shoji, K., Yoneda, M., Fujiyuki, T., Amagai, Y., Tanaka, A[1]., Matsuda, A[2]., Ogihara, K[3]., Naya, Y[3]., Ikeda, F., Matsuda, H[2]., Sato, H. and Kai, C.: [1]Laboratories of Comparative Animal Medicine, Division of Animal Life Science, Tokyo University of Agriculture and Technology, Japan [2]Laboratories of Veterinary Molecular Pathology and Therapeutics, Division of Animal Life Science, Tokyo University of Agriculture and Technology, Japan [3]Department of Pathology, School of Life and Environmental Science, Azabu University, Japan

Oncolytic virotherapy is a promising treatment strategy for cancer. We previously generated a recombinant measles virus (rMV-SLAMblind) that selectively uses a poliovirus receptor-related 4 (PVRL4/Nectin4) receptor, but not signaling lymphocyte activation molecule (SLAM). We demonstrated that the virus exerts therapeutic effects against human breast cancer cells. In this study, we further examined the applicability of rMV-SLAMblind to treating canine mammary cancers (CMCs). We found that the susceptibilities of host cells to rMV-SLAMblind were dependent on canine Nectin-4 expression. Nectin-4 was detected in four of nine CMC cell lines. The rMV-SLAMblind efficiently infected those four Nectin-4-positive cell lines and was cytotoxic for three of them (CF33, CHMm, and CTBm). In vivo experiment showed that the administration of rMV-SLAMblind greatly suppressed the progression of tumors in mice xenografted with a CMC cell line (CF33). Immunohistochemistry revealed that canine Nectin-4 was expressed in 45% of canine mammary tumors, and the tumor cells derived from one clinical specimen were efficiently infected with rMV-SLAMblind. These results suggest that rMV-SLAMblind infects CMC cells and displays antitumor activity in vitro, in xenografts, and ex vivo. Therefore, oncolytic virotherapy with rMV-SLAMblind can be a novel method for treating CMCs.

Eukaryotic elongation factor 1-beta interacts with the 5' untranslated region of the M gene of Nipah virus to promote mRNA translation.

Uchida, S., Sato, H., Yoneda, M. and Kai, C.

Nipah virus belongs to the genus Henipavirus in the family Paramyxoviridae, and its RNA genome is larger than those of other paramyxoviruses because it has long untranslated regions (UTRs) in each gene. However, the functions of these UTRs

are not fully understood. In this study, we investigated the functions of the 5' UTRs and found that the 5' UTR of the M gene upregulated the translation of a reporter gene. Using an RNA pull-down assay, we showed that eukaryotic elongation factor 1-beta (EEF1B2) interacts with nucleotides 81-100 of the M 5' UTR and specifically enhances its translation efficiency. Our results suggest that the M 5' UTR promotes the production of M protein and viral budding by recruiting EEF1B2.

Infectious progression of canine distemper virus from circulating cerebrospinal fluid into the central nervous system.

Takenaka, A., Sato, H., Ikeda, F., Yoneda, M. and Kai, C.

In this study, we generated recombinant chimeric canine distemper viruses (CDVs) by replacing the hemagglutinin (H) and/or phosphoprotein (P) gene in an avirulent strain expressing enhanced green fluorescent protein (EGFP) with those of a mouse-adapted neurovirulent strain. An in vitro experimental infection indicated that the chimeric CDVs possessing the H gene derived from the mouse-adapted CDV acquired infectivity for neural cells. These cells lack the CDV receptors that have been identified to date (SLAM and Nectin-4), indicating that the H protein defines infectivity in various cell lines. The recombinant viruses were administered intracerebrally to 1-week-old mice. Fatal neurological signs of disease were observed only with a recombinant CDV that possessed both the H and P genes of the mouse-adapted strain, similar to the parental mouse-adapted strain, suggesting that both genes are important to drive virulence of CDV in mice. Using this recombinant CDV, we traced the intracerebral propagation of CDV by detecting EGFP. Widespread infection was observed in the cerebral hemispheres and brainstems of the infected mice. In addition, EGFP fluorescence in the brain slices demonstrated a sequential infectious progression in the central nervous system: CDV primarily infected the neuroependymal cells lining the ventricular wall and the neurons of the hippocampus and cortex adjacent to the ventricle, and it then progressed to an extensive infection of the brain surface, followed by the parenchyma and cortex. In the hippocampal formation, CDV spread in a unidirectional retrograde pattern along neuronal processes in the hippocampal formation from the CA1 region to the CA3 region and the dentate gyrus. Our mouse model demonstrated that the main target cells of CDV are neurons in the acute phase and that the virus spreads via neuronal transmission pathways in the hippocampal formation.

Publications

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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, Shinichi Yokota, Motonori Ohno[1], Naoko Oda-Ueda[2], Takahito Chijiwa[1], Aichi Yoshida[3], Yoshihiro Hayashi[4], and Tomohisa Ogawa[5]: [1]Department of Applied Life Science, Faculty of Bioscience, Sojo University, [2]Department of Biochemistry, Faculty of Pharmaceutical Science, Sojo University, [3]School of Health Science, Faculty of Medicine, Kagoshima University, [4]National Museum of Nature and Science, Tokyo, [5]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 de-

creased. 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. Comparative mitogenomic analysis in *Protobothrops genus snakes*

Hiroki Shibata[6], Takahito Chijiwa, Shosaku Hattori, Koki Terada[7], and Motonori Ohno: [6]Medical Institute of Bioregulation, Kyushu University, [7]Okinawa Prefectural Institute of Health and Environment

We determined the complete mtDNA sequence of 43 specimens of Protobothrops collected from 12 Japanese subtropical islands. As a result, *P. flavoviridis* and *P. tokarensis* form a distinct species group in the phylogeny of 12 Protobothrops species. We observed a significant genetic divergence of *P. flavoviridis* between the Amami Clade and the Okinawa Clade. All specimen of *P. tokarensis* are included in the Amami Cluster, suggesting the paraphyly of *P. flavoviridis*. The divergence time of the two clusters, 6.5 MYA, vastly precedes the geological vicariance of the islands, 1.5 MYA. As expected from the limited mobility of terrestrial reptiles including snakes, we observed high genetic divergence in Habu mtDNA among Japanese subtropical island populations.

3. Reproduction of squirrel monkeys and owl monekys.

Shinichi Yokota, Shosaku Hattori, 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. Three newborns were given in reproductive groups of squirrel monkeys in 2016. Two of 4 newborns were nursed by laboratory staffs because of neglect of their mothers. On the other hand, the newborn of owl monkeys was not given in 2016.

4. Comparison of histocytological specificities of adrenal cortex in the New World Monkeys, Aotus lemurinus and Saimiri boliviensis

Toru Tachibana[8], Ken Takeshi Kusakabe[8], Sayuri Osaki[8], Takeshi Kuraishi, Shosaku Hattori, Midori Yoshizawa[9], Chieko Kai, and Yasuo Kiso[8]: [8]Joint Faculty of Veterinary Science, Yamaguchi University, [9]Graduate School of Agricultural Science, Utsunomiya University

The New World monkey Aotus spp. (night monkeys) are expected for use of valuable experimental animal with the close species of Saimiri spp. (squirrel monkeys). Saimiri is known to show spontaneous hypercortisolemia, although few reports in Aotus. We compared basic states of blood steroid hormones and histological structure of the adrenal glands in two monkeys. Serum cortisol and ACTH levels were statistically lower in Aotus than Saimiri. Conversely, Aotus adrenocortical area showed significant enlargement, especially at the zona fasciculata. Electron microscopic observation at Aotus fasciculata cells revealed notable accumulation of large lipid droplets and irregular shapes of the mitochondrial cristae. These results suggest potential differences in cellular activities for steroidogenesis between Aotus and Saimiri and experimental usefulness in adrenocortical physiology and pathological models.

5. Estimation of the therapeutic effect of transplantation of DFAT cells sheet on corneal epithelial defects in squirrel monkey (*Saimiri boliviensis*)

Shinichi Yokota, Shosaku Hattori, Akane Tanaka[10], Hiroshi Matsuda[10], and Chieko Kai: [10]Division of animal life science, Institute of agriculture, Tokyo University of Agriculture and Technology

Dedifferentiated fat (DFAT) cells are seemed to be a good candidate source of adult stem cells in regenerative medicine, because these cells exhibit multilineage potential as adipose-derived stem/stromal cells (ASCs). We isolated squirrel monkey DFAT cells from a small amount of adipose tissue by the ceiling culture method, and confirmed multilineage differentiation potential similar to mesenchymal stem cells. The DFAT cells derived from five different squirrel monkeys were expanded to sufficient numbers for transplantation as cell sheets, and were applied to the in vivo model of corneal epithelial wound healing in an autologous manner. In conclusion, it was demonstrated that the DFAT cell sheets promoted the healing process of corneal epithelium defect in squirrel monkey.

Publications

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Laboratory of Molecular Genetics
遺伝子解析施設

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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 293hde 12 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) as well as FLP/frt system (Nakano *et al*., Nucleic Acids Res. 29: e40, 2001; Kondo *et al*., Nucleic Acids Res. 31:e 76, 2003; Kondo *et al*., Microbiol. Immunol., 50: 831-843, 2006; Kondo et al., J. Molec. Biol., 2009). These methods continuously promote studies of various fields of molecular biology and medicine.

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

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

The research activities in 2016 are shown below. They include the studies of hepatitis B virus (HBV) and the application of AdVs in the field of genome editing.

1. Single-type adenovirus vector simultaneously expressing multiplex shRNAs for suppression of HBV replication

Mariko Suzuki, Aya Maekawa, Yumi Kanegae[1] and Izumu Saito.: [1]Core Research Facilities of Basic Science (Molecular Genetics), Research Center for Medical Science, Jikei University School of Medicine, Tokyo

The short-hairpin RNAs (shRNAs) have been expected as a future curative medicine for HBV as well as hepatitis C virus (HCV). Pei *et al.* reported that, interestingly, doubly-infected shRNA-expressing AdVs suppressed replication of HCV genome replication more efficiently than the singly-expressing AdV even in the same total dose (Pei et al., Sci Rep, 2013). However, when AdVs expressing different shRNAs are co-infected, simultaneous induction of all of the shRNAs to a single cell is difficult to achieve. Also, the total infectious dose of AdVs must be increased to maintain the same expression level of each shRNA per cell, and high doses of AdVs may induce immune responses. Therefore, the development of a "single-type" AdV that simultaneously expresses multiplex shRNAs is desirable.

We previously developed a method connecting multiple copies of small identical DNA fragments (Saito and Stark, PNAS, 1986). A modified method was used in this study: both the restriction enzymes producing non-palindromic termini and subsequent PCR were applied to construct the DNA fragments consisting of multiplex expression units. A modified method could also be used to generate the "single-type" AdV that simultaneously expresses multiplex shRNAs.

HCV genome is a plus-strand single-chain RNA. In contrast, there are two forms of full-length HBV genome: pregenomic RNA (pgRNA) is a plus-strand single-chain RNA, which is converted to covalently-closed circular DNA (ccc DNA) genome via reverse transcription. The ccc DNA genome is very stable and cause the resistance to current ant-HBV drugs. The precise mechanism of the ccc-DNA formation is still not understood. Therefore, we want to examine whether the single-type AdV expressing multiplex shRNAs can efficiently knock-down pgRNA and also reduce ccc DNA genome.

We constructed six AdVs, each of which singly expresses a different shRNA targeted at the different positions of HBV genome, and three shRNAs were identified that showed higher suppression efficiencies than others. Then, we constructed a single-type AdV simultaneously expressing these best-three shRNAs, and compared its efficiency with that of the mixture of the three AdVs expressing each shRNA. We observed that, as expected, the single-type AdV simultaneously expressing three shRNAs reduced the amount of pgRNA much more efficiently then the mixture of the three AdVs. However, although the amount of ccc genome were indeed decreased by the infection of this single-type AdV, the reduction level was lower than that of pgRNA. The result may imply that there could be a some mechanism which controls the copy number of ccc DNA genome.

2. Efficient elimination of HBV DNA using adenovirus vector expressing transiently expressed Cas9 and multiplex guide RNAs

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

HBV infection increases the risk of liver cirrhosis and hepatocellular carcinoma. Current therapeutic treatments for HBV infection cannot eliminate ccc DNA efficiently and thus complete clearance of HBV is difficult. The CRISPR/Cas9 system could resolve this problem, because it can disrupt HBV ccc DNA directly. We have reported a new method for efficient generation of the replicating HBV genome using AdV (HBV103AdV system). In this study, we specifically detected the replicating HBV genome using this system and showed the efficient reduction of ccc DNA using an improved CRISPR/Cas9 system.

We have established a method for construction of AdVs bearing three multiplex-guide RNAs (gRNAs) targeting HBV genome. This AdV efficiently disrupted the HBV genome generated by HBV103AdV system when co-infected with Cas9-expressing AdV (Cas9-AdV): the HBV genome was cleaved at the three different positions. Because simultaneous cleavages of two or more positions result in irreversible deletion, the HBV genome was efficiently disrupted by this AdV. However, although this result is promising, Cas9-AdV is stable and continues to injure cell chromosomal DNA due to the off-target effect. Therefore, transient expression of Cas9 is desirable rather than continuous expression as long as sufficient disruption of HBV genome is achieved.

We have developed "excisional expression" system, where the Cas9-expression is turned on only when this expression unit is excised and circularized by Cre. Because the circular DNA molecule is unstable compared with AdV genome, the Cas9 expression is transient in this system. Therefore, we applied this system to disrupt HBV genome in or-

der to achieve transient expression of Cas9. We constructed a pair of AdVs: one contains excisional expression unit of Cas9, and the other contains both the expression units of Cre and three-multiplex gRNAs targeting HBV genome. Coinfection of the two AdVs resulted in the disruption of HBV genome almost the same efficiency as coinfection of direct Cas9 expression as observed using AdV directly expressing Cas9. Although the specificity of Cre is rather low, FLP shows higher specificity. Therefore, the excisional expression system of Cas9 may offer safer choice when used together with FLP in the application to gene therapy.

Publications

Suzuki R., Saito K., Matsuda M., Sato M., Kanegae Y., Shi G, Watashi K., Aizaki H., Chiba J., Saito I., Wakita T. and Suzuki T. Single-domain intrabodies against hepatitis C virus core inhibit viral propagation and core-induced NFκB activation. J. Gen. Virol., 97: 887-892, 2016.

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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 integrative proteomic analyses from a physicochemical, structural and systems biology point of view. Currently, we mainly focus on functional protein-protein interaction networks related to a variety of diseases including cancer and infection. We are also engaged in collaborative researches regarding mass spectrometry and electron microscopy, which 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], Kouhei Tsumoto, Jun-ichiro 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. System-wide perturbation of the proteome and phosphoproteome dynamics in glioblastoma stem cells through mTOR signaling inihibition

Hiroko Kozuka-Hata, Tomoko Hiroki, Ryo Koyama-Nasu[1], Kouhei Tsumoto, Jun-ichiro Inoue, Tetsu Akiyama[1] and Masaaki Oyama.

As glioblastoma is the most common and aggressive brain tumor with poor prognosis, systematic elucidation of signaling networks causally linked to the tumorigenesis is very crucial for developing more effective treatments for this intractable cancer. In our previous study, we applied a high-resolution mass spectrometry-based proteomics technology in combination with SILAC quantitative methods to understand EGF-dependent phosphoproteome dynamics in patient-derived glioblastoma stem cells. We demonstrated that the phosphorylation levels of the representative mTOR signaling molecules such as RPS6 and PRAS40 were dramatically up-regulated upon EGF stimulation. As EGFR signaling has been reported to play a pivotal role in regulating the maintenance of cancer stem cells, we next carried out mTOR inhibitor-dependent signaling perturbations to unravel stemness-related pathways at the network level.

In the present study, we identified a total of 3,726 proteins including 49 up-regulated and 436 down-regulated factors by Torin 1 treatment. Interestingly, we found that one of the well-known cancer stem cell markers was significantly down-regulated through mTOR signaling inhibition. Our in-depth phosphoproteome analysis also led to identification of 6,250 unique phosphopeptides derived from 2,221 proteins and unveiled a variety of dynamic changes regarding phosphorylation levels of cancer and neural stem cell markers in a comprehensive manner. The integrative view of the mTOR inhibitor-dependent proteome and phosphoproteome dynamics in glioblastoma stem cells presents us with further prospects towards understanding previously unrecognized regulations at the system level.

3. Integrative Network Analysis Combined with Quantitative Phosphoproteomics Reveals Transforming Growth Factor-beta Receptor type-2 (TGFBR2) as a Novel Regulator of Glioblastoma Stem Cell Properties

Yuta Narushima, Hiroko Kozuka-Hata, Ryo Koyama-Nasu[1], Kouhei Tsumoto, Jun-ichiro Inoue, Tetsu Akiyama[1] and Masaaki Oyama.

Glioblastoma is one of the most malignant brain tumors with poor prognosis and their development and progression are known to be driven by glioblastoma stem cells. Although glioblastoma stem cells lose their cancer stem cell properties during cultivation in serum-containing medium, little is known about the molecular mechanisms regulating signaling alteration in relation to reduction of stem cell-like characteristics. To elucidate the global phosphorylation-related signaling events, we performed a SILAC-based quantitative phosphoproteome analysis of serum-induced dynamics in glioblastoma stem cells established from the tumor tissues of the patient. Among a total of 2876 phosphorylation sites on 1584 proteins identified in our analysis, 732 phosphorylation sites on 419 proteins were regulated through the alteration of stem cell-like characteristics. The integrative computational analyses based on the quantified phosphoproteome data revealed the relevant changes of phosphorylation levels regarding the proteins associated with cytoskeleton reorganization such as Rho family GTPase and Intermediate filament signaling, in addition to transforming growth factor-β receptor type-2 (TGFBR2) as a prominent upstream regulator involved in the serum-induced phosphoproteome regulation. The functional association of transforming growth factor-β receptor type-2 with stem cell-like properties was experimentally validated through signaling perturbation using the corresponding inhibitors, which indicated that transforming growth factor-β receptor type-2 could play an important role as a novel cell fate determinant in glioblastoma stem cell regulation.

4. Quantitative phosphoproteomics-based molecular network description for high-resolution kinase-substrate interactome analysis

Yuta Narushima, Hiroko Kozuka-Hata, Kouhei Tsumoto, Jun-ichiro Inoue and Masaaki Oyama.

Phosphorylation-dependent cellular signaling is known to play a diverse role in regulating multiple cellular processes such as proliferation, differentiation and apoptosis. Recent technological advances in mass spectrometry-based phosphoproteomics have enabled us to measure network-wide signaling dynamics in a comprehensive and quantitative manner. As conventional protein-protein interaction (PPI) information-based network analysis is insufficient to systematically analyze phosphorylation site-dependent complex interaction dynamics, here we develop and evaluate a platform to provide a high-resolution molecular network description for kinase-substrate interactome analysis. In this study, we developed a Cytoscape-based bioinformatical platform named "Post Translational Modification mapper (PTMapper)" to integrate PPI data with publicly available kinase-substrate relations at the resolution of phosphorylated amino acid residues. The previous phosphoproteome data on EGF-induced cellular signaling in glioblastoma stem cells was applied to evaluate our platform, leading to discovery of phosphorylation-dependent crucial signaling modulation in the p70S6K1-related pathway. Our study revealed that high-resolution cellular network description of phosphorylation-site de-

pendent kinase-substrate signaling regulation should accelerate phosphoproteomics-based exploration of novel drug targets in the context of each disease-related signaling.

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

Hiroko Kozuka-Hata, Masato Suzuki[2], Kotaro Kiga[2], Shinya Tasaki, Jun-ichiro Inoue, Tadashi Yamamoto[3], Chihiro Sasakawa[2] and Masaaki Oyama: [2]Division of Bacterial Infection, Department of Microbiology and Immunology, IMSUT, [3]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.

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

Masaaki Oyama, Hiroko Kozuka-Hata, Sumio Sugano[4], Tadashi Yamamoto[3] and Jun-ichiro Inoue: [4]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. Disruption of cell adhesion by an antibody targeting the cell-adhesive intermediate (X-dimer) of human P-cadherin

Kudo S, Caaveiro JM, Nagatoishi S, Miyafusa T, Matsuura T, Sudou Y and Tsumoto K.

Human P-cadherin is a cell adhesion protein of the family of classical cadherins, the overexpression of which is correlated with poor prognosis in various types of cancer. Antibodies inhibiting cell-cell adhesion mediated by P-cadherin show clear therapeutic effect, although the mechanistic basis explaining their effectiveness is still unclear. Based on structural, physicochemical, and functional analyses, we have elucidated the molecular mechanism of disruption of cell adhesion by antibodies targeting human P-cadherin. Herein we have studied three different antibodies, TSP5, TSP7, and TSP11, each recognizing a different epitope on the surface of the cell-adhesive domain (EC1). Although all these three antibodies recognized human P-cadherin with high affinity, only TSP7 disrupted cell adhesion. Notably, we demonstrated that TSP7 abolishes cell adhesion by disabling the so-called X-dimer (a kinetic adhesive intermediate), in addition to disrupting the strand-swap dimer (the final thermodynamic state). The inhibition of the X-dimer was crucial for the overall inhibitory effect, raising the therapeutic value of a kinetic intermediary not only for preventing, but also for reversing, cell adhesion mediated by a member of the classical cadherin family. These findings should help to design more innovative and effective therapeutic solutions targeting human P-cadherin.

2. Differential Effect of Membrane Composition on the Pore-Forming Ability of Four Different Sea Anemone Actinoporins

García-Linares S, Rivera-de-Torre E, Morante K, Tsumoto K, Caaveiro JM, Gavilanes JG, Slotte JP and Martínez-Del-Pozo Á.

Sea anemone actinoporins constitute a protein family of multigene pore-forming toxins (PFT). Equinatoxin II (EqtII), fragaceatoxin C (FraC), and sticholysins I and II (StnI and StnII, respectively), produced by three different sea anemone species, are the only actinoporins whose molecular structures have been studied in depth. These four proteins show high sequence identities and practically coincident three-dimensional structures. However, their pore-forming activity can be quite different depending on the model lipid system employed, a feature that has not been systematically studied before. Therefore, the aim of this work was to evaluate and compare the influence of several distinct membrane conditions on their particular pore-forming behavior. Using a complex model membrane system, such as sheep erythrocytes, StnII showed hemolytic activity much higher than those of the other three actinoporins studied. In lipid model systems, pore-forming ability when assayed against 4 : 1 1,2 - dioleoyl - sn - glycero - 3 - phosphocholine (DOPC)/sphingomyelin (SM) vesicles, with the membrane binding being the rate-limiting step, decreased in the following order: StnI > StnII > EqtII > FraC. When using 1:1:1 DOPC/SM/cholesterol LUVs, the presence of Chol not only enhanced membrane binding affinities by ~2 orders of magnitude but also revealed how StnII was much faster than the other three actinoporins in producing calcein release. This ability agrees with the proposal that explains this behavior in terms of their high sequence variability along their first 30 N-terminal residues. The influence of interfacial hydrogen bonding in SM- or dihydro-SM-containing bilayers was also shown to be a generalized feature of the four actinoporins studied. It is finally hypothesized that this observed variable ability could be explained as a consequence of their distinct specificities and/or membrane binding affinities. Eventually, this behavior can be modulated by the nature of their natural target membranes or the interaction with not yet characterized isotoxin forms from the same sea anemone species.

3. Structural basis for broad neutralization of HIV-1 through the molecular recognition of 10 E8 helical epitope at the membrane interface

Rujas E, Caaveiro JM, Partida-Hanon A, Gulzar N, Morante K, Apellániz B, García-Porras M, Bruix M, Tsumoto K, Scott JK, Jiménez MÁ and Nieva JL.

The mechanism by which the HIV-1 MPER epitope is recognized by the potent neutralizing antibody 10E8 at membrane interfaces remains poorly understood. To solve this problem, we have optimized a 10E8 peptide epitope and analyzed the structure and binding activities of the antibody in membrane and membrane-like environments. The X-ray crystal structure of the Fab-peptide complex in detergents revealed for the first time that the epitope of 10E8 comprises a continuous helix spanning the gp41 MPER/transmembrane domain junction (MPER-N-TMD; Env residues 671-687). The MPER-N-TMD helix projects beyond the tip of the heavy-chain complementarity determining region 3 loop, indicating that the antibody sits parallel to the plane of the membrane in binding the native epitope. Biophysical, biochemical and mutational analyses demonstrated that strengthening the affinity of 10E8 for the TMD helix in a membrane environment, correlated with its neutralizing potency. Our research clarifies the molecular mechanisms

underlying broad neutralization of HIV-1 by 10E8, and the structure of its natural epitope. The conclusions of our research will guide future vaccine-design strategies targeting MPER.

4. Improved brain expression of anti-amyloid β scFv by complexation of mRNA including a secretion sequence with PEG-based block catiomer

Perche F, Uchida S, Akiba H, Lin CY, Ikegami M, Dirisala A, Nakashima T, Itaka K, Tsumoto K and Kataoka K.

Amyloid β aggregates have been proposed as central effectors in Alzheimer's disease, as evidenced by several passive immunotherapies in clinical trials. We used mRNA encoding anti-amyloid β (Aβ) scFv for passive immunotherapy. These scFvs could recognize amyloid oligomers with significant affinity, delay the aggregation of amyloid β in vitro, and dissociate Aβ aggregates in vitro. Intra-cerebroventricular injection of pDNA and mRNA encoding the scFv resulted in detectable scFv levels in the brain, with higher in vivo expression from mRNA. Notably, injection of mRNA decreased Aβ burden in an acute amyloidosis model. scFv production in situ from mRNA may represent a novel immunotherapy approach, and a safer alternative compared to the administration of anti-Aβ antibodies or adenovirus encoding antibodies.

5. Adhesive Dimerization of Human P-Cadherin Catalyzed by a Chaperone-like Mechanism

Kudo S, Caaveiro JM and Tsumoto K.

Orderly assembly of classical cadherins governs cell adhesion and tissue maintenance. A key event is the strand-swap dimerization of the extracellular ectodomains of two cadherin molecules from apposing cells. Here we have determined crystal structures of P-cadherin in six different conformational states to elaborate a motion picture of its adhesive dimerization at the atomic level. The snapshots revealed that cell-adhesive dimerization is facilitated by several intermediate states collectively termed X-dimer in analogy to other classical cadherins. Based on previous studies and on the combined structural, kinetic, thermodynamic, biochemical, and cellular data reported herein, we propose that the adhesive dimerization of human P-cadherin is achieved by a stepwise mechanism analogous to that of assembly chaperones. This mechanism, applicable to type I classical cadherins, confers high specificity and fast association rates. We expect these findings to guide innovative therapeutic approaches targeting P-cadherin in cancer.

6. In-Cell Enzymology To Probe His-Heme Ligation in Heme Oxygenase Catalysis

Sigala PA, Morante K, Tsumoto K, Caaveiro JM and Goldberg DE.

Heme oxygenase (HO) is a ubiquitous enzyme with key roles in inflammation, cell signaling, heme disposal, and iron acquisition. HO catalyzes the oxidative conversion of heme to biliverdin (BV) using a conserved histidine to coordinate the iron atom of bound heme. This His-heme interaction has been regarded as being essential for enzyme activity, because His-to-Ala mutants fail to convert heme to biliverdin in vitro. We probed a panel of proximal His mutants of cyanobacterial, human, and plant HO enzymes using a live-cell activity assay based on heterologous co-expression in Escherichia coli of each HO mutant and a fluorescent biliverdin biosensor. In contrast to in vitro studies with purified proteins, we observed that multiple HO mutants retained significant activity within the intracellular environment of bacteria. X-ray crystallographic structures of human HO1 H25R with bound heme and additional functional studies suggest that HO mutant activity inside these cells does not involve heme ligation by a proximal amino acid. Our study reveals unexpected plasticity in the active site binding interactions with heme that can support HO activity within cells, suggests important contributions by the surrounding active site environment to HO catalysis, and can guide efforts to understand the evolution and divergence of HO function.

7. Identification of a Membrane-bound Prepore Species Clarifies the Lytic Mechanism of Actinoporins

Morante K, Bellomio A, Gil-Cartón D, Redondo-Morata L, Sot J, Scheuring S, Valle M, González-Mañas JM, Tsumoto K and Caaveiro JM.

Pore-forming toxins (PFTs) are cytolytic proteins belonging to the molecular warfare apparatus of living organisms. The assembly of the functional transmembrane pore requires several intermediate steps ranging from a water-soluble monomeric species to the multimeric ensemble inserted in the cell membrane. The non-lytic oligomeric intermediate known as prepore plays an essential role in the mechanism of insertion of the class of β-PFTs. However, in the class of α-PFTs, like the actinoporins produced by sea anemones, evidence of membrane-bound prepores is still lacking. We have employed single-particle cryo-electron microscopy (cryo-EM) and atomic force microscopy to identify, for the first time, a prepore species of the actinoporin fragaceatoxin C bound to lipid vesicles.

The size of the prepore coincides with that of the functional pore, except for the transmembrane region, which is absent in the prepore. Biochemical assays indicated that, in the prepore species, the N terminus is not inserted in the bilayer but is exposed to the aqueous solution. Our study reveals the structure of the prepore in actinoporins and highlights the role of structural intermediates for the formation of cytolytic pores by an α-PFT.

8. Structural basis for amino acid export by DMT superfamily transporter YddG

Tsuchiya H, Doki S, Takemoto M, Ikuta T, Higuchi T, Fukui K, Usuda Y, Tabuchi E, Nagatoishi S, Tsumoto K, Nishizawa T, Ito K, Dohmae N, Ishitani R and Nureki O.

The drug/metabolite transporter (DMT) superfamily is a large group of membrane transporters ubiquitously found in eukaryotes, bacteria and archaea, and includes exporters for a remarkably wide range of substrates, such as toxic compounds and metabolites. YddG is a bacterial DMT protein that expels aromatic amino acids and exogenous toxic compounds, thereby contributing to cellular homeostasis. Here we present structural and functional analyses of YddG. Using liposome-based analyses, we show that Escherichia coli and Starkeya novella YddG export various amino acids. The crystal structure of S. novella YddG at 2.4Å resolution reveals a new membrane transporter topology, with ten transmembrane segments in an outward-facing state. The overall structure is basket-shaped, with a large substrate-binding cavity at the centre of the molecule, and is composed of inverted structural repeats related by two-fold pseudo-symmetry. On the basis of this intramolecular symmetry, we propose a structural model for the inward-facing state and a mechanism of the conformational change for substrate transport, which we confirmed by biochemical analyses. These findings provide a structural basis for the mechanism of transport of DMT superfamily proteins.

9. Assessment of the Protein-Protein Interactions in a Highly Concentrated Antibody Solution by Using Raman Spectroscopy

Ota C, Noguchi S, Nagatoishi S and Tsumoto K.

PURPOSE: To investigate the protein-protein interactions of a highly concentrated antibody solution that could cause oligomerization or aggregation and to develop a better understanding of the optimization of drug formulations.

METHODS: In this study, we used Raman spectroscopy to investigate the structure and interactions of a highly concentrated antibody solution over a wide range of concentrations (10-200 mg/mL) with the aid of a multivariate analysis.

RESULTS: Our analysis of the amide I band, I 856 /I 830 of Tyr, and the relative intensity at 1004 cm(-1) of the Phe and OH stretching region at around 3000cm(-1) showed that across this wide range of concentrations, the secondary structure of the IgG molecules did not change; however, short-range attractive interactions around the Tyr and Phe residues occurred as the distance between the IgG molecules decreased with increasing concentration. Analysis of the OH stretching region at around 3000cm(-1) showed that these short-range attractive interactions correlated with the amount of hydrated water around the IgG molecules.

CONCLUSIONS: Our data show that Raman spectroscopy can provide valuable information of the protein-protein interactions based on conformational approaches to support conventional colloidal approaches, especially for analyses of highly concentrated solutions.

10. Mitochonic Acid 5 Binds Mitochondria and Ameliorates Renal Tubular and Cardiac Myocyte Damage

Suzuki T, Yamaguchi H, Kikusato M, Hashizume O, Nagatoishi S, Matsuo A, Sato T, Kudo T, Matsuhashi T, Murayama K, Ohba Y, Watanabe S, Kanno S, Minaki D, Saigusa D, Shinbo H, Mori N, Yuri A, Yokoro M, Mishima E, Shima H, Akiyama Y, Takeuchi Y, Kikuchi K, Toyohara T, Suzuki C, Ichimura T, Anzai J, Kohzuki M, Mano N, Kure S, Yanagisawa T, Tomioka Y, Toyomizu M, Tsumoto K, Nakada K, Bonventre JV, Ito S, Osaka H, Hayashi K and Abe T.

Mitochondrial dysfunction causes increased oxidative stress and depletion of ATP, which are involved in the etiology of a variety of renal diseases, such as CKD, AKI, and steroid-resistant nephrotic syndrome. Antioxidant therapies are being investigated, but clinical outcomes have yet to be determined. Recently, we reported that a newly synthesized indole derivative, mitochonic acid 5 (MA-5), increases cellular ATP level and survival of fibroblasts from patients with mitochondrial disease. MA-5 modulates mitochondrial ATP synthesis independently of oxidative phosphorylation and the electron transport chain. Here, we further investigated the mechanism of action for MA-5. Administration of MA-5 to an ischemia-reperfusion injury model and a cisplatin-induced nephropathy model improved renal function. In in vitro bioenergetic studies, MA-5 facilitated ATP production and reduced the level of mitochondrial reactive oxygen species (ROS) without affecting activity of mitochondrial complexes I-IV. Additional assays revealed that MA-5 targets the mitochondrial protein

mitofilin at the crista junction of the inner membrane. In Hep3B cells, overexpression of mitofilin increased the basal ATP level, and treatment with MA-5 amplified this effect. In a unique mitochondrial disease model (Mitomice with mitochondrial DNA deletion that mimics typical human mitochondrial disease phenotype), MA-5 improved the reduced cardiac and renal mitochondrial respiration and seemed to prolong survival, although statistical analysis of survival times could not be conducted. These results suggest that MA-5 functions in a manner differing from that of antioxidant therapy and could be a novel therapeutic drug for the treatment of cardiac and renal diseases associated with mitochondrial dysfunction.

<Group III>

1. Development of new methods for analyzing 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 systematically to make up complex neural circuits. Characteristics of the neural cell 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 retinal neurons, are not enough. Until recently to collect ultrathin serial sections and observe in transmission electron microscope (TEM) were the only way to reveal the connectivity of actual neural cells morphologically. But the technical difficulties discouraged us from such a troublesome studies. Recent progress in scanning electron microscope (SEM) equipment allowed us to develop a new method to observe ultrathin TEM sections in SEM. Samples were specifically treated to enhance electron contrast and serial thin TEM sections were collected on the smooth conductive matrix. By using SEM to observe thin TEM sections, it became possible to analyze much wider areas than by using TEM. We have analyzed about 500 serial thin sections of zebrafish retinal outer plexiform layer by this method and succeeded in tracing thin processes of bipolar cells into the photoreceptor terminal. In the course of this study, it became obvious that thinner and larger numbers of section are needed to reveal the accurate connectivity especially for thinner processes. This year, we developed a new equipment to collect huge number of serial sections stably and efficiently. By using this equipment, it became possible to cut more than 1000 serial sections of less than 30nm thickness much easier.

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, 20 projects in 14 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[1]'s laboratory: [1]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.

a-2. Essential role of docosahexaenoic acid in visual function by formation of photoreceptor cells.

We have been performing several studies also with research groups in Dr. Watanabe[2]'s laboratory: [2]Project Division of Molecular and Developmental Biology. This year, we evaluated the need of do-

cosahexaenoic acid (DHA) in retinal development. With the analysis of the retina by electron microscopy, we estimated that DHA-containing phospholipids might be more essential for disc morphogenesis and/or maintenance than for new disc formation at the basal OS. In another work with Dr. Watanabe's laboratory, we analyzed the function of SYMD 5 (SET and MYND domain-containing protein 5) in zebrafish retinal development. SYMD1-4 are known to have critical function in carcinogenesis and/or in the development of heart and skeletal muscle, but our analysis of SYMD5 indicated that SYMD5 play critical role in hematopoiesis rather than muscle development (ref. Fujii[2] *et al*).

Some other collaborative research works using thin section electron microscopy and/or immuno-electron microscopy were performed with Dr. Yasuda[3], [3]Department of Integrated Biosciences, Graduate School of Frontier Sciences, Tokyo University, concerning the effects of radiation on central nervous system, Dr. Inoue[4]'s group, in [4]Division of Cellular and Molecular Biology, Dr. Taketani[5] in [5]Department of Ophthalmology, The University of Tokyo, regarding the morphology of the mouse cornea, and so on.

b. Negative staining techniques

Negative staining techniques are simple and quick method to observe the morphology of the macro-molecules. This year, negative staining techniques combined with scanning electron microscopy were used to analyze the function of a protein during in vitro formation of collagen fibers in collaboration with Dr. Tsumoto[6] *et al* in [6]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. Kobayashi[7]'s group, in [7]Department of Systems Innovation, School of engineering, about the generation of electricity using bacteria. Scanning electron microscopy was also used to analyze the morphological changes of collagen fibers as a collaborative work with Tsumoto[6] *et al*.

Publications

<Group I>

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<Group II>

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