Affiliated Facilities

Laboratory Animal Research Center
実験動物研究施設

Professor	Chieko Kai, D.V.M., Ph.D.	教　授	農学博士	甲　斐　知恵子
Assistant Professor	Misako Yoneda, D.V.M., Ph.D.	助　教	農学博士	米　田　美佐子
Assistant Professor	Hiroki Sato, Ph.D.	助　教	理学博士	佐　藤　宏　樹
Assistant Professor	Kentaro Fujita D.V.M., Ph.D.	助　教	農学博士	藤　田　賢太郎

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 induces cell-type specific changes in gene expression

Hiroki Sato, Reiko Honma[1], Misako Yoneda, Ryuichi Miura, Kyoko Tsukiyama-Kohara[2], Fusako Ikeda, Takahiro Seki, Shinya Watanabe[1] and Chieko Kai: [1]Clinical Informatics, Tokyo Medical and Dental University. [2]Faculty of Medical and Pharmaceutical Sciences, Kumamoto University.

Measles virus (MV) causes various responses including the induction of immune responses, transient immunosuppression and establishment of long-lasting immunity. To obtain a comprehensive view of the effects of MV infection on target cells, DNA microarray analyses of two different cell-types were performed. An epithelial (293SLAM; a 293 cell line stably expressing SLAM) and lymphoid (COBL-a) cell line were inoculated with purified wild-type MV. Microarray analyses revealed significant differences in the regulation of cellular gene expression between these two different cells. In 293SLAM cells, upregulation of genes involved in the antiviral response was rapidly induced; in the later stages of infection, this was followed by regulation of many genes across a broad range of functional categories. On the other hand, in COBL-a cells, only a limited set of gene expression profiles was modulated after MV infection. Since it was reported that V protein of MV inhibited the IFN signaling pathway, we performed a microarray analysis using V knockout MV to evaluate V protein's effect on cellular gene expression. The V knockout MV displayed a similar profile to that of parental MV. In particular, in COBL-a cells infected with the virus, no alteration of cellular gene expression, including IFN signaling, was observed. Furthermore, IFN signaling analyzed in vitro was completely suppressed by MV infection in the COBL-a cells. These results reveal that MV induces different cellular responses in a cell-type specific manner. Microarray analyses will provide us useful information about potential mechanisms of MV pathogenesis.

Phosphorylation of measles virus nucleoprotein upregulates the transcriptional activity of minigenomic RNA

Kyoji Hagiwara, Hiroki Sato, Yoshihisa Inoue, Akira Watanabe, Misako Yoneda, Fusako Ikeda, Kentaro Fujita, Hiroyuki Fukuda[1], Chizuko Takamura[1], Hiroko Kozuka-Hata[1,2], Masaaki Oyama[1,2], Sumio Sugano[2], Shinobu Ohmi[1], Chieko Kai: [1]Medical Proteomics Laboratory, The Institute of Medical Science, The University of Tokyo, [2]Department of Medical Genome Sciences, The Institute of Medical Science, The University of Tokyo.

The nucleoprotein (N) of MV is known to be the most abundant protein in infected cells; it constructs the N-RNA complex (nucleocapsid) and supports transcription and replication of viral genomic RNA. To determine the role of phosphorylation of the N protein, we expressed the N protein of the HL strain of MV in mammalian cells and purified the nucleocapsid. After separation of the C-terminal region from the core region, phosphorylated amino acids were assayed using MALDI-TOF/TOF and ESI-Q-TOF MS analyses. Two amino acids, S479 and S 510, were shown to be phosphorylated by both methods of analysis. Metabolic labeling of the N protein with ^{32}P demonstrated that these two sites are the major phosphorylated sites within the MV-N protein. In transcriptional analysis using negative-strand minigenomic RNA containing the ORF of the luciferase gene, mutants of each phosphorylation site showed approximately 80% reduction in luciferase activity compared with the wild-type N, suggesting that the phosphorylation of N protein is important in the activation of the transcription of viral mRNA and/or replication of the genome in vivo.

Pathological and phylogenetic features of prevalent canine distemper viruses in wild masked palm civets in Japan

Ikuyo Takayama, Masahito Kubo[1], Akiko Takenaka, Kentaro Fujita, Takaaki Sugiyama, Tetsuro Arai, Misako Yoneda, Hiroki Sato, Tokuma Yanai[1] and Chieko Kai: [1]Laboratories of Veterinary Pathology, Department of Veterinary Medicine, Faculty of Applied Biological Sciences, Gifu University.

Ten wild masked palm civets infected with canine distemper virus (CDV), captured in Japan from 2005 to 2007, were histopathologically and phylogenetically analyzed. Phylogenetic analysis based on the amino acid sequences of the H protein of two CDV isolates from masked palm civets revealed that the two isolates were classified into the clade of recent isolates in Japan. Histopathologically marked lesions of virus encephalitis were present in the brain, whereas gastrointestinal lesions were absent or at a mild degree. The distribution of the lesions resembles that of recent CDV cases in dogs. Therefore, recent CDV infections in masked palm civets could be caused by recently prevalent CDV in dogs. The possibility of the masked palm civet as a spreader of CDV among wildlife is also discussed.

Inhibition of host protein synthesis in B95a cells infected with the HL strain of measles virus

Yoshihisa Inoue, Kyoko Tsukiyama-Kohara[1], Misako Yoneda, Hiroki Sato and Chieko Kai: [1] Faculty of Medical and Pharmaceutical Sciences, Kumamoto University

The shut-off of host protein synthesis in virus-infected cells is one of the important mechanisms for viral replication. In this report, we showed that MV-HL as well as other field isolates, which were isolated from human blood lymphocytes using B95a cells, induce the shut-off in B95a cells. Since the Edmonston srrain of MV failed to induce the shut-off in B95a cells, the ability to induce the shut-off was considered to be dependent on virus strains. Although, the modification of eukaryotic translation initiation factors (eIF) including eIF4G, eIF4E, and 4E-BP1 was reported for shut-off by various viruses, the involvement of these eIFs was not observed in MV-HL-infected B95a cells. Instead, the accumulation of phosphorylated eIF2α was found to coincide to the decrease of host protein synthesis, suggesting the involvement of phosphorylation of eIF2α in inhibition of translation as one of the mechanisms of the shut-off.

Publications

Hagiwara, K., Sato, H., Inoue, Y., Watanabe, A., Yoneda, M., Ikeda, F., Fujita, K., Fukuda, H., Takamura, C., Kozuka-Hata, H., Oyama, M., Sugano, S., Ohmi, S. and Kai, C. Phosphorylation of measles virus nucleoprotein upregulates the transcriptional activity of minigenomic RNA. *Proteomics*, 8, 1871-1879, 2008.

Sato, H., Honma, R., Yoneda, M., Miura, R.,

Tsukiyama-Kohara, K., Ikeda, F., Seki, T., Watanabe, S., Kai, C. Measles virus induced cell-type specific changes in gene expression. *Virology*, 321-330, 2008.

Terao-Muto, Y., Yoneda, M., Seki, T., Watanabe, A., Tsukiyama-Kohara, K., Fujita, K. and Kai, C. Heparin-like glycosaminoglycans prevent the infection of measles virus in SLAM-negative cell lines. *Antiviral Res*., 80: 370-376, 2008.

Takayama, I., Kubo, M., Takenaka, A., Fujita, K., Sugiyama, T., Arai, T., Yoneda, M., Sato, H., Yanai, T. and Kai, C. Pathological and phylogenetic features of prevalent canine distemper viruses in wild Masked palm civets in Japan. *Comp. Immunol. Microb*., in press, 2008

Yasui, F., Kai, C., Kitabatake, M., Inoue, S., Yoneda, M., Yokochi, S., Kase, R., Sekiguchi, S., Morita, K., Hishima, T., Suzuki, H., Karamatsu, K., Yasutomi, Y., Shida, H., Kidokoro, M., Mizuno, K., Matsushima, K. and Kohara, M. Prior immunization with SARS-CoV nucleocapsid protein causes severe pneumonia in mice infected with SARS-CoV. *J. Immunol*., 18(9): 6337-48, 2008

Inoue, Y., Tshukiyama-Kohara, K., Yoneda, M., Sato, H. and Kai, C. Inhibition of host protein synthesis in B95a cells infected with HL strain of measles virus. *Comp. Immunol. Microb*., 32: 29-41, 2009.

Affiliated Facilities

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

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

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

1. Research on the Habu control

Shosaku Hattori, Takeshi Kuraishi, Motonori Ohno[1], Naoko Ueda[1], Takahito Chijiwa[1], Aichi Yoshida[2], Yoshihiro Hayashi[3], Michihisa Toriba[4], and Tomohisa Ogawa[5],: [1]Department of Applied Life Science, Faculty of Bioscience, Sojo University, [2]School of Health Science, Faculty of Medicine, Kagoshima University, [3]Department of Veterinary Anatomy, Faculty of Agriculture, University of Tokyo, [4]The Japan Snake Institute, [5]Faculty of Agriculture, Tohoku university

Snake bites by the venomous snake Habu, *Protobothrops flavoviridis*, have been reported annually about 80 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. Morphological comparisons between three species of the Ryukyu spiny rats

Hideki Endo[6], Shosaku Hattori, Yoshihiro Hayashi[3], and Kimiyuki Tsuchiya[7]: [6]University Museum, University of Tokyo, [7]Department of Animal Science, Faculty of Agriculture, Tokyo University of Agriculture

Skulls of the three species of Ryukyu spiny rats (*Tokudaia osimensis, T. tokunoshimennsis*, and *T. muenninki*) were osteometrically compared. In all measurements the size tendencies in which *T. tokunoshimennsis* was the largest, *T. muenninki* middle, and *T. osimensis* the smallest were confirmed. However, the lengths of the molar row were exceptionally the largest in *T. muenninki*, middle in *T. tokunoshimennsis* and the smallest in *T. osimensis.* The raw measurement data obviously indicated significant differences in size among the three species. We demonstrated that the neurocranium was larger in *T. osimensis* than in *T. muenninki* from the results of the proportional analyses. The principle component analyses using the raw measurement data also pointed out that the skull from the three species were obviously distinguished in the charts, although the plots were not completely separated by the principal component analysis using the proportional indices. The CT images showed that both maxilla and palatine bone were thinner near the greater palatine foramen area and pointed out that the characteristic palatine structure fixed the position of the greater palatine foramen in each species of *Tokudaia.*

3. 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 newborns are given annually by 25 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.

4. The diet of dogs in the Amami-Oshima Island forest, with special attention to predation on endangered animals

Yuya Watari[8], Yumiko Nagai[9], Fumio Yamada[10], Taku Sakoda[9], Takeshi Kuraishi, Shintaro Abe[9] and Yoshimi Satomura[11]: [8] Graduate school of Agricultural and life science, The University of Tokyo, [9]Amami wildlife conservation center, [10]Forestry and forest product research institute, [11]Amami mammalogical society

We analyzed 135 dog fecal pellets sampled in a forest on Amami-Oshima Island, Japan. Rare mammals, including the Amami rabbit (*Pentalagus furnessi*; 45.2%), Amami spinous rat (*Tokudaia osimensis*; 23.7%), and long-haired rat (*Diprothrix legata*; 20.0%), occurred in fecal pellets at high frequencies, indicating that these species are highly vulnerable to dog predation. Dog reproduction in the forest area has not been confirmed. However, many pets or hunting dogs appear to be abandoned or unsupervised, indicating that the morals of pet owners may largely influence the dog population and, hence, its impact on native species. We propose the following management strategy. 1) Long term: preventing dogs from entering the forest. Enforcing current laws regarding supervision of dogs and educating pet owners is required. These are fundamental actions that will reduce the abundance of dogs in the forest; thus, this should be given high priority. 2) Short term: rapid action against detected dogs. A system to report dog sightings

is necessary, together with a system to quickly catch reported dogs.

Publications

Endo, H., Hattori, S., Hayashi, Y., and Tsuchiya, K. Morphological comparisons between three species of the Ryukyu spiny rats. Mammal Study, 33: 1-10, 2008.

服部正策，倉石武．病理実験部会報告．平成19年度奄美ハブ毒免疫機序研究報告書．（鹿児島県）．pp. 43 - 50，2008.

服部正策．野外調査部会報告．平成19年度ハブ動態制御研究報告書．（鹿児島県）．pp. 9-43，2008.

服部正策，倉石武．ハブ高密度地域の環境調査．平成19年度ハブ動態制御研究報告書．（鹿児島県）．pp. 49-52，2008.

Affiliated Facilities

Laboratory of Molecular Genetics
遺伝子解析施設

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

This laboratory has two main activities, development of efficient expression vectors for gene therapy, especially for anti-cancer, 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. 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 aiming at gene therapy. We are maintaining more than 20 collaborations within and outside of this institute. In these collaborations, we offer and supply our efficient method to construct adenovirus vector (AdV) expressing various genes efficiently. And recently we developed the new cosmid cassette for AdV construction, which can choose not only very efficient COS-TPC method (Miyake *et al*., PNAS 93: 1320-1324, 1996) but also an easier method using a full-length viral genome with intact viral termini (Fukuda *et al*., Microbiol. Immunol. 50: 643-654, 2006). This new 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: e 40, 2001; Kondo *et al*., Nucleic Acids Res. 31: e 76, 2003; Kondo et al., Microbiol. Immunol., 50: 831-843, 2006). The methods will promote studies of various fields of molecular biology and medicine and may open a new field of "intracellular gene manipulation". We recently identified adenovirus pIX gene as a main cause of inflammation observed in AdV infection (Nakai *et al*., Hum Gene Ther. 18: 925-936, 2007). The research activities in 2008 were shown below.

1. Development of enhanced and cell-specific "excisional-expression" system using adenovirus vector

Miho Terashima, Zheng Pei, Saki Kondo, Izumu Saito and Yumi Kanegae

Adenovirus vectors (AdV) are valuable because of high transduction efficiency to broad range of cell types but, conversely, do not show cell specificity. Therefore, for cell-specific expression this vector must be used together with an-

other technique, such as use of cell-specific promoter. However, expression level of a cell-specific promoter is much lower, about several hundred times less, than a versatile potent promoter such as CAG and EF1α promoters and, if this problem could be solved, cell-specific promoters must become much more useful in many *in vivo* studies. Here, we provide a new strategy using a novel "excisional-expression" system on a single AdV genome. This method uses a "switch" unit under control of cancer-specific, α-fetoprotein (AFP) promoter as an example of a cell-specific promoter, and a "target" unit, which consists of a line as loxP, purpose gene, polyA signal, EF1α promoter and second loxP in that order. In normal cells, Cre can not be expressed because of strict specificity of AFP promoter, however in the hepatocellular carcinoma cells, Cre expression is induced and a target unit is excised as a circle, where the purpose gene can be connected with EF1α promoter and at last expressed.

However, during preparation of the vector, a deleted AdV lacking the target unit was generated because of a leak expression of Cre in E. coli and in 293 cells. To overcome this problem in 293 cells, we constructed a dominant-negative Cre (dnCreRY), which efficiently suppressed the leak expression of Cre. We generated a cell line which constitutively express dnCreRY and found that improved preparations of this vector were able to be achieved using this 293 cell line. In addition, excised circular target DNA contaminated in the viral stock and being expressed in AFP (-) cells, was able to be removed through virus purification by CsCl centrifugation. Although there are still some problems to be solved, this method will offer high-level expression while maintaining high specificity and will not only provide a novel strategy of suicide gene therapy using AdV, but also be very valuable for many studies requiring cell-specific expression.

2. Construction of "humanized" FLP recombinases aiming for efficient generation of helper-dependent adenovirus vector

Saki Kondo, Miho Terashima, Pei Zheng, Yumi Kanegae and Izumu Saito

Recently, a helper-dependent adenovirus vector (HD vector), which lacks all viral genome except for viral packaging signal and ITRs (inverted terminal repeats), is expected to solve drawbacks of first-generation adenovirus vector. However, amplification of HD vector is sometimes difficult because of severe contamination of helper virus. In general, a helper virus has a viral packaging signal flanked by a pair of loxPs to be excised in Cre-expressing 293 cells, and only HD viral genome can be packaged into a capsid. However, because the Cre mediated excision of packaging signal may be not complete, some helper viruses may escape and be packaged. Cre is more commonly used than FLP, which derived from *Saccharomyces cerevisiae*, because of its high recombination efficiency especially in mammalian cells. However, our preliminary observation showed that, in mammalian cells, FLP recombination proceeded nearly completely, while Cre recombination reached a plateau level possibly because a reversed reaction occurred.

In this study, we generated several FLP mutants aiming to obtain a better performance of recombinase, and characterized in mammalian cells. It is well known that one of the reasons of low recombination efficiency of FLP is its poor thermo-stability, and thermo-stable FLP (FLPe) containing four-amino acid mutation has been reported. The activity of FLPe has been believed to be higher than that of wild-type (wt) FLP in mammalian cells and animals. Unexpectedly, however, we here showed that enzymatic activity of FLPe protein was less efficient than that of wtFLP protein even in mammalian cells but that its thermo-stability overcame the lower activity. One way to improve the virtual efficiency of FLP in mammalian cells is to obtain efficient translation. So, we generated another mutant FLPe, "humanized" FLPe (hFLPe), in which the codon usage was optimized for use in humans. Then we compared FLP recombination efficiency in HeLa cells. We observed that hFLPe-expressing plasmid was able to produce 10-fold more FLPe protein than FLPe-expressing plasmid. So, we established FLP-expressing 293 cell lines using hFLPe, FLPe and wtFLP, and expressed FLP recombination activity were measured. hFLPe-expressing cell lines showed a highest excision efficiency. Therefore, an hFLPe-expressing 293 cell line that we cloned here, named hde12, may be valuable for HD vector generation, and further examinations are now under way.

3. Examination of regulation using Cre and three loxPs and development of a cassette for serial gene expression of rapid-OFF type

Shoko Saegusa, Saki Kondo, Miho Terashima, Yumi Kanegae and Izumu Saito

Cre excises a DNA between two loxPs when the two loxPs are present on the same DNA molecule in the same orientation. Gene expres-

sion using Cre excision reaction is utilized in many fields especially because its OFF-regulation is very strict. So far, two OFF-regulation systems has been developed: cDNA expression type (cDNA type) and two-loxP excision type (2L type). In the cDNA type, expression is rapidly turned off because cDNA of the target gene is excised out as a circular DNA but a promoter and polyA site remains in the chromosome. The remaining promoter sometimes causes a trouble in the expression experiment because of its enhancer effect. Meanwhile, in the L2 type, loxPs flank an entire expression unit of a promoter, the cDNA and a polyA and, therefore, L2 type is more useful because no sequences except a loxP remain in the chromosome. However, the expression turns OFF only slowly because the excised circular expression unit still expresses the target gene.

Therefore, we construct 3L OFF-regulation cassette, where the entire expression unit is flanked by two loxPs as 2L type, but it contains the third loxP between the promoter and cDNA. After supplying with Cre with AdV infection, the promoter must be excised as a circle and, at the same time, the target cDNA plus polyA must also be excised. Consequently the expression must be turned off rapidly and the unit must be excised out from the chromosome and we expected that both problems should be overcome. We used CV-1 cells, Transfast for transfections of GFP and AdV for supply Cre, and amounts of GFP RNA were measured three days after transfection. The remained GFP RNA level in the L2 type was reduced to about one third from that in the L3 type, and the data of Southern analysis confirmed these results. Therefore, we concluded that the L3 type construction containing three loxPs surely improved the commonly-used L2 type construction and brought about quicker OFF expression. The reason why a significant level of GFP RNA still remained is that the experiment above used transfection and that Cre must process very many copies of transfected target molecules; much better result is expected if the target gene would be present in a cell chromosome.

Because this work showed the utility of L3 type OFF-regulation, we then construct a serial gene expression cassette, pcLCAFNFL5Lr, which can perform a given OFF-expression unit into a chromosome using the neo selection, ON-regulation of target gene by FLP-expressing AdV and then quick OFF-regulation by excision of entire expression unit. The cassette is available on request. This work was the graduation study of S.S. in Kitasato University.

Publications

1. Kondo, S., Tanaka, Y., Nakano, M., Saito, I. and Kanegae, Y. Activities of various FLP recombinases expressed by adenovirus vectors in mammalian cells. J. Molec. Biol. *in press*, 2009.

Affiliated Facilities

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

Professor	Jun-ichiro Inoue, Ph.D.		教授(兼)	薬学博士	井 上 純一郎
Associate Professor	Shinobu Imajoh-Ohmi, D.Sc.		准教授	理学博士	大 海 忍
Assistant Professor	Hiroshi Sagara, Ph.D.		助 教	医学博士	相 良 洋
Project Assistant Professor	Masaaki Oyama, Ph.D.		特任助教	医学博士	尾 山 大 明

The mission of our laboratory is to develop technologies for protein research that enable us to analyze complex cellular systems leading to a variety of diseases such as cancer and infection. We mainly focus on the researches based on advanced technologies regarding mass spectrometry and electron microscopy for precise measurement of dynamic behaviors of functional protein networks.
We are also engaged in collaborative researches regarding electron microscopy, mass spectrometry, peptide synthesis and purification of proteins and their functional analyses and have made a substantial contribution to many scientific achievements.

〈Group I〉

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. 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 polymerize to F-actin. The 40-kDa actin-derived fragment is apparently related to spontaneous apoptosis of neutrophils. To investigate the role of actin proteolysis, we have made a cleavage-site-directed antibody (#1090 pAb) 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.

b. 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.

2. Establishment of novel antibodies as tools available for in situ analyses of post-translational modification of proteins

After biosynthesis proteins undergo various post-translational modifications, and their functions are modulated. In order to understand such biochemical reactions in a single cell, we have been making modification-specific antibodies as probes for such in situ analyses; cleavage-site-directed antibodies for proteolysis , phosphorylation-site-specfic antibodies, myristoilated peptide-specific antibodies , ubiquitination-specific antibodies , inhibitor-bound enzyme-specific antibodies etc. These antibodies should be useful tools for research in cellular biochemistry.

a. Evaluation of polyclonal cleavage-site directed antibodies and their fractionation into more easy-to use probes

Tsuyoshi Katagiri, Chizuko Takamura, Nozomi Ichikawa and Shinobu Imajoh-Ohmi

Cleavage-site directed antibodies are convenient tool for in situ analysis of proteolysis, since they do not bind unproteolyzed native proteins that retain the same sequence internally. To obtain such antibodies, peptides corresponding to the terminal regions around the cleavage are synthesized chemically and used for haptens, where molecular design of the peptides is critical for quality of the antibodies. Too short peptide results in generation of useless antibodies recognizing the short peptide but not the terminus of cleaved proteins. On the other hand, when a longer sequence is selected for immunogenic peptide, antibodies raised bind unproteolyzed proeins as well as the cleaved ones. Thus, an evaluation system is necessary for cleavage-site directed antibodies. Phage display liblaries were used for evaluation of antigenic specificity of cleavage-site directed antibodies. Randomized sequences of synthetic oligonucleotide were introduced into phage DNA in order that a fusion protein with randomized sequences of amino- or carboxyl-terminal region. A library was applied to immobilized antibodies, and phages bound were subjected to sequence analysis for terminal regions. When antigenic specificity of a cleavage-site derected antibody was examined by this method, the antibody was found to be a mixture of three types of antibodies that bind to terminal and two internal regions of the peptide used for immunogen. Quality of the antibody was successfully improved by affinity chromatography immobilized three peptides according to the evaluation method.

b. A novel method for hunting substrates of caspases in apoptotic cells

Maiko Okada, Chidzuko Takamura, Hiroyuki

Fukuda, Masahiko Kato and Shinobu Imajoh-Ohmi

Caspases catalyze limited proteolysis of many proteins in apoptotic cells. Hundreds of substrates have been identified as targets of caspases so far. Previously, nonmuscle myosin heavy chain-A and a component of DNA-dependent proein kinase, Ku80, are found to be cleaved during apoptosis in human Jurkat T cells. We used first a cleavage-site derected antibody against the amino-terminal fragment of caspase 3/7-catalyzed calpastatin. Carboxyl-terminal region of caspase-proteolyzed fragments resemble each other, and such antibodies are expected to misrecognize the target molecules. We further investigated the apoptotic Jurkat cells for the anitobody-stained polypeptides. Cells were selectively extracted with salt- and denaturant-containing buffers, and extracts were subjected to two-dimensional gel electrophoresis / immunoblotting . Candidate polypeptides stained with antibodies were digested with trypsin and analyzed by mass spetrometer. Isoforms of ribonucleoprotein were thus identified.

c. Identification of cysteine proteases in *Caenorhabditis elegans*

Yohei Kato and Shinobu Imajoh-Ohmi

E64c, [L-3-trans-carbonyloxirane-2-carbonyl]-L -leucine(3-methylbutyl)amide, is a synthetic inhibitor for cysteine proteases such as cathepsins B, H, L and calpain. To inhibit intracellular cysteine proteases E64d, [L-3-trans-ethoxycarbonyloxirane-2-carbonyl] -L-leucine (3-methylbutyl)amide, a membrane-permeable derivative of E64c is used instead of E64c. E64d penetrates into the cell membranes where cellular esterases convert it to E64c that covalently binds to the SH group of active center in enzymes. Thus, anti-E64c antibody is a useful probe for *in vivo* analysis of cysteine proteases.

We have succeeded in making an antibody to E64c. First, we tried to establish an antibody against E64c-bound calpain. A peptide corresponding to the active center of calpain was synthesized by using the multiple-antigen peptide system. E64c was chemically introduced into the SH group of active center cysteine under reducing conditions. Rabbits were immunized with the E64c-conjugated calpain-derived peptide without further conjugation with a carrier protein. Unexpectedly, an antibody thus prepared reacted not only with E64c-inactivated calpain but also with E64c-bound other cysteine proteases such as papain and cathepsins. Low antigenicity of peptide region in the immunogen may result in such broad specificity of the antibody. Our antibody is expected to be used for identification of E64c-targeted novel proteases. When cells were treated with E64d, cell growth was suppressed and several proteins were labeled by E64c that is visualized with this antibody on immunoblotting. Structural analysis of these proteins may lead identification of novel cysteine proteases.

Homogenates of *C. elegans* were treated with E64c in the presence or absence of calcium ion, and subjected to electrophoresis/immunoblotting using an anti-E64c antibody. A 55-kDa polypeptide (p55) was labelled with E64c in a calcium ion-dependent manner. In *C. elegans* several calpain-related gene products were identified at the mRNA level, but their physiological function remains to be elucidated

3. Myosin IIC-BRCA2 complex is localized to the midbody of cytokinesis.

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

Cytokinesis is the final step of the M phase of the cell cycle and completed by scission of the midbody, a narrow intercellular bridge between daughter cells. We reported that human nonmuscle myosin heavy chain (NMHC) IIC was recruited between both of the plus-ends of microtubules of the midbody and using specific RNA interference to decrease NMHC IIC in A 549 cells resulted in failure to complete a functional cleavage of the midbody. Here, we demonstrate that NMHC IIC interacts with BRCA2. BRCA2 is localized mainly in the nucleus where it plays an important role in DNA damage repair mediated by homologous recombination. Some BRCA2 protein is also present in the midbody. However, the role of BRCA2 in the midbody has been unclear. In order to determine whether endogenous BRCA2 forms a complex, a lysate derived from HeLa S3 cells was fractioned by glycerol density gradient centrifugation. Each fraction was immunoblotted with the anti-BRCA2 antibody. Although the molecular weight of the BRCA2 protein is 380 kDa, the majority of endogenous BRCA2 was observed to sediment at approximately 700–800 kDa. In an effort to identify the proteins that physically interacted with BRCA2, BRCA2 was immunoprecipitated using the anti-BRCA2 antibody. The immunoprecipitates were digested with trypsin, and the cleaved fragments were analyzed using

tandem mass spectrometry. Following a database search, NMHC IIC was identified as a candidate for the BRCA2-associated protein. Next, we examined the relationship between BRCA2 and NMHC IIC. In order to determine whether endogenous BRCA2 interacts with NMHC IIC, we performed a co-precipitation analysis. BRCA 2 was immunoprecipitated from A549 cells, and analysis of anti-BRCA2 immunoprecipitates with anti- NMHC IIC antibody demonstrated the presence of NMHC IIC in the immunoprecipitated complexes. No band was observed when precipitation was performed using anti-NMHC IIB as one of NMHC II isoforms and mouse IgG. These results indicate that there is a specific direct or indirect interaction between NMHC IIC and BRCA2. We next conducted an immunostaining analysis of A549 cells using an anti-BRCA2 antibody in combination with an antibody against NMHC IIC. The merged image showed that BRCA2 localized to the midbody region and as foci in the nuclei. An immunostaining analysis of NMHC IIC and BRCA2 distribution identified a staining pattern throughout the midbody. These findings demonstrate that NMHC IIC-BRCA2 complex may play an essential role in the terminal phase of cytokinesis.

4. Role of chemokines in experimental autoimmune encephalomyelitis

Taku Kuwabara[3] and Terutaka Kakiuchi[3]: [3]Depertment of Immunology, Toho University School of Medicine

CC chemokine ligand (CCL) 19 and CCL21 are thought to be critical for experimental autoimmune encephalomyelitis (EAE) induction, but their precise role is unknown. We examined the role of these chemokines in inducing EAE. C57 BL/6 mice lacking expression of these chemokines (plt/plt mice) or their receptor CCR7 were resistant to EAE induced with myelin oligodendrocyte glycoprotein peptide 35-55 (MOG35-55) and pertussis toxin. However, passive transfer of pathogenic T cells from wild type mice induced EAE in plt/plt mice, suggesting a defect independent of the role of CCR7-ligands in the migration of immune cells. Examination of draining lymph node (DLN) cells from MOG35-55-immunized plt/plt mice found decreased IL-23 and IL-12 production by plt/plt dendritic cells (DCs) and a concomitant defect in Th17 cell and Th1 cell generation. In contrast, production of the Th17 lineage commitment factors IL-6 and TGF-β were unaffected by loss of CCR7-ligands. The adoptive transfer of in vitro generated Th17 cells from DLN cells of MOG35-55-immunized plt/plt mice, developed EAE in wild type recipient mice, whereas that of Th1 cells did not at all. Th17 cell generation was restored in plt/plt DLN with addition of exogenous IL-23 or CCL 19/CCL21, and could be reversed by inclusion of anti-IL-23 mAb in cultures. Exogenous CCL19 /CCL21 induced IL-23p19 expression and IL-23 production by plt/plt or wild type DCs. Therefore, CCR7-ligands have a novel function in stimulating DCs to produce IL-23 and are important in the IL-23-dependent generation of pathogenic Th17 cells in EAE induction.

〈Group II〉

1. Time-resolved analysis of global phosphoproteome dynamics for network-wide exploration of novel drug targets against breast cancer

Masaaki Oyama, Hiroko Kozuka-Hata, Noriko Yumoto[1], Takeshi Nagashima[1], Yoko Kuroki[1], Satoshi Inoue[2], Mariko Hatakeyama[1] and Hiroaki Kitano[3]: [1]Advanced Science Institute, RIKEN, [2]Research Center for Genomic Medicine, Saitama Medical University, [3]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 a proteomics framework for obtaining time-resolved description of global phosphoproteome dynamics using highly sensitive nanoLC-MS/MS system in combination with the Stable Isotope Labeling by Amino acids in Cell culture (SILAC) technology. Through shotgun identification and quantification of phosphorylated molecules enriched with anti-phosphotyrosine antibodies/Phos-tag reagents, we obtained a global view of the dynamics regarding breast cancer-related signaling networks upon estrogen (E2) or heregulin (HRG) stimulation in human MCF-7 cells, which clearly revealed the network activation status common to or different between these two signaling pathways. Integration of our data with time-course gene expression profiles should provide an initial basis for constructing a comprehensive network model to analyze ER/ErbB receptor-mediated signaling dynamics at the system-level. We consider that our integrative model for breast cancer ER/ErbB signaling will facilitate the analysis of their potential cross-talk machinery which might be involved in the ac-

quisition of drug resistance property against an ER antagonist, tamoxifen.

2. Time-resolved description of tyrosine-phosphoproteome signaling dynamics reveals Src family-mediated global regulatory networks

Hiroko Kozuka-Hata, Masaaki Oyama, Shinya Tasaki, Kentaro Semba[4], Seisuke Hattori[5,6], Sumio Sugano[7], Jun-ichiro Inoue and Tadashi Yamamoto[8]: [4]Department of Life Science and Medical Bio-Science, Waseda University, [5]Division of Cellular Proteomics (BML), IMSUT, [6]Department of Biochemistry, School of Pharmaceutical Sciences, Kitasato University, [7]Laboratory of Functional Genomics, Department of Medical Genome Sciences, Graduate School of Frontier Sciences, The University of Tokyo, [8]Division of Oncology, Department of Cancer Biology, IMSUT

It is well-known that signal transduction system within a cell leads to the determination of diverse cell fates, such as proliferation, differentiation, or apoptosis. As phosphotyrosine-dependent networks play a key role in transmitting signals, time-resolved description of their dynamics provides a fundamental platform for analyzing the regulatory mechanisms at the system level. Here we established a mass spectrometry-based framework for analyzing tyrosine-phosphoproteome dynamics through temporal network perturbation and applied our methodology to the signaling networks that worked in human A431 cells as a model system. The dynamic behavior upon EGF stimulation revealed the property of multi-phase network activation, comprising spike signal transmission within 1 min followed by prolonged activation of multiple Src-related molecules. Temporal perturbation of Src-family kinases with the corresponding inhibitor PP2 in the prolonged activation phase enabled us to clearly distinguish between sensitive and robust pathways to this treatment, providing a system-level view of Src function in EGF signaling of A431 cells. Our methodology enables us to refine literature-based network structure into cell type-specific architecture. We expect that mathematical analyses on cell type-specific network models will lead us to efficient identification of potential drug targets in each disease condition and also enable us to theoretically estimate the effect of their corresponding drugs on a network-wide scale prior to clinical application.

3. Phosphoproteomics-based computational modeling defines the critical regulatory mechanisms of the aberrant EGFR signaling

Shinya Tasaki, Masao Nagasaki[9], Hiroko Kozuka-Hata, Kentaro Semba[4], Noriko Gotoh[10], Seisuke Hattori[5,6], Jun-ichiro Inoue, Tadashi Yamamoto[8], Satoru Miyano[9], Sumio Sugano[7] and Masaaki Oyama: [9]Laboratory of DNA Information Analysis, Human Genome Center, IMSUT, [10]Division of Systems Biomedical Technology, IMSUT

Mutation of epidermal growth factor receptor (EGFR) is known to trigger the deregulation of the signal transduction system and is strongly associated with a number of diseases. However, the mechanism by which mutated EGFR alters the downstream signaling is not yet fully understood at the system level. Here, we applied a computational methodology for characterizing the regulatory mechanism of aberrant EGFR signaling based on phosphoproteomics and network modeling. As a model system, we analyzed the system-wide effect of single point mutation at Y992, one of the "hub" autophosphorylation sites of EGFR. First, we measured EGF-induced temporal activation of protein tyrosine phosphorylation in two NIH3T3-derived cells expressing either wild-type EGFR (WT) or mutant EGFR with substitution of tyrosine to phenylalanine at position 992 (Y992F). Our comparative analysis of tyrosine-phosphoproteome dynamics in these two cell types characterized an unbiased landscape of the aberrant Y992F signaling. Based on the quantitative profiles, our computational analysis enabled us to rediscover already-known properties of Y992 and further gain model-driven insights into the effect of cellular content and the regulation of EGFR degradation. Our kinetic model also defined critical reactions to reconstruct the diverse effects of the mutation on the phosphoproteome dynamics. This is the first theoretical description of the regulatory mechanism of mutated EGFR signaling, which could provide a systematic strategy toward controlling disease-related cell signaling.

4. Large-scale identification of small proteins by 2DnanoLC-MS/MS system defines increased complexity of the human short ORFeome

Masaaki Oyama, Hiroko Kozuka-Hata, Sumio Sugano[7], Tadashi Yamamoto[8] and Jun-ichiro Inoue

In parallel with the human genome projects,

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

〈Group III〉

The main activity of this group is to offer supports for the research projects those need electron microscopic techniques. The electron microscopic 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 can not be acquired by other methods. In addition to the supportive work, we have been examining to develop new methods to reveal the electron microscopic localization of GFP and other fluorescent markers which are indispensable for biological studies. Another project to develop the methods to get serial thin sections and to observe with both transmission and scanning electron microscope are in progress.

1. Thin section electron microscopy and immuno-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. Localization of the Kid proteins during chromosome compaction.

Ohsugi M[1], Tokai-Nishizumi N[1], Sagara H and Yamamoto T[1]: [1]Division of Oncology, Department of Cancer Biology

In this study, localization of the chromokinesin Kid/kinesin-10 during mitosis were examined with thin section immuno-electron microscopy. The results showed that Kid localized to the boundaries of anaphase and telophase chromosomes and contributed to the chromosome compaction. These results were published in the journal Cell (ref. Ohsugi *et al*).

b. Ultrastructural analysis of entry and assembly of Herpes Simplex Virus.

Sugimoto K[2], Uema M[2], Arii J[2], Sagara H and Kawaguchi Y[2]: [2]Department of Infectious Disease Control, International Research Center for Infectious Diseases.

We have been performing several studies with Dr. Kawaguchi's laboratory groupes regarding to the infection processes of herpes simplex viruses. This year, two studies were published. In the work regarding the assembly process, cells

infected with triply fluorescent-tagged viruses were studied with thin section electron microscopy. We showed that multiple compartments correlated with the assembly of virus particles were formed near the base of the cells (ref Sugimoto *et al*.). In another project, a viral entry pathway dependent on the paired immunoglobulin-like type 2 receptor (PILR) α was analyzed with electron microscopy. We showed that the entry of the herpes virus into the PILRα transduced chinese hamster ovary cells was via virus-cell fusion at the cell surface. This and other results indicated that expression of PILRα produced an alternative HSV-1 entry pathway (ref. Arii *et al*).

Some other collaborative research works using thin section electron microscopy and/or immuno-electron microscopy were done with Dr. Nishiwaki[3] *et al*, in [3]Developmental Neurobiology Unit, Okinawa Institute of Science and Technology Promotion Corporation (ref. Nishiwaki *et al*), Dr. Goto[1] *et al* (ref. Goto *et al*), Dr. Chida[4] *et al*, in [4]Department of Pathology, Research Institute, Internal Medical Center of Japan (ref. Chida et al), and Dr. Nagatsuma[5] *et al*, in [5]Department of Pathology, The Jikei University School of Medicine (ref. Nagatsumaa *et al*). Also, other works are now in progress with Dr. Noda[6] *et al* in [6]Division of Virology, Department of Microbiology and Immunology, with Dr. Terao[7] et al in [7]Laboratory Animal Center, and with Dr. Nochi[8] in [8]Division of Mucosal Immunology, Department of Microbiology and Immunology.

2. Negative staining techniques

Negative staining techniques are simple and quick method to observe the morphology of the macro molecules. This year, the negative staining method were used in collaborative work with Dr. Hagiwara[7] *et al*. at [7]Laboratory Animal Center. In this study, negative staining techniques were used to analyze the roles of canine distemper virus or influenza virus proteins in nucleocapsid formation.

3. 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. Uchida[9] *et al*, [9] Department of Histology, School of life Dentistry, The Nippon Dental University, to analyze the factors those affect the bone regeneration processes (ref Uchida *et al*). Other works are in progress with Dr. Sanada[10] *et al*, [10]Department of Gerontological Nursing, Division of Health Science and Nursing, Graduate School of Medicine, to analyze the effects of diabetes or bacterial infection during wound repair.

Publications

〈Group I〉

Furuta T, Imajoh-Ohmi S, Fukuda H, Kano S, Miyake K and Watanabe N. Mast cell-mediated immune responses through IgE antibody and Toll-like receptor 4 by malarial peroxiredoxin. Eur J Immunol 38: 1-10, 2008.

Arimoto K, Fukuda H, Imajoh-Ohmi S, Saito H and Takekawa M. Formation of stress granules inhibits apoptosis by suppressing stress-responsive MAPK pathways. Nat Cell Biol 10: 1324-1332, 2008.

Kuribayashi F, Tsuruta S, Yamazaki T, Nunoi H, Imajoh-Ohmi S, Kanegasaki S and Nakamura M. Cell adhesion markedly increases lucigenin -enhanced chemiluminescence of the phagocyte NADPH oxidase. Genes Cells, in press.

Nakamura M, Morisawa H, Imajoh-Ohmi S, Takamura C, Fukuda H and Toda T. Proteomic analysis of protein complexes in human SH-SY5Y neuroblastoma cells by using blue-native gel electrophoresis: An increase in lamin A/C associated with heat shock protein 90 in response to 6-hydroxydopamine-induced oxidative stress Experimental Gerontology, in press.

〈Group II〉

Mathivanan S, Ahmed M, Ahn NG, Alexandre H, Amanchy R, Andrews PC, Bader JS, Balgley BM, Bantscheff M, Bennett KL, Björling E, Blagoev B, Bose R, Brahmachari SK, Burlingame AS, Bustelo XR, Cagney G, Cantin GT, Cardasis HL, Celis JE, Chaerkady R, Chu F, Cole PA, Costello CE, Cotter RJ, Crockett D, DeLany JP, De Marzo AM, DeSouza LV, Deutsch EW, Dransfield E, Drewes G, Droit A, Dunn MJ, Elenitoba-Johnson K, Ewing RM, Van Eyk J, Faca V, Falkner J, Fang X, Fenselau C, Figeys D, Gagné P, Gelfi C, Gevaert K, Gimble JM, Gnad F, Goel R, Gromov P, Hanash SM, Hancock WS, Harsha HC, Hart G, Hays F, He F, Hebbar P, Helsens K, Hermeking H, Hide W, Hjernø K, Hochstrasser DF, Hofmann O, Horn DM, Hruban RH, Ibarrola N, James P, Jensen ON, Jensen PH, Jung P, Kandasamy K, Kheterpal I, Kikuno RF, Korf U, Körner R, Kuster B, Kwon MS, Lee HJ, Lee YJ, Lefevre M, Lehvaslaiho M, Les-

cuyer P, Levander F, Lim MS, Löbke C, Loo JA, Mann M, Martens L, Martinez-Heredia J, McComb M, McRedmond J, Mehrle A, Menon R, Miller CA, Mischak H, Mohan SS, Mohmood R, Molina H, Moran MF, Morgan JD, Moritz R, Morzel M, Muddiman DC, Nalli A, Navarro JD, Neubert TA, Ohara O, Oliva R, Omenn GS, Oyama M, Paik YK, Pennington K, Pepperkok R, Periaswamy B, Petricoin EF, Poirier GG, Prasad TS, Purvine SO, Rahiman BA, Ramachandran P, Ramachandra YL, Rice RH, Rick J, Ronnholm RH, Salonen J, Sanchez JC, Sayd T, Seshi B, Shankari K, Sheng SJ, Shetty V, Shivakumar K, Simpson RJ, Sirdeshmukh R, Siu KW, Smith JC, Smith RD, States DJ, Sugano S, Sullivan M, Superti-Furga G, Takatalo M, Thongboonkerd V, Trinidad JC, Uhlen M, Vandekerckhove J, Vasilescu J, Veenstra TD, Vidal-Taboada JM, Vihinen M, Wait R, Wang X, Wiemann S, Wu B, Xu T, Yates JR, Zhong J, Zhou M, Zhu Y, Zurbig P and Pandey A. Human Proteinpedia enables sharing of human protein data. Nat Biotechnol 26: 164-167, 2008.

Hagiwara K, Sato H, Inoue Y, Watanabe A, Yoneda M, Ikeda F, Fujita K, Fukuda H, Takamura C, Kozuka-Hata H, Oyama M, Sugano S, Ohmi S and Kai C. Phosphorylation of measles virus nucleoprotein upregulates the transcriptional activity of minigenomic RNA. Proteomics 8: 1871-1879, 2008.

Nagashima T*, Oyama M*, Kozuka-Hata H, Yumoto N, Sakaki Y and Hatakeyama M. (*; equal contribution) Phosphoproteome and transcriptome analyses of ErbB ligand-stimulated MCF-7 cells. Cancer Genomics Proteomics 5: 161-168, 2008.

Oyama M*, Kozuka-Hata H*, Tasaki S, Semba K, hattori S, Sugano S, Inoue J and Yamamoto T. (*; equal contribution) Temporal perturbation of tyrosine-phosphoproteome dynamics reveals the system-wide regulatory networks. Mol Cell Proteomics, in press.

Oyama M. Comprehensive description of signal transduction networks by quantitative proteomics and systems biology. Current Bioinformatics, in press.

〈Group III〉

Ohsugi M, Adachi K, Horai R, Kakuta S, Sudo K, Kotaki H, Tokai-Nishizumi N, Sagara H, Iwakura Y and Yamamoto T. Kid-mediated chromosome compaction ensures proper nuclear envelope formation. Cell 132: 771-782, 2008.

Goto J, Tezuka T, Nakazawa T, Sagara H and Yamamoto T. Loss of Fyn tyrosine kinase on the C57BL/6 genetic background causes hydrocephalus with defects in oligodendrocyte development. Mol Cell Neurosci 38: 203-212, 2008.

Sugimoto K, Uema M, Sagara H, Tanaka M, Sata T, Hashimoto Y and Kawaguchi Y. Simultaneous tracking of capsid, tegument, and envelope protein localization in living cells infected with triply fluorescent herpes simplex virus 1. J Virol 82: 5198-5211, 2008.

Chida D, Hashimoto O, Kuwahara M, Sagara H, Osaka T, Tsubone H and Iwakura Y. Increased fat: carbohydrate oxidation ratio in Il1 ra (-/-) mice on a high-fat diet is associated with increased sympathetic tone. Diabetologia 51: 1698-1706, 2008.

Nishiwaki Y, Komori A, Sagara H, Suzuki E, Manabe T, Hosoya T, Nojima Y, Wada H, Tanaka H, Okamoto H and Masai I. Mutation of cGMP phosphodiesterase 6 alpha'-subunit gene causes progressive degeneration of cone photoreceptors in zebrafish. Mech Dev 125: 932-946, 2008.

Nagatsuma K, Hayashi Y, Hano H, Sagara H, Murakami K, Saito M, Masaki T, Lu T, Tanaka M, Enzan H, Aizawa Y, Tajiri H and Matsuura T. Lecithin: retinol acyltransferase protein is distributed in both hepatic stellate cells and endothelial cells of normal rodent and human liver. Liver Int, in press.

Uchida M, Agata H, Sagara H, Shinohara Y, Kagami H and Asahina I. Mixing conditions for cell scaffolds affect the bone formation induced by bone engineering with human bone marrow stromal cells, beta-tricalcium phosphate granules, and rhBMP-2. J Biomed Mater Res A, in press.

Arii J, Uema M, Morimoto T, Sagara H, Akashi H, Ono E, Arase H and Kawaguchi Y. Entry of herpes simplex virus 1 and other alphaherpesviruses via the paired immunoglobulin-like type 2 receptor α. J Virol, in press.