Affiliated Facilities

Laboratory Animal Research Center
実験動物研究施設

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Research associate	Misako Yoneda D.V.M., Ph.D.	助　手	農学博士	米　田　美佐子
Research associate	Hiroki Sato Ph.D.	助　手	理学博士	佐　藤　宏　樹

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.

Establishment of a Nipah virus rescue system

Misako Yoneda, Vanessa Guillaume[1], Fusako Ikeda, Yuki Sakuma, Hiroki Sato, T. Fabian Wild[1], and Chieko Kai: [1]Institut National de la Sante et de la Recherche Medicale, Lyon, France

Nipah virus (NiV), a paramyxovirus, was first discovered in Malaysia in 1998 in an outbreak of infection in pigs and humans and incurred a high fatality rate in humans. Fruit bats, living in vast areas extending from India to the western Pacific, were identified as the natural reservoir of the virus. However, the mechanisms that resulted in severe pathogenicity in humans (up to 70% mortality) and that enabled crossing the species barrier were not known. In this study, we established a system that enabled the rescue of replicating NiVs from a cloned DNA by co-transfection of a constructed full-length cDNA clone and supporting plasmids coding virus nucleoprotein, phosphoprotein, and polymerase with the infection of the recombinant vaccinia virus, MVAGKT7, expressing T7 RNA polymerase. The rescued NiV (rNiV), by using the newly developed reverse genetics system, showed properties *in vitro* that were similar to the parent virus and retained the severe pathogenicity in a previously established animal model by experimental infection. A recombinant NiV was also developed, expressing enhanced green fluorescent protein (rNiV-EGFP). Using the virus, permissibility of NiV was compared with the presence of a known cellular receptor, ephrin B2, in a number of cell lines of different origins. Interestingly, two cell lines expressing ephrin B2 were not susceptible for rNiV-EGFP, indicating that additional factors are clearly required for full NiV replication. The reverse genetics for NiV will provide a powerful tool for the analysis of the molecular mechanisms of pathogenicity and cross-species infection.

Host range and receptor utilization of canine distemper virus analyzed by recombinant viruses: Involvement of heparin-like molecule in CDV infection

Kentaro Fujita, Ryuichi Miura, Misako Yoneda, Fusako Shimizu, Hiroki Sato, Yuri Muto, Yasuyuki Endo, Kyoko Tsukiyama-Kohara and Chieko Kai,

We constructed recombinant viruses expressing enhanced green fluorescent protein (EGFP) or firefly luciferase from cDNA clones of the canine distemper virus (CDV) (a Japanese field isolate, Yanaka strain). Using these viruses, we examined susceptibilities of different cell lines to CDV infection. The results revealed that the recombinant CDVs can infect a broad range of cell lines. Infectivity inhibition assay using a monoclonal antibody specific to the human SLAM molecule indicated that the infection of B95a cells with these recombinant CDVs is mainly mediated by SLAM but the infection of 293 cell lines with CDV is not, implying the presence of one or more alternative receptors for CDV in non-lymphoid tissue. Infection of 293 cells with the recombinant CDV was inhibited by soluble heparin, and the recombinant virus bound to immobilized heparin. Both F and H proteins of CDV could bind to immobilized heparin. These results suggest that heparin-like molecules are involved in CDV infection.

Morbillivirus nucleoprotein possesses a novel nuclear localization signal and a CRM1-independent nuclear export signal

Hiroki Sato, Munemitsu Masuda, Ryuichi Miura, Misako Yoneda and Chieko Kai

Morbilliviruses, which belong to the Mononegavirales, replicate its RNA genome in the cytoplasm of the host cell. However, they also form characteristic intranuclear inclusion bodies, consisting of nucleoprotein (N), in infected cells. To analyze the mechanisms of nucleocytoplasmic transport of N protein, we characterized the nuclear localization (NLS) and nuclear export (NES) signals of canine distemper virus (CDV) N protein by deletion mutation and alanine substitution of the protein. The NLS has a novel leucine/isoleucine-rich motif (TGILISIL) at positions 70-77, whereas the NES is composed of a leucine-rich motif (LLRSLTLF) at positions 4-11. The NLS and NES of the N proteins of other morbilliviruses, that is, measles virus (MV) and rinderpest virus (RPV), were also analyzed. The NLS of CDV-N protein is conserved at the same position in MV-N protein, whereas the NES of MV-N protein is located in the C-terminal region. The NES of RPV-N protein is also located at the same position as CDV-N protein, whereas the NLS motif is present not only at the same locus as CDV-N protein but also at other sites. Interestingly, the nuclear export of all these N proteins appears to proceed via a CRM1-independent pathway.

Sequence analysis of VP2 gene of canine parvovirus isolated from domestic dogs in Japan in 1999 and 2000.

Miho Doki, Kentaro Fujita, Ryuichi Miura, Misako Yoneda, Yoshihisa Ishikawa[1], Akira Taneno[1] and Chieko Kai.: [1]Intervet K.K., Central Research Laboratories, Ibaraki, Japan

Seven strains of canine parvovirus (CPV) were isolated from affected dogs in Japan between 1999 and 2000, and their VP2 genes were genetically analyzed. Comparison of the predicted amino acid sequences of VP2 suggested that three field isolates corresponded to CPV type 2 a, while the other four to CPV type 2b. The phylogenetic tree constructed from the VP2 genes showed that the newly isolated strains are classified into the cluster consisting of recent Japanese and Taiwanese field isolates, which are distinct from Vietnamese isolates, United States Isolates, or classical CPV type 2. These results suggest that the CPV transmission occurred between Japan and Taiwan in 1990s, and the offspring are still circulating in both countries.

Publications

Cosby, S.L., Kai, C. and Yamanouchi, K. Immunology of rinderpest-an immunosuppression but a life long viccine protection. In Rinderpest and Peste des Ruminants, Academic Press, pp 196-221, 2005

Masuda, M., Sato, H., Kamata, H., Katsuo Tomoe, Takenaka, A., Miura, R., Yoneda, M., Tsukiyama-Kohara, K., Mizumoto, K. and Kai, C. Characterization of monoclonal antibodies directed against the canine distemper virus nucleocapsid protein. Comp. Immunol. Microb., 29, 157-165, 2006.

Sato, H., Masuda, M., Miura, R., Yoneda, M. and Kai, C. Morbillivirus nucleoprotein possesses a novel nuclear localization signal and a CRM1-independent nuclear export signal. Virology 352, 121-130, 2006.

Doki, M., Fujita, K., Miura, R., Yoneda, M., Ishikawa, Y Taneno, A. and Kai, C. Sequence analysis of VP2 gene of canine parvovirus isolated from domestic dogs in Japan in 1999 and 2000. Comp. Immunol. Microb., 29 (4),

199-206, 2006.
Inoue, Y., Nomura, Y., Haishi, T., Yoshikawa, K., Seki, T., Tsukiyama-Kohara, K., Kai, C., Okubo, T., and Ohtomo, K. Imaging of Living Mice Using a 1-T Compact Magnetic Resonance Imaging System. J. Mag. Res. Imag. 2006. in press.
Yoneda, M., Guillaume, V., Ikeda, F., Sakuma, Y., Sato, H., Wild, T.F. and Kai, C. Establishment of a Nipah virus rescue system. Proc. Natl. Acad. Sci., USA, 103 (44), 16508-16513, 2006.
Fujita, K., Miura, R., Yoneda, M., Shimizu, F., Sato, H., Muto, Y., Endo, Y., Tsukiyama-Kohara, K. and Kai, C. Host range and receptor utilization of canine distemper virus analyzed by recombinant viruses: involvement heparin-like molecule in CDV infection. Virology, 2006 in press

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.	助教授	農学博士	服　部　正　策
Research Associate	Takeshi Kuraishi, Ph.D.	助　手	農学博士	倉　石　　武

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

1. Research on the Habu control

Shosaku Hattori, Yuko Katakai, Hiroshi Kihara[1], Motonori Ohno[2], Naoko Ueda[2], Shigenari Terada[3], Hiro Yonezawa[4], Yoshihiro Hayashi[5], Michihisa Toriba[6],i, and Tomohisa Ogawa[7],: [1]Bioscience research Institute, Takara Shuzo Co., Ltd., [2]Departmen of Applied Life Science, Faculty of Bioscience, Sojo University, [3]Department of Biochemistry, Faculty of Science, Fukuoka University, [4]Department of biochemistry, Faculty of Science, Kagoshima University, [5]Department of Veterinary Anatomy, Faculty of Agriculture, University of Tokyo, [6]The Japan Snake Institute, [7]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, *Trimeresu-*

rus 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. Discovery of novel [Arg^{49}] phospholipase A_2 isozymes from *Protobothrops elegans* venom and regional evolution of Crotalinae snake venom phospholipase A_2 isozymes in the southwestern islands of Japan and Taiwan.

Takahito Chijiwa[8], Emi Tokunaga[8], Ryo Ikeda[8], Koki Terada[9], Tomohisa Ogawa[7], Naoko Oda-Ueda[2], Shosaku Hattori, Masatoshi Nozaki[9] and Motonori Ohno[2]: [8]Department of Applied Life Science, Faculty of Bioscience, Sojo University, [9]Department of Health Science, Okinawa Prefectural Institute of Health and Environment,

Protobothrops (formerly *Trimeresurus*) *elegans*, a Crotalinae snake, inhabits Ishigaki and Iriomote islands of the Sakishima Island of Japan which are located between Okinawa island of Japan and Taiwan. Two phospholipase A_2 (PLA_2) isozymes were purified to homogeneity from *P. elegans* venom and sequenced. This led to a discovery of novel PLA_2 isozymes with Arg at position 49, that is, [Arg^{49}] PLA_2 forms, named *Pe*BP(R)-I and *Pe*BP(R)-II. They are polymorphic at position 3, Val for *Pe*BP(R)-I and Ile for *Pe*BP (R)-II. The cDNAs encoding *Pe*BP(R)-I and *Pe*BP (R)-II were cloned. The cDNA encoding an [Arg^{49}] PLA_2 named *Pe*PLA_2 was also obtained. In contrast to PLA_2 isozymes from *Protobothrops* genus with 122 amino acid residues, *Pe*BP(R)-I and *Pe*BP(R)-II are composed of 121 amino acid residues due to lack of Pro at position 90. They exhibited necrotic and edema-inducing activities but no hemorrhagic activity was detected. A phylogenetic tree constructed for venom PLA_2 isozymes of *Protobothrops* genus and of related genera in the southwestern islands of Japan and Taiwan revealed that *Pe*BP(R)-I and *Pe*BP(R)-II of *P. elegans* are evolutionarily much closer to *Pm*K49PLA_2, a [Lys^{49}] PLA_2, from *P. mucrosquamatus* (Taiwan) than BPI and BPII, both [Lys$_{49}$] PLA_2 forms, from *P. flavoviridis* (Amami-Oshima and Tokunoshima islands of Japan). Such evolutionary relationships are also seen in neutral [Asp^{49}] PLA_2 isozymes from the three *Protobothrops* species. Thus, *P. elegans* is the speices much closer to *P. mucrosquamatus* than *P. flavoviridis*. Their evolutionary distances seem to be well related to geological history of the islands where they have lived. In addition, it was cleary noted that *Ovophis okinavensis* (Amami-Oshima), which had formerly belonged to the *Trimeresurus* genus, and *Trimeresurus stejnegeri* (Taiwan) are the species fairly distant from *Protobothrops* genes.

3. Reproduction of squirrel monkeys.

Shosaku Hattori, Takeshi Kuraishi, Yuko Katakai[10], Mamoru Ito[11], and Chieko Kai[12]: [10]Corporation for production and research of laboratory primates, [11]Laboratory of Immunology, Central Institute of Experimental Animals, [12]Laboratory Animal Research Center

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 5 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. Research of wild mammals.

Shosaku Hattori, Takeshi Kuraishi, Takashi

Suzuki[13] and Fumio Yamada[14]: [13]Japan Wildlife Research Center, [14]Wildlife Ecology Laboratory, Forestry & Forest Products Research Institute

Amami-oshima Island is a habitat of animals and plants indigenous to the Nansei Islands. These animals occur originally in the Oriental region of Asia and include the Amami rabbit, *Pentalagus furnessi*, the Ryukyu spiny rat, *Tokudaia osimensis*, the Okinawa long-haired rat, *Diplothrix legata*, the Watase's shrew, *Crocidura watasei*, and the Musk shrew, *Suncus murinus*. These mammals are used for researches on comparative anatomy, taxonomy, and development of experimental animals. Besides, these mammals are valuable species biologically as survivors from the Miocene about 10,000,000 years ago. We have initiated the investigation for these species to protect from extinction. We have documented the feasibility of recovering large numbers of oocytes from the Watase's shrew, and some of oocytes can be induced to mature in vitro.

Recently, the Java mongoose, *Herpetologica javanicus* grew in the wild as invasive carnivore in the Amami-Oshima Island. The population of the mongoose increases every year and the habitat range is extending to south area in the Island. It is necessary to remove the invader to defend nature. Then we are investigating the influence which the mongoose gives to wildlife in the Island. Since hairs such as Amami rabbit, Ryukyu spiny rat, Akahige were confirmed from the excrement of the mongoose, the necessity of the urgent ridding countermeasure of the mongoose was indicated. From 2000, the capture project of the mongoose was started by Ministry of Environment in order to protect Amami-Oshima's endemic species.

Publications

Chijiwa, T., Tokunaga, E., Ikeda, R., Terada, K., Ogawa, T., Oda-Ueda, N., Hattori, S., Nozaki, M. and Ohno, M. Discovery of novel [Arg[49]] phospholipase A_2 isozymes from *Protobothrops elegans* venom and regional evolution of Crotalinae snake venom phospholipase A_2 isozymes in the southwestern islands of Japan and Taiwan. Toxicon. 48: 672-682, 2006.

服部正策．奄美の自然とハブとヒト―撲滅から共存へ―．Biophillia，2(1): 50‐54，2006.

服部正策，倉石武．動物実験部会報告．平成17年度ハブ毒阻害因子応用開発研究報告書．（鹿児島県）：pp. 41−51，2006.

服部正策，倉石武．野外調査部会報告．平成17年度ハブ動態調査研究報告書．（鹿児島県）：pp. 11−45，2006.

Affiliated Facilities

Laboratory of Molecular Genetics
遺伝子解析施設

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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 under the safety guideline.

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 under the safety guideline. 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 recombinant adenovirus (rAd) expressing various genes efficiently. And recently we developed the new cosmid cassette for construction of rAd which can choose not only COS-TPC method but also an easier method using a full-length viral genome with intact viral termini. 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/*lox*P 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). The method will promote many fields of molecular biology and medicine and may open a new field of "intracellular gene manipulation". The research activities in 2006 were shown below.

1. Efficient sequential gene-regulation via FLP- and Cre- recombinase using adenovirus vector in mammalian cells including mouse ES cells

Saki Kondo, Yuzuka Takahashi, Seiji Shiozawa[1], Hirotake Ichise[1], Nobuaki Yoshida[1], Yumi Kanegae and Izumu Saito: [1]Laboratory of Gene Expression and Regulation, Institute of Medical Science, University of Tokyo

After elucidation of genome sequences, studies of gene function become increasingly important. For this purpose, overexpression of a gene in cell lines and generation of transgenic and knockout animals have been widely performed. However, these approaches sometimes raise difficulties depending on the characteristic of the genes being expressed. Because some genes have functions deleterious or inhibitory to cell proliferation, gene activation/inactivation systems, especially the OFF-regulation approach,

have been developed. The site-specific recombinase-mediated systems using the Cre and FLP have been used for these purposes. Cre can regulate gene expression very strictly and efficiently not only in cultured cells but also in animals, although it exerts the regulatory action only once and cannot regulate either repeatedly or sequentially. However, to elucidate the functions of unknown genes more precisely, it is necessary to examine the phenotypes newly emerging after expressions of the genes turned OFF and ON sequentially with a given timing (OFF-ON-OFF or ON-OFF-ON), comparable to the natural processes of development and regeneration. Moreover, genome/proteomics studies are progressed recently and it becomes more and more important to precisely compare the expression profiles of genes among these ON- or OFF-regulated cells. Combining two site-specific recombinases would theoretically allow sequential regulation but had been impractical because the recombination efficiency of FLP is much lower than that of Cre. However, this can be problematic because not only rAd infection in a high dose but also Cre itself is toxic to cells. Therefore, it is valuable to determine the range of infectious doses that do not cause any toxicity to the cells infected serially with both FLP- and Cre-expressing rAds.

Recently, rAds are applied for ES cell investigation, to eliminate a drug-resistance gene using Cre-expressing rAd for generating transgenic/knockout mice, for example. Furthermore, it was difficult to control the amount of rAd for infection because, in general, ES cells were cultured on feeder cells and were infected together with feeder cells. Because the feeder cells were competitively infected with rAd, excess amounts of rAd were necessary for sufficient transduction into ES cells. To apply FLP-expressing rAd for elimination of the drug-resistance gene in ES cells seems to be especially useful when generating knockout mice conditioned by Cre. However, the recombination efficiency of FLP is less than that of Cre, and therefore an infection method, which is more efficient and easier to control the viral amount, is desired.

We demonstrated efficient "sequential" gene-regulation using Cre- and FLP-expressing rAd in two different monitor cell lines, for regulation of one gene (OFF-ON-OFF) and for two genes (ON-OFF and OFF-ON, independently). Generally, serial use of Cre- and FLP-expressing rAd tends to cause significant cytotoxicity, but we here described optimum dose of the rAds for serial regulation. We also established an efficient method of rAd infection to mouse ES cell lines after removing feeder cells, showing that this system is useful for removal of FRT-flanked drug-resistance gene cassette from recombinant ES cells prior to introduction of ES cells into blastocytes for chimeric mice production. Removal of the drug-resistance gene by FLP in ES cells is very simple and useful for efficiently generation of Cre-mediating conditional knockout mice. Because our sequential gene regulation system offers efficient purpose-gene regulation and strict OFF-regulation, it is potentially valuable for elucidating not only novel gene functions using cDNA microarray analysis but also for "gene switching" in development and regeneration research. This work has been published in *Microbiology and Immunology*, 50: 831-843, 2006.

2. Possible mechanism of adenovirus generation from a cloned viral genome tagged with nucleotides at its ends

Hiromitsu Fukuda, Miho Terashima, Michiko Koshikawa, Yumi Kanegae and Izumu Saito

Human adenovirus type 5 (Ad5) is often used as an efficient expression vector in various fields of study in basic research and gene therapies, and was historically studied as the first model of DNA replication in mammalian cells. The virus has a double-stranded, linear DNA genome of 36-kilobases (kb) and the 5′ ends of the genome are covalently linked to a viral-coded terminal protein (TP) followed by the sequence starting 5′-CATCAT... Each end of the viral genome carries an identical sequence of 103 base pairs (bp), called the inverted terminal repeat (ITR), and the viral genome replication origin is located at each end. Because the viral genome is linear rather than circular, manipulation of the genome is not simple and *in vivo* study using the full-length viral genome has been very limited.

Therefore, early studies were performed using cloned a tagged or nibbled left-end fragment of the adenovirus (Ad) type 2 or 5 genome ligated with the large right fragment including intact right end. From these works, it was believed that the left end sequence is correctly repaired by using the intact sequence of the right-end ITR. However, it was reported that the entire cloned Ad5 genome was able to generate infectious virus when transfected into 293 cells, only after both ends of the genome had been exposed by *Eco*RI cleavage at the terminal-tagged linkers. The oligonucleotides derived from the tagged linker appeared to have been removed in the restored viral genome but the presented data were very limited. The efficiency of viral generation using cloned genome DNA was appreciable, but was 50-fold lower than that using proteinase K-

treated, TP-removed viral DNA. Therefore, a question arose how the viruses generate when their both ends are tagged with oligonucleotides, i.e. when they lacks any native end. Nevertheless, this method was employed for generation of adenovirus vector by exposing the genome ends via digestion by *Pac*I, because no *Pac*I cleavage site is present in the Ad5 vector genome. So far, the mechanism by which the virus restores proper sequences at both ends has not yet been investigated. In addition, this method cannot be used if the insert DNA contains a *Pac*I site.

We here showed in our semi-quantitative study that the generation efficiency of virus clones decreases possibly depending on the length of nucleotide tags at the both ends and that both the oligonucleotide tags were precisely removed during virus generation with restoration of the proper terminal sequences. Interestingly the viral genome of which one end was tagged, while the other was attached about 12-kb sequences, did generate intact viral clones at a reduced but significant efficiency. From these results, we here propose a possible mechanism whereby the terminal-protein-deoxycytidine complex enters from the enzyme-cleaved end and reaches deoxyguanine at the initiating position of DNA synthesis *in vivo*. A replication origin at one end, embedded deeply in double-stranded DNA, can be activated by two cycles of one-directional full-length DNA synthesis initiated by the other exposed replication origin about 30 kilobases away. We also describe new cassette cosmids which can use not only *Pac*I but also *Bst*BI for construction of an adenovirus vector, without reducing construction efficiency. This work has been published in *Microbiology and Immunology*, 50: 643-654, 2006.

3. Keratinocyte growth factor gene transduction ameliorates acute lung injury and mortality in mice

Yasuko Baba[1,2], Takuya Yazawa[3], Yumi Kanegae[1], Seiko Sakamoto[2], Izumu Saito[1], Naoto Morimura[2], Takahisa Goto[2], Yoshitsugu Yamada[2] and Kiyoyasu Kurahashi[2]: [1]Laboratory of Molecular Genetics, Institute of Medical Science, University of Tokyo; Departments of [2]Anesthesiology and Critical Care Medicine and [3]Applied Pathobiology, Yokohama City University Graduate School of Medicine

Critically ill patients with acute lung injury (ALI) or acute respiratory distress syndrome (ARDS) have a high mortality. ALI/ARDS is a common clinical syndrome that occurs in both medical and post-surgical patients; its features include a rapid onset, progressive lung edema, and severe hypoxemia. Histopathologically, disruption of alveolar epithelium and capillary injury cause increased permeability of alveolar septa and protein-rich pulmonary edema. Despite advances in airway management and improvements in protective ventilation strategies, the mortality rate of ALI/ARDS patients is 35% to 60%. No effective pharmacological therapy for ALI/ARDS exists. Thus, a novel approach that strengthens lung cells against further insults or promotes the repair of injured epithelial cells to restore lung tissue integrity could be clinically useful for treating ALI/ARDS.

Lung tissue repair may be promoted by endogenous factors, such as keratinocyte growth factor (KGF) that is also known as a FGF-7. KGF is an epithelial-specific growth factor and has been studied with respect to its effect in various lung injury models. KGF stimulates type II pneumocyte proliferation, increases surfactant protein production, increases alveolar epithelial fluid transport, decreases apoptosis, and promotes DNA repair. Although the effectiveness of recombinant KGF protein has been investigated in different lung injury models, the effect of recombinant human KGF (rhKGF) protein was reported to be lost within days. In some studies, repeated administration of rhKGF was required to obtain beneficial results.

KGF gene transduction is a potentially useful method for obtaining an adequate effective duration of action to overcome the critical phase of ALI/ARDS. Therefore, we constructed a KGF-expressing rAd using the CAG promoter expecting that it may give a significant therapeutic effect with a single and relatively small vector dose, hence causing less inflammation, while serving strong KGF expression in an ALI model. We investigated the effectiveness of intratracheal KGF gene transduction using rAd in ALI. We constructed an rAd expressing mouse KGF, and 1.0×10^{9} plaque-forming units of mKGF cDNA expressing and control rAd were intratracheally instilled using a microsprayer into anesthetized BALB/c mice. Three days later, the mice were exposed to >90% oxygen for 72 hours, and the effect of KGF on hyperoxia-induced lung injury was examined. In the KGF-expressing rAd group, KGF was strongly expressed in the airway epithelial cells, and peribronchiolar and alveolar inflammation caused by rAd instillation was minimal. The KGF overexpression not only induced proliferation of surfactant protein-C-positive cuboidal cells, especially in the terminal bronchiolar and alveolar walls, but also prevented lung injury including intra-alveolar exudation/hemorrhage, albumin permeability increase, and pulmonary edema. The arterial oxy-

gen tension and the survival rate were significantly higher in the KGF-transfected group. These findings suggest that KGF gene transduction into the airway epithelium is a promising potential treatment for ALI. The work also presented an important example of rAd usage expressing a growth or differentiating factor for gene therapy and basic study, because its expression is limited to be transient and because the vector genome will vanish with time. This work has now been published in *Human Gene Therapy*, 18: 130-141, 2007.

Publications

1. Ueda, S., Fukamachi, K., Matsuioka, Y., Takasuka, N., Takeshita, F., Naito, A., Iigo, M., Alexander, DB., Moore, MA., Saito, I., Ochiya, T., Tsuda, H. Ductal origin of pancreatic adenocarcinomas induced by conditional activation of a human Ha-ras oncogene in rat pancreas. Carcinogenesis. *27*: 2497-2510, 2006.
2. Cheong, J., Yamada, Y., Yamashita, R., Irie, T., Kanai, A., Wakaguri, H., Nakai, K., Ito, T., Saito, I., Sugano, S., Suzuki, Y. Diverse DNA methylation statuses at alternative promoters of human genes in various tissues. DNA Research. *13*: 155-167, 2006.
3. Kondo, S., Takahashi, Y., Shinozawa, S., Ichise, H., Yoshida, N., Kanegae, Y., and Saito, I. Efficient sequential gene regulation via FLP - and Cre - recombinase using adenovirus vector in mammalian cells including mouse ES cells. Microbiol Immunol. *50*: 831-843, 2006.
4. Fukuda, H., Terashima, M., Koshikawa, M., Kanegae, Y., and Saito, I. Possible Mechanism of adenovirus generation from a viral genome tagged with nucleotides at its ends. Microbiol Immunol. *50*: 643-654, 2006.

Affiliated Facilities

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

Chief, Professor	Tadashi Yamamoto, Ph.D.	
Associate Professor	Shinobu Imajoh-Ohmi, D.Sci.	
Research Associate	Hiroshi Sagara, D.M.Sc.	

ラボラトリー長（教授・併任）		
	理学博士	山　本　　雅
助教授	理学博士	大　海　　忍
助　手	医学博士	相　良　　洋

In the fiscal year of 2006, the Institute of Medical Science, University of Tokyo (IMSUT) has established Medical Proteomics Laboratory by reorganizing core facilities of Fine Morphology, Laboratory Center for Proteomics Research, and Culture Media Section, and Division of Molecular Biology (2). The Laboratory provides high quality of support for the proteomics research in IMSUT, and carries out of itself modern proteomics research.

Mission

Proteins are integral component of the cells' structure and function, and thus are the major players of various biological phenomena. Proteins after produced in the cells are often modified by sugar, lipid, phosphate, and etc, and/or processed by cleavage, and/or form protein complexes. Proteins are also transported to specific sites within a cell. Disruption of the sophisticated processing, or defects of expression, or structural alteration of proteins is causative to various human diseases. Recent revolutionary progress of genome sciences contributed largely to our understanding on the nature of life and the cause of various diseases. Nevertheless, further studies at the level of proteins are no doubt required for dissecting the mechanisms of various processes of biological events as well as development of diseases. Proteomics researches by the use of highly developed techniques of imaging, mass spectrometry, and X-ray crystallography are key issues to clarify intracellular localization, movement, complex formation, intracellular signaling network, and structure of proteins. The mission of this laboratory is to polish up and provide highly qualified techniques and equipments for the electron microscopic and mass spectrometric analysis to the researchers of IMSUT who have been conducting biomedical studies on infection, cancer and other intractable disease. We will also provide support for designing peptides for the use of various biochemical and cell biological analysis that includes production of anti-peptide antibodies. This laboratory also copes with any consultation on protein chemistry as well as proteomics.

1. Research activities of Dr. Ohmi's group are listed elsewhere.

2. Comprehensive analysis of phosphotyrosine-dependent signaling network dynamics by quantitative proteomics

Masaaki Oyama, Hiroko Kozuka-Hata, Shinya Tasaki[1], Ayumu Saito[2], Masao Nagasaki[2], Seiya Imoto[2], Ryo Yoshida[2], Kentaro Semba[3], Sumio Sugano[1], Satoru Miyano[2] and Tadashi Yamamoto: [1]Laboratory of Functional Genomics, Department of Medical Genome Sciences, Graduate School of Frontier Sciences, The Uni-

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Signal transduction system is known to widely regulate complex biological events such as cell proliferation and differentiation. As phosphotyrosine-dependent signaling networks play a key role in transmitting the signal, comprehensive description of their dynamics surely makes a substantial contribution to understanding the regulatory mechanism that results in each biological effect. Here we have developed a highly sensitive and simple method for making a temporal quantitative analysis of phosphotyrosine-related proteins. Cells differentially-labeled with stable isotopes of arginine were stimulated with growth factors for different time periods, followed by affinity-purification of signaling molecules with anti-phosphotyrosine antibodies. After direct digestion of these proteins, shotgun protein identification was performed by nanoLC -MS/MS analysis. In order to perform automatic quantitation based on large volumes of LC-MS/MS proteome data, we also developed software called AYUMS. Using this software, we obtained activation profiles of the signaling molecules that worked in A431 cells upon EGF stimulation, providing detailed quantitative information with high time-resolution necessary for dynamic Bayesian network modeling in a high-throughput fashion. We also investigated signaling networks in NIH3T3 cells transfected with either of wild type or mutated EGFR to analyze the effect of the mutation on their signaling network dynamics. In order to perform in silico simulation based on our proteome data, we have constructed a simulation model of the EGFR pathway using Cell Illustrator (Tasaki et al., in press). Our simulation model provided knowledge about some possible regulation that had been previously inferred or confirmed experimentally.

3. Finding of novel short coding sequences from human mRNAs by mass spectrometry

Masaaki Oyama, Hiroko Kozuka-Hata, Kentaro Semba, Sumio Sugano[1], Tadashi Yamamoto

In parallel with the human genome projects, accumulation of human full-length cDNA data has also been intensively performed. Large-scale analysis of their 5′-UTRs revealed that about half of them had a short ORF upstream of the coding region. Experimental verification as to whether translation of such upstream ORFs occurs 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 proteomic 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 make a more comprehensive search for unpredicted short coding sequences from human mRNAs, we have constructed an automated two-dimensional nano-liquid chromatography (2DnanoLC) system coupled with a high-resolution hybrid tandem mass spectrometer. Separation of peptide mixtures through on-line multi-dimensional LC enables us to perform large-scale identification of highly complex biological samples, such as cell lysates. In this study, we have applied this technology for shotgun identification of the components in small protein-enriched fractions prepared from human cultured cells. For the samples prepared from human K562 and HEK293 cells, fully automated 2DLC separation of the tryptic peptides led to the generation of 11,310 and 11,127 MS/MS spectra, respectively. As a result of a database search against the RefSeq human protein database (as of July 3, 2006), 1,859 peptides were identified from the sample of human K562 cells and 2,337 peptides were identified from that of HEK293 cells. The identified peptides were ultimately assigned to 350 and 341 protein sequences, respectively. They included 134 and 162 proteins with a theoretical mass of less than 20 kDa, which resulted in 197 non-redundant short proteins. Additional 10 peptides were also identified from regions besides the annotated CDSs in three reading frames of RefSeq mRNAs (as of July 3, 2006).

4. The main activity of Dr. Sagara's group is to offer supports for the research projects using electron microscopes. The electron microscopic techniques available are the conventional thin section transmission electron microscopy, immuno-electron microscopy, negative staining techniques and scanning electron microscopy. By using these individual techniques or by combining some of these, we can obtain direct visual evidence that can not be acquired by other methods.

4-1. Thin section electron microscopy and immuno-electron microscopy

Thin section electron microscopy is the most widely used technique to observe the fine struc-

ture of the cells and tissues. 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 methods and then incubated with the antibody recognizing the epitope exposed on the surface of the sections. Then the localization of the antibodies are marked by incubating with the secondly antibody labeled with colloidal gold particles. This year, thin section electron microscopy combined with immuno-electron microscopy was used in many collaborative works.

4-1-a. Architecture of ribonucleoprotein complexes in influenza A virus particles

Noda T[1]., Sagara H., Yen A[1]., Takada A[1]., Kida H[1]., Cheng R.H[1]. and Kawaoka Y[1]: [1]Division of Virology, Department of Microbiology and Immunology

In this study, the structure of the influenza A virus particles budding from the host cells were examined in detail by using thin section electron microscopy. We revealed that the individual influenza viruses contained "eight" ribonucleoprotein complexes with different length. These data indicate that the eight kinds of ribonucleoprotein are selectively incorporated into the influenza A virus particles. These results were published in the journal, "Nature" (ref : Noda *et al*).

4-1-b. Assembly and Budding of Ebolavirus.

Noda T[1]., Ebihara H[1]., Muramoto Y[1]., Fujii K[1]., Takada A[1]., Sagara H., Kim JH[1]., Kida H[1]. and Kawaoka Y[1].

In this study, some proteins of Ebola virus were expressed individually or in combination and examined ultrastructurally. The matrix protein VP40 was found to be critical for transport of nucleocapsid (NC)s to the cell surface and for the incorporation of NCs into virions, where interaction between nucleoprotein and the matrix protein VP40 is likely essential for these processes. Examination of virus-infected cells revealed that virions containing NCs mainly emerge horizontally from the cell surface, whereas empty virions mainly bud vertically, suggesting that horizontal budding is the major mode of Ebolavirus budding. These data were published in the "PLoS Pathogens". (ref. Noda *et al*).

4-1-c. Some other collaborative research works using thin section electron microscopy and/or immuno-electron microscopy are now in progress with Dr. Kawaguchi and Dr. Nakagawa, both are in Department of Infectious Disease Control, International Research Center for Infectious Diseases, and with Dr. Honda, in Division of Stem Cell Engineering.

5. Negative staining techniques

Negative staining techniques provided by Dr. Sagara's group are simple and quick method to observe the morphology of the macro molecules. This year, the negative staining method is used in two collaborative works. One is with Dr. Morita-Ishihara *et al*. at Division of Structural Biology, Department of Basic Medical Sciences. In this study, negative staining methods were used to reveal the structural components of the invasive bacteria, *Shigella* (ref. Morita-Ishihara *et al*). The other is with Dr. Suhara *et al*. at Material Science laboratory, NTT Basic Research Laboratories. In this study, negative staining techniques combined with immuno-electron microscopy were used to prove that the inositol 1,4,5-trisphosphate receptor molecules are enriched in the membrane fraction obtained by transfection and following biochemical procedures (ref. Suhara *et al*.).

6. Scanning electron microscopy

Scanning electron microscopy in Dr. Sagara's group 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. Sumita *et al*, Division of Stem Cell Engineering, to observe the structure of the mineral scaffold to be used to regenerate the teeth (ref Sumita *et al*). Other works are also in progress with Dr. Iizumi, Division of Bacterial Infection, to observe the changes in the surface structure of the bacteria infected cells.

Publications

Tasaki, S., Nagasaki, M., Oyama, M., Hata, H., Ueno, K., Yoshida, R., Higuchi, T., Sugano, S., and Miyano, S. Modeling and Estimation of Dynamic EGFR Pathway by Data Assimilation Approach using Time Series Proteomic Data. Genome Inform., in press.

Takeda, J., Suzuki, Y., Nakao, M., Barrero, R.A., Koyanagi, K.O., Jin, L., Motono, C., Hata, H.,

Isogai, T., Nagai, K., Otsuki, T., Kuryshev, V., Shionyu, M., Yura, K., Go, M., Thierry-Mieg, J., Thierry-Mieg, D., Wiemann, S., Nomura, N., Sugano, S., Gojobori, T., and Imanishi, T. Large-scale identification and characterization of alternative splicing variants of human gene transcripts using 56,419 completely sequenced and manually annotated full-length cDNAs. Nucleic Acid Res. 34: 3917-3928, 2006.

Suhara, W., Kobayashi, M., Sagara, H., Hamada, K., Goto, T., Fujimoto I., Torimitsu K. and Mikoshiba, K. Visualization of inositol 1,4,5-trisphosphate receptor by atomic force microscopy. Neuroscience Letters 391: 102-107, 2006

Noda, T., Sagara, H., Yen, A., Takada, A., Kida, H., Cheng, R.H. and Kawaoka, Y Architecture of ribonucleoprotein complexes in influenza A virus particles. Nature 439: 490-492, 2006

Morita-Ishihara, T., Ogawa, M., Sagara, H., Yoshida, M., Katayama, E., and Sasakawa, C. *Shigella* SPA33 is an essential C-ring component of type III secretion machinery. Journal of Biological Chemistry 281: 599-607, 2006

Sumita Y., Honda MJ., Ohara T., Tsuchiya S., Sagara H., Kagami H. and Ueda M. Performance of collagen sponge as a 3-D scaffold for tooth-tissue engineering. Biomaterials. 27: 3238-48, 2006

Noda T., Ebihara H., Muramoto Y., Fujii K., Takada A., Sagara H., Kim JH., Kida H., Feldmann H. and Kawaoka Y. Assembly and budding of Ebolavirus. PLoS Pathogens. 2: e99, 2006

Honda MJ., Tsuchiya S., Sumita Y., Sagara H. and Ueda M. The sequential seeding of epithelial and mesenchymal cells for tissue-engineered tooth regeneration. Biomaterials. in press