*Affiliated Facilities*

# Laboratory Animal Research Center
## 実験動物研究施設


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*Morbilliviruses in the family Paramyxoviridae including measles virus, rinderpest virus and canine distemper virus are highly infectious among their natural hosts. We have succeeded in establishing a system of reverse genetics for these three morbilliviruses, using originally isolated strains. The studies on the functions of viral proteins in replication and species-specificities have been performed. In addition, more than 30,000 mice, mainly transgenic and gene-targeted ones, are always kept for researches of IMSUT and the technical staffs contribute to their maintenance and breeding.*


### 1. Regulation mechanism of foreign gene expression from recombinant canine distemper virus vector.


**Uema M., Fujita K., Miura R., Kai C.**


In mononegavirus genome, viral transcription is considered to be regulated by two major factors, the polar attenuation and the gene regulatory sequences at the viral gene junctions. In this study, we investigated the regulation mechanism of foreign gene expression in CDV using our recombinant CDVs expressing the firefly luciferase gene under control of 6 kinds of gene signals, rCDV-luc (signal), and with insertion of the luciferase gene at each gene junction, rCDV-luc (position). We constructed 11 recombinant CDV cDNA full genomic plasmids including 6 kinds of rCDV-luc (signal)s carrying the luciferase gene after the N gene and 5 kinds of rCDV-luc (position)s carrying the luciferase gene at 5 gene junctions N-P, P-M, M-F, F-H and H-L. In order to recover 11 recombinant CDVs, reverse genetics method was used. The expression levels of mRNA and activities of luciferase protein were measured using northern blot analysis and luciferase assay, respectively. The growth kinetics of all recombinant CDVs in B95a cells were also analyzed. We successfully recovered the 11 recombinant CDVs expressing the firefly luciferase gene and demonstrated that i) the expression level of a foreign gene was not affected by the putative gene regulatory sequences of CDV, ii) the expression level of a foreign gene could be controlled by its inserted position, iii) the insertion of a foreign gene had little effect on virus growth, and iv) all recovered viruses showed CPE typical of CDV infection in B95a cells as well as their parental CDV-Yanaka strain. It was demonstrated that the variable gene regulatory sequences of CDV had similar re-initiation capacity and that we could control the expression level of foreign gene using the different insertion sites in CDV cDNA vector in vitro. Our studies provided useful information on development of CDV as a gene transfer vector or live attenuated multivalent vaccines.

## 2. Measles Virus Causes the Host Shut-off by eIF2α Phosphorylation

**Yoshihisa Inoue, Kyoko Tukiyama-Kohara, and Chieko Kai**

Measles virus (MV) induces a shut-off of host protein synthesis in the infected cells, where the virus proteins are dominantly synthesized. The mechanism, however, is not well-known. In the case of other virus infections, it is reported that the shut-off phenomena occur at the level of translation. In all the case of infection of picornaviruses, influenza virus and vesicular stomatitis virus, the modification of translation initiation factors (eIFs) and inhibition of translation at the initiation step were induced. We therefore focused on the modification of eIFs concerning the cap-dependent translation for clarification of the mechanisms of MV-induced shut-off of host protein synthesis. B95a cells were infected with a field isolated MV HL strain and harvested at indicated times. The effects of MV infection on eIFs were analyzed by Western Blotting using antibodies against eIF4G, eIF4E, 4EBP-1 or eIF2 α. The phosphorylation states of eIF4E and eIF2 α were analyzed by the antibodies against phosphorylated eIF4E or eIF2α. We first characterized eIF4G, eIF4E and 4EBP-1 during the time course of MV infection. The eIF4G was not cleaved throughout the MV infection and phosphorylation states of 4EBP-1 and eIF4E were not influenced by MV infection until 36 h p.i. Thus, we analyzed the phosphorylation state of eIF2α in MV-infected B95a cells. The eIF2α was phosphorylated at 12 h p.i. and not dephosphorylated until the end of the observation. To analyze the possibility that the phosphorylation of eIF2α plays a crucial role in MV-induced host shut-off, an expression plasmid of eIF2α, of which the phosphorylation site was mutated, was produced and designated as eIF2α S51A. B 95a cells expressing eIF2α S51A (B95a-2αS51A) were established. In B95a-2αS51A cells, MV-induced host shut-off was delayed and weakened as compared with that in B95a cells. In MV -infected B95a cells, eIF2α was phosphorylated. Because the host shut-off was inhibited by the expression of eIF2α S51A, phosphorylation of eIF2α should play a crucial role in this phenomenon. Other factors, however, could also be involved in the host shut-off because complete inhibition of the shut-off effect was not induced by the mutant expression.

## 3. Involvement of heparan sulfate during the cell attachment process of morbillivirus infection.

**Y. Muto, K. Fujita, Miura, M. Yoneda, K. Kohara-Tsukiyama, C. Kai.**

Morbilliviruses (measles virus: MV, canine distemper virus: CDV, and rinderpest virus) are known to use SLAM as a cellular receptor. However, as the viruses can also infect SLAM-negative cells, it is believed that some other molecules must contribute to the attachment of virus to such cells. In this study, we investigated whether glycosaminoglycan (GAG) is involved in the viral attachment and infection. Recombinant CDV and MV expressing enhanced green fluorescence protein (EGFP) were prepared using our previously established reverse genetics system with a field isolate strain. B95a cells or 293 cells were incubated with chondroitins or soluble heparin and then were inoculated with the viruses. The infectivity was determined by the number of EGFP positive cells as analyzed using flow cytometry. The cells were also pretreated with GAGs lyases. Heparan sulfate-deficient CHO cell lines (pgsA-745 and pgsD-677) and CHO-K1 were also used for the analysis of viruses' infectivities. In addition, purified CDV-EGFP was incubated with heparin-or BSA-agarose beads and the bound virus was analyzed by Western blot using an anti-CDV N MAb. Pre-treatments of B95a cells with the GAGs induced no significant inhibition of the infectivity CDV-EGFP and MV-EGFP for any of the chondroitins. In contrast, treatment of 293 cells with soluble heparin produced a significant inhibition of the viral infectivity in a dose dependent manner. Pre-treatment of 293 cells with heparitinase remarkably decreased the infectivity. The infectivities of the viruses to pgsA-745 and pgsD-677 cells were lower than those to the parental CHO-K1 cells. In the binding assay with heparin-agarose or BSA-agarose, CDV-EGFP was detected only after the incubation with heparin-agarose. These results strongly indicate that heparan sulfate is implicated in the attachment process of morbillivirus infection to the cells. The fact that B95a cells highly express SLAM on the surface and the viruses use it as a receptor with a strong affinity for them, may be the reason why the pre-treatment with heparan sulfate did not affect the viruses' infectivity to them but did affect their infectivity to 293 cells. Thus, it is suggested that heparan sulfate may play an important role in the virus binding to SLAM negative cells.

## 4. Characterization of Canine Distemper Virus Propagation in Dog Hippocampal Slice Culture.

**T. Kooriyama, R. Miura, K. Fujita, H. Sagara, H. Sato, K. Kohara-Tsukiyama and C. Kai**

Canine distemper virus (CDV) infection of dogs causes an acute systemic disease and often induces central nervous system (CNS) symptoms. The reverse genetics system of CDV was established in our laboratory in 1999 and recombinant CDV expressing enhanced green fluorescence protein (EGFP-CDV) was also rescued. In this study, the author analyzed the mechanism of CDV propagation and spread in dog hippocampal slices in place of whole brain culture by using EGFP-CDV. Hippocampal slices were prepared from 6-month-old dog and inoculation was taken place by dropping EGFP-CDV. EGFP fluorescence was analyzed for over 6 months with a confocal microscope. Immunohistochemistry were performed to detect the EGFP positive cells with GFAP, CNP and MAP2 cell markers and also taken place with anti CDV envelope M, F and H protein specific antibodies to analyze their distributions at EGFP-CDV infected cells. Electron microscopic analysis was also performed to analyze the transmission to next cells at synapse. The CDV infection and propagation mainly occurred in cornus ammonis (CA) regions of hippocampus. The EGFP-CDV is favorable for neuronal infection than astrocytes or oligodendrocytes and the expression of EGFP was observed for more than 6 months without cytotoxicity and syncytium formation of neurons. The EGFP-CDV was observed to transport from a neuron to the other through synapses, and a part of synapses of infected neuron mediated the transmission. The viral envelop proteins F and H were distributed in the whole neurons but M protein is mainly located at soma and partially at dendrites and synapses. Electron microscopic analysis demonstrated that ribonucleocapsid protein (RNP) is sorted to synaptic terminals and viral particles were visualized at extrasynaptic regions. Confocal microscopic analysis with EGFP-CDV showed that CDV infection and propagation mainly occurred in neurons at cornus ammonis and transmission from a neuron to the neighbors via synapses. Immunohistochemical analysis showed that viral envelope proteins are well sorted to dendrites and synapses, and RNPs were also accumulated at synapses. The selective transmission may be related with localization of M protein in neural cells.

## 5. Significance of gene order of P and M genes in canine distemper virus.

**Hiroki Sato, Takanori Kooriyama, Kentaro Fujita, Ryuichi Miura, Misako Yoneda, Kyoko Tsukiyama-Kohara & Chieko Kai**

The gene order of the *Mononegavirales* genome is highly conserved. The genes that are located closest to the 3́ promoter are transcribed most abundantly, and those located more distantly from the promoter are transcribed in successively smaller amounts (polar attenuation). The resulting molar ratio of expressed viral proteins has been thought to be important for efficient virus replication. To investigate the importance of the gene order in virus replication, we produced the gene-order-rearranged recombinant canine distemper virus (CDV) by a reverse genetics system using the CDV-Yanaka strain. We replaced the order of P and M genes in a full-length cDNA clone of the CDV genome and succeeded in rescuing the clone, which is designated as rCDV-MP. In rCDV-MP-infected cells, the expression level of P protein was lower than that of M protein, as predicted by the polar attenuation model. Interestingly, the yield of infective rCDV-MP was significantly low, whereas M protein and F protein showed co-localization in rCDV-MP-induced syncytia. Sucrose-gradient ultracentrifugation revealed that rCDV-MP-infected cells released particles that may not have infectivity. These results suggested that the gene order of the CDV virus genome is significant for the appropriate transcriptional rate of each viral gene, which is required for efficient replication of CDV, and that an excessive amount of M protein may hamper the efficient construction of infectious virus particles.

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*Affiliated Facilities*

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


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| Associate Professor | Shosaku Hattori, D.V.M., Ph.D. | 助教授　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, Yuko Katakai, Hiroshi Kihara[1], Motonori Ohno, Naoko Ueda[2], Shigenari Terada[3], Hiro Yonezawa4, Yoshihiro Hayashi[5], Michihisa Toriba[6], i, and Tomohisa Ogawa[7]: [1]Bioscience research Institute, Takara Shuzo Co., Ltd., [2]Departmen of Applied Microbiology, Kumamoto Institute of Technology, [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, *Trimeresurus 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 A2 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 A2 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. Research on endocrine of the Habu

**Takeshi Kuraishi, Misako Yoneda[8], Shosaku Hattori, and Chieko Kai[8]: [8]Laboratory Animal Research center**

We determined the cDNA sequence encoding the prolactin (PRL) gene of Habu. The full-length cDNA of Habu PRL consists of 989 bp. The open reading frame encodes a protein of 229 amino acids, which consists of a putative signal peptide of 30 residues and a mature protein of 199 amino acids. The amino acid sequence of Habu PRL deduced from its nucleotide sequence showed higher homologies with those PRL sequences of tetrapod animals than with those of teleosts. Northern blot analysis revealed the Habu PRL mRNA size to be approximately 1.1 kb. The expression of PRL gene in different tissues (brain, pituitary, venom, heart, lung, liver, pancreas, spleen, kidney, testis, ovary, muscle) was determined using northern blot analysis. The high levels of PRL gene expression was only detected in pituitary. PRL is known to have a wide spectrum of functions. In submammalian vertebrates, PRL acts as an osmo -regulatory hormone. The initial step in the action of PRL, like all other hormones, is the binding to a specific membrane receptor, the PRL receptor (PRL-R). In reptiles, presence of PRL-R from kidney membranes of the turtle and snake were reported. The presence of PRL-R in the snake kidney suggests a putative osmoregulatory role for PRL in this reptile. It is thought that Habu PRL is also involved in water and electrolyte balance.

## 3. Reproduction of squirrel monkeys.

**Shosaku Hattori, Takeshi Kuraishi, Yuko Katakai[9], Mamoru Ito[10], and Chieko Kai[8]: [9]Corporation for production and research of laboratory primates, [10]Laboratory of Immunology, Central Institute of Experimental Animals**

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. Experimental infection with equine herpesvirus 9 (EHV-9) in common marmosets *(Callithrix jacchus)*

**Atsushi Kodama[11], Tokuma Yanai[11], Kayoko Yomemaru[11], Hiroki Sakai[11], Toshiaki Masegi[11], Souichi Yamada[11], Hideto Fukushi[11], Takeshi Kuraishi, Shosaku Hattori and Chieko Kai[8]: [11]Department of Veterinary Pathology and Microbiology, Gifu University**

To assess the infectivity of equine herpesvirus 9 (EHV-9), four common marmosets were inoculated with a new neurotropic equine herpesvirus, $10^6$ plaque-forming units EHV-9, by the nasal route. All of the inoculated animal exhibited various neurological signs and finally collapsed. Histologically, the affected animals had severe encephalitis characterized by neuronal degeneration and necrosis with intra-nuclear inclusions,

which extended from the olfactory bulb to the rhinencephalon and piriform lobe. Immunohistochemistry to EHV-9 antigen revealed intense positive reaction in degenerating neuronal cells. The nasal cavity had severe necrotizing rhinitis with prominent intra-nuclear inclusion bodies in the olfactory mucosa. These findings indicate that non-human primates as represented by the marmoset might be susceptible to EHV-9, and concerns regarding a possible outbreak are legitimate.

### 5. 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.

In this laboratory, a sex determination method using DNA extracted from feces has been developed for Amami rabbit in order to apply fecal DNA analysis to field study of this rabbit. We determined a partial sequence of the ZFX and SRY gene of Amami rabbit. The ZFX gene exists on the X chromosome in both females and males, while the SRY gene exists only on the Y chromosome in males. Based on a partial sequence of the ZFX or SRY gene of Amami rabbit, we designed primers which could amplify DNA fragments of the ZFX or SRY gene. PCR products were detected in 5 out of 7 fecal samples collected from carcass of Amami rabbit and the sexes estimated by the present method were perfectly matched with the actual sexes. Further research is needed to verify from Amami rabbit feces collected in the field. By applying this method in combination with individual identification using fecal DNA analysis to the ecological study of Amami rabbit, it is hoped that we can obtain more information (genetic structure of population, home range, population density) which is necessary for planning appropriate wildlife management in Amami-oshima Island.

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*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 be chosen 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/*loxP* system (Kanegae *et al*., Nucleic Acids Res. 23: 3816-3821, 1995; Kanegae *et al*., Gene 181: 207-212, 1996) as well as FLP/FRT system (Nakano *et al*., Nucleic Acids Res. 29: e40, 2001). 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 2005 were shown below.

## 1. Preparation of cosmid aiming efficient cancer-specific expression vector.


**Hiromitsu Fukuda, Hiyori Haraguchi[1], Saki Kondo , Yuzuka Takahashi, Yumi Kanegae and Izumu Saito: [1]School of Science, Kitasato University**


Cancer-specific promoter that are active only cancer cells, such as the α-fetoprotein (AFP) and the carcinoembryonic antigen (CEA) etc., are possibly be valuable for specific therapy of cancer. However, such promoters are generally not strong enough to fulfill effective cancer therapy. We previously developed a "double infection method" that allows highly-enhanced expression with maintaining promoter specificity (Sato et al., BBRC, 244: 455-462, 1998). Briefly, we constructed a "regulator rAd" that expresses Cre recombinase under the control of the AFP promoter. We also prepared a "target rAd" that expresses a therapeutic protein under the control

of a very potent CAG promoter, while the CAG promoter is arranged so as to switch on by Cre recombinase. When both the rAds are infected into the same cancer cells, Cre is expressed and switches on the CAG promoter in the target virus. Because the CAG promoter is about 500 times more powerful than the AFP promoter, very strong expression was observed specifically in hepatocellular carcinoma-derived cells.

Although the double infection method gave about 50-fold more expression of target gene while maintaining its specificity, the method has a drawback to be improved. Because the regulator unit and the target unit are located on the different viral genome, the designed expression occurs only when both the viruses are infected into the same cell. Consequently, the expression sharply decreased when the concentration of the two viruses becomes lower in the target tissue. To overcome the problem, we are trying to construct an rAd that containing both the regulator unit and the target unit in the one single virus genome (a double-unit rAd).

To construct rAd containing both the units in one genome, we must use a helper-dependent (HD) vector (see later) that has capacity enough to do so. Another problem to be overcome is that, when Cre gene and a pair of loxP sequences are cloned in the same cosmid, Cre expression was unexpectedly observed and Cre-mediated deletion between a pair of loxPs was significantly observed, even during the propagation of cosmid in E. coli. We solved the problem by designing the cosmid so that the cosmid lacking the sequence between the two loxPs cannot be packaged because the size of such a deleted cosmid is less than that of the lower limit of the packaging. Such a deletion is expected not only in the E. coli growth but also in 293 cells during the HD-virus growth. So we constructed several Cre mutant that work as dominant-negatives and suppress Cre function. We identified such Cre mutants and are trying their application in the system described above.

## 2. Characterization of the recombination mediated by FLP-expressing rAds in mammalian cells

**Saki Kondo, Yuki Takata[2], Yuzuka Takahashi, Yumi Kanegae and Izumu Saito: [2]School of Pharmaceutical Sciences, Kitasato University**

Although the site-specific recombinase FLP can be applied for gene regulation in mammalian cells, the recombination efficiency of FLP is much lower than that of Cre even at 30℃, which is optimum temperature of FLP. If FLP variants showing higher activity could be obtained, the application of FLP would become much more popular. Until last year we compared the recombination activity of hFLPe with that of wild-type (wt)-FLP using adenovirus expression vector in mammalian cells. The hFLPe-expressing rAd, AxCAhFLPe, gave much higher level of expressed hFLPe proteins in mammalian cells than the wtFLP-expressing rAd, AxCAFLP. Unexpectedly, however, the recombination efficiency using AxCAhFLPe was no higher than that using AxCAFLP. In addition, we observed cell toxicity when using AxCAhFLPe, that was never observed using AxCAFLP even at a very high dose. To examine the cell toxicity caused by hFLPe, we newly constructed "hFLP" gene, of which the codon usage was "humanized" but the amino-acid sequences were identical to wtFLP, and compared the recombination activities among wtFLP, hFLPe, and FLPe by transfecting each FLP-expressing plasmid to mammalian cells. The western blot analysis showed that hFLP-expressing plasmid gave higher level of FLP protein than FLP-expressing plasmid in mammalian cells. However, the recombination efficiency of hFLP was again no higher than that of FLP. The results suggested that the cell toxicity may not be due to alternation of protein structure by the four amino-acid substitution that stabilize FLP.

To examine further the recombination efficiency and cell toxicity of these FLP variants in mammalian cells, we newly constructed hFLP- and FLPe-expressing rAds. Hovever, we found that it was difficult to obtain the hFLP-expressing rAd because it is rather unstable. In mammalian cells, the FLPe-expressing Ad yielded a few times more FLP than that of wtFLP but it was a few times less than that of hFLPe. However, in spite of the differences of steady-state expression levels, the three rAds gave almost similar recombination efficiency in mammalian cells. These results suggested that obtaining a high level expression of FLP is not a simple work. We are now comparing the three FLPs from the various points of views to determine which one is the most convenient and practical for general use.

## 3. Characterization of 293 cell lines expressing site-specific recombinases

**Yumi Kanegae, Yuzuka Takahashi, Saki Kondo, Hiromitsu Fukuda, Michiko Koshikawa and Izumu Saito**

The HD adenovirus vector, also called gutted vector, has recently been developed. The vector lacks almost all the viral genome DNA of 36 kb, only containing small DNA regions at the both

ends of the genome, 0.4 kb at the left end and 0.2kb at the right end. All the viral proteins supporting production of functional HD virus is supplied *in trans* by co-infected helper adenovirus. The helper virus cannot be packaged into the viral empty shell because the genome of helper virus loses its packaging signal sequence that is flanked with a pair of recombinase target sequences. After entry of the viral genome into the recombinase-expressing 293 cells, its packaging signal is excised out from the genome. Therefore helper virus genome cannot be packaged and only HD virus genome can be. At the each passage and expansion step of the HD vector, helper virus is co-infected and the passages are carried out in recombinase-expressing cell lines. The characters of the cell lines are an important factor for efficiency of HD vector production.

We so far established numbers of recombinase -expressing 293 cell lines and identified three cell lines, each of which show the highest recombination efficiency containing each recombinase-expressing gene in its chromosomes. The recombinase genes introduced were wtFLP (cell line F13), hFLPe (H24) and NLS-tagged Cre (N11). The results showed that the FLP- and hFLPe-expressing cell lines showed very high excision efficiency about 100% when using the analysis of the rAds containing a pair of recombinase targets (FRTs), while the correspondent experiments using Cre-expressing cell lines gave 80% of efficiency at best . Therefore, FLP-expressing cell lines appeared better than that expressing Cre. Then we compared efficiency of propagation of an HD vector in cell lines expressing wtFLP and hFLPe. The result showed that hFLPe cell line gave higher efficiency of producing HD virus than wtFLP cell line. Detailed comparison of these two cell lines and improvement of HD vector production using hFLPe cell line are under way.

## Publications


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3. Kido, T., Arata, S., Suzuki, R., Hosono, T., Nakanishi, Y., Miyazaki, J., Saito, I., Kuroki, T., and Shioda, S. The testicular fatty acid binding protein PERF15 regulates the fate of germ cells in PERF15 transgenic mice. Dev Growth Differ, 47: 15-24, 2005.
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*Affiliated Facilities*

# Laboratory of Fine Morpholology
# コアラボラトリー微細形態室

Research Associate Hiroshi Sagara, Ph.D.

助 手 医学博士 相 良 洋

*This laboratory was newly established by separating from the Division of Structural Biology. The main activity of this laboratory 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 have been expected by biochemical experiments.*

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

**Hiroshi Sagara**

Thin section electron microscopy is the most widely used technique to observe the fine structure 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 the immuno-electron microscopic techniques, thin sections are obtained by similar methods, and them incubated with the antibody recognizing the epitope revealed 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 were used in many collaborative works.

### a. Escape of intracellular *Shigella* from autophagy

**Ogawa, M[1]., Yoshimori, T[1]., Suzuki, T[1]., Sagara, H., Mizushima, N[1]., and Sasakawa, C[1].: [1]Division of Bacterial Infection, Department of Microbiology and Immunology**

By using thin section electron microscopy combined with immuno-electron microscopy, we clarified that *Shigella*, an invasive bacteria, are trapped into the auto-phagosome, and analyzed the mechanism they escape from it. These results were published in the journal, "Science" (ref: Ogawa *et al*).

### b. Architecture of ribonucleoprotein complexes in influenza A virus particles

**Noda, T[2]., Sagara, H., Yen, A[2]., Takada, A[2]., Kida, H[2]., Cheng, R.H[2]. and Kawaoka, Y[2]: [2]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 are now in press in the journal, "Nature" (ref: Noda *et al*).

### c. Nucleocapsid-like structures of Ebola virus reconstructed using electron tomography.

**Noda, T[2]., Aoyama, K[2]., Sagara, H., Kida, H[2]. and Kawaoka, Y[2].**

In this study, we used the newly developed method, electron tomography. Using electron tomography, we can obtain high resolution three-dimensional structure by reconstructing a tilt series of images. Nucleocapsid-like tubules were observed in the cytoplasm of Evola virus infected cells. The structure of these tubules were examined by using electron tomography methods and revealed the left-handed helices within these tubules. This showed the structural similarity between Ebola virus and other non-segmented negative-stranded RNA viruses, which suggests the common mechanism in the replicating cycles. These data were published in the journal of Veterinary Medical Science (ref Noda *et al*).

Some other collaborative research works using thin section electron microscopy and / or immuno-electron microscopy are now proceeding.

## 2. Negative staining techniques

**Hiroshi Sagara**

Negative staining techniques are simple and quick method to observe the morphology of the macro molecules. This year, the negative staining method are used in two collaborative works.

### a. *Shigella* SPA33 is an essential C-ring component of type III secretion machinery.

**Morita-Ishihara, T[1]., Ogawa, M[1]., Sagara, H., Yoshida, M[1]., Katayama, E[3]., and Sasakawa, C[1].: [3]Division of Structural Biology, Department of Basic Medical Sciences**

In this study, negative staining methods were used to reveal the structure of the type III secretion machinery and C-ring of the invasive bacteria, *Shigella*, and showed that the SPA33, a bacterial protein, is the structural component of the C-ring and essential for the formation of Type III secretion machinery. This work will be published in the "Journal of Biological Chemistry". (Morita-Ishihara *et al*)

### b. Visualization of inositol 1,4,5-trisphosphate receptor by atomic force microscopy.

**Suhara, W[4]., Kobayashi, M[5]., Sagara, H., Hamada, K[6]., Goto, T[5]., Fujimoto I[4]., Torimitsu K[4]. and Mikoshiba, K[7].: [4]Division of Neural Signal Information, NTT-IMSUT, [5]Material Science laboratory, NTT Basic Research Laboratories, [6]Calcium Oscillation Project, ICORP, JST, [7]Division of Developmental Neurobiology**

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. After that, the structure of the inositol 1,4,5-trisphosphate receptor molecules embedded in the membrane and in the aqueous solution were examined by atomic force microscopy. This work was published in the Neuroscience Letters (ref Suhara *et al*)

## 3. Scanning electron microscopy

**Hiroshi Sagara**

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 are now in progress with Dr. Iizumi, Division of Bacterial Infection, to observe the , changes in the surface structure of the bacteria infected cells. Other work with Dr. Utida, Division of Stem Cell Engineering, to observe the structure of the mineral scaffold to be used to regenerate the bone.

## Publications

Ogawa, M., Yoshimori, T., Suzuki, T., Sagara, H., Mizushima, N., and Sasakawa, C. Escape of intracellular *Shigella* from autophagy. Science 307: 727-31, 2005.

Noda, T., Aoyama, K., Sagara, H., Kida, H. and Kawaoka, Y. Nucleocapsid-like structures of Ebola virus reconstructed using electron tomography. Journal of Veterinary Medical Science 67: 325-8, 2005

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 (in press)

Morita-Ishihara, T., Ogawa, M., Sagara, H., Yoshida, M., Katayama, E., and Sasakawa, C. *Shigella* SPA33 is an essential C-ring compo-

nent of type III secretion machinery. Journal of Biological Science (in press)
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 (in press)