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INFORMATION

Hokkaido University confered the degree of Bachelor of Veterinary Medicine to the following 36 graduates of the School of Veterinary Medicine on March 23, 2007.

The authors summaries of their theses are as follows :

Expression of hepatocyte nuclear factor 4 alpha (Hnf 4α) associates with nephrogenesis in mouse kidney

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Hepatocyte nuclear factor 4 alpha (Hnf 4α) is expressed in several embryonic tissues including liver and york sack, in which Hnf 4α contributes to their developments. Since homozygous mutation of Hnf 4α gives rise to the result of embryonic lethality before initiation of kidney development, the function of Hnf4α during renal organogenesis is almost unknown. In this study, expression and localization of Hnf4α in the kidney of C57BL/6 mouse embryos were analyzed by immunohistochemical and molecular biological techniques. In addition, RNA interference strategy was applied to investigate the influence of Hnf 4α repression for nephrogenesis using the metanephros organ culture system.

Expression of Hnf 4α mRNA became detectable in the kidney on 12.5 embryonic day, the first period of nephrogenesis in both intact and cultured organs. Immunoreactions for Hnf 4α were detected in part of comma and/or S-shaped bodies in the foetal kidney, as well as in epithelial cells of proximal tubules in the adult kidney. Repression of Hnf4α resulted in massive apoptosis in the condensed mesenchyme around ureteric buds.

These results suggest that Hnf4α showing active expression during kidney development is an important role for the survival of the condensed mesenchyme belonging to the early nephrogenesis. As a reason appearing apoptosis in the condensed mesenchyme, the following two possibilities were considered. They were included ; 1) Hnf4α expressed weakly in the condensed mesenchyme is directly associated with the regulation for their differentiation/proliferation surrounding the ureteric buds prior mesenchyme-epithelial transformation. 2) Hnf4α produced from epitherialized Comma and/or S-shaped bodies maintains indirectly the survival of the condensed mesenchyme.

Identification of proinsulin C-peptide binding protein on the surface of HL-60 human monocytoid cells

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A study on brown adipose tissue in rabbit (*Oryctolagus cuniculus*) and pika (*Ochotona dauurica*)

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Nonshivering thermogenesis is a major component of facultative thermogenesis in small rodents or hibernators. They have a special thermogenic organ, known as brown adipose tissue (BAT), which is characterized by expressing uncoupling protein 1 (UCP1) as a heat producing protein. Defferent from these animals, adult rabbits do not have BAT in macroscopic examination, while the other lagomorph, pikas, living in a cold environment through their lifetime have BAT. In the present study, I examined UCP1 expression and its function in neonatal and mature rabbits, and further characterized molecular structure of pika UCP1, to elucidate the role of UCP1 in the regulatory thermogenesis in lagomorphs.

Macroscopic examination revealed that neonatal rabbits have BAT around the scapular regions, and two masses of BAT were distinguishable by their position. One (osBAT) was located over the scapula, the other (isBAT) was present in interscapular region. Interestingly, isBAT expressed more amounts of UCP1 protein than osBAT. Similarly, adipocytes isolated from isBAT consumed more oxygen, an indicator of heat generation, in response to sympathetic agonists than those from osBAT. In contrast, adipocytes isolated from adult adipose tissue present in the same region of osBAT and isBAT did not enhance the oxygen consumption even at supraphysiological dose of the agonists. These results suggest that rabbit BAT functions as a heat generator for the neonatal period, but decreases its function and expression of UCP1 during growth, possibly due to BAT atrophy and replacement by white adipose tissue.

Next, I have cloned pika UCP1 cDNA containing the full-length of coding sequence (GenBank accession number: AB283043). The deduced amino acid sequence indicates that pika UCP1 is most closely related to rabbit UCP1 and conserved all characteristic domains being documented to be important in UCP1 function. UCP1 was exclusively expressed in BAT of pika. When pikas were exposed to cold, BAT tended to become smaller with small lipid droplets and more UCP1.

These findings implicate that there are

some differences in postnatal development of BAT between rabbits and pikas, but if present, BAT functions as a heat-generating organ through the activity of UCP1 in both species.

NPY induces site specific STAT3 phosphorylation through MAP kinase pathway

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Neuropeptide Y (NPY) is one of the most abundant peptides in both central and peripheral nervous systems. Central NPY may be involved in the regulations of food intake, anxiety, seizures, circadian rhythm, memory, and pain. Peripheral NPY is stored and released along with norepinephrine in sympathetic nerve ending to induce a long-lasting vasoconstriction and increase vascular permeability and re-vascularization of ischemic tissues.

NPY mediates its effects through five subtypes of the receptor, all of which are coupled with pertussis toxin-sensitive G protein to inhibit adenylate cyclase, mobilize Ca^{2+} from intracellular stores and activate extracellular signal-regulated kinase (ERK) and other effectors.

In the present study, I examined the effects of NPY on phosphorylation and function of signal transducer and activator of transcription 3 (STAT3) in mouse lung microvascular endothelial (LE Ⅱ) cells. LE Ⅱ cells treated with NPY induced ERK phosphorylation in concetration-and time-dependent manners. The cells treated with NPY also increased STAT3 phosphorylation at serine 727, but not tyrosine 705, the latter being constitutively phosphorylated. The cells pre-treated with PD98059, an ERK inhibitor, abrogated the NPY-induced phosphorylation of ERK and STAT3 at serine 727, while it did not affect phosphorylation of STAT3 at tyrosine 705. Furthermore, the cell treated with NPY increased the expression of *egr 1* and *suppressor of cytokine signaling (SOCS3)* genes, target genes of STAT3. The present results suggest that NPY phosphorylates STAT3 at serine 727 via ERK dependent pathway and probably increases the gene expressions by modulating STAT3 functions.

Characterization of voltage-dependent channels in cells of porcine vomeronasal epithelium

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I examined the property of voltage-dependent channels in the cells of porcine vomeronasal sensory epithelium of sliced vomeronasal organ with a whole-cell patch-clamp technique.

Vomeronasal tissues were immunostained

with protein gene product9.5(PGP9.5) as a neural cell marker. PGP9.5-positive cell bodies were mainly found in the side of the basal membrane, and their dendrites extended to the apical side of the vomeronasal organ. There are many PGP9.5-negative cells in the apical and middle regions of the vomeronasal sensory epithelium.

Using K^+ electrodes, depolarizing pulses from a holding potential of -90mV produced transient inward currents followed by outward currents in the cells of apical and basal sides of sensory epithelium. Inward and outward currets in the cells at the basal side, vomeronasal neurons, were larger in amplitude than those at the apical side. Using Cs^+ electrodes, outward currents disappeared.

Tetrodotoxin (TTX) dose-dependently inhibited the inward currents of the vomeronasal neurons. The inactivation curves of inward currents were obtained from cells at the apical and basal sides. The membrane potentials producing half inactivation were -71 ± 2 mV for the vomeronasal neurons at the basal side and -97 ± 2 mV for the cells at the apical side. Similar results were obtained with Cs^+ electrodes.

Voltage-dependent K^+ channel currents began to be activated by depolarizing pulses to -40mV in cells at both sides. The outward K^+ currents were increased linearly with increasing depolarization in the vomeronasal neurons but showed outward rectification in cells at the apical side. Tetraethylammonium (TEA) and 4-aminopyridine dose-dependently inhibited the K^+ currents in both types of cells.

These results suggest that there is a different type of cells in characteristics of voltage-dependent Na^+ channels from neurons in porcine vomeronasal sensory epithelium and that these cells are supporting cells having glial chracteriristics and lower voltage-dependent channel density than vomeronasal neurons.

Development of influenza vaccine prepared from H7 virus isolates from ducks

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Outbreaks of highly pathogenic avian influenza (HPAI) caused by H5N1 viruses have spread worldwide. H7 influenza viruses also gave a rise to outbreaks of HPAI in European countries, Pakistan, and North Korea, bringing about serious economic losses. In the present study, influenza vaccine was prepared from H7 virus strains isolated from migratory ducks and evaluated for its potency.

During 2001-2004, 41 H7 influenza viruses (2 H7N1 and 39 H7N7 strains) were isolated from fecal samples of feral water birds in Hokkaido and Mongolia, and their hemagglutinins (HAs) were analyzed antigenically and genetically. Phylogenetic analysis of the H7 HA genes revealed that all isolates were classified into Eurasian lineage, and the deduced amino acid sequence at the cleavage sites of the HAs represented apathogenic profile. Antigenic analysis of H7 viruses with monoclonal antibodies to the H7 HA and chicken antiserum to H7 strains revealed that the HAs of these isolates antigenically closely related with those of H7 HPAI viruses, A/

chicken/Netherlands/2586/03 (H7N7), A/chicken/Pakistan/447/95 (H7N3) and A/turkey/England/63 (H7N3), and less close relationship with A/turkey/Italy/4580/99 (H7N1) and A/duck/Taiwan/Ya103/93 (H7N7).

Vaccine was prepared as follows; genetic reassortant virus, A/duck/Hokkaido/Vac-2/04 (H7N7) generated between A/duck/Mongolia/736/02 (H7N7) and A/duck/Hokkaido/49/98 (H9N2) to confer property of high growth in chicken embryo, was inactivated and adjuvanted with water-in-oil emulsion. It was demonstrated that the present vaccine conferred immunity for chickens to protect manifestation of disease signs against H7 HPAI virus challenge from eight days post vaccination.

The present results indicate that isolates from water birds are useful for vaccine against H7 influenza virus infection.

Detection of antibodies to leptospires in pigs in Japan

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Leptospirosis is a zoonotic infection caused by *Leptospira interrogans*. It is known that, in sows, the infection with leptospires causes reproductive disorders including abortion. However, little is known on the epidemiology of swine leptospirosis in Japan.

Therefore, in the present study, a serological surveillance was conducted to investigate the prevalence of leptospires in pig populations in Japan. Serum samples were collected from 938 pigs of 24 farms in Hokkaido, Kagoshima, and Okinawa prefectures. Enzyme-linked immunosorbent assay (ELISA) was used for the detection of antibodies to LipL32 antigen which is common in leptospires of *L. interrogans*. Samples positive in ELISA were investigated by microscopic agglutination test to identify causal leptospires. Antibodies specific to leptospires of serovars Copenhageni, Bratislava, Australis, and Javanica were detected in serum samples from each of the three districts. The present study revealed that leptospires of multiple serovars prevail in pig population in Japan.

Experimental infection in miniature pigs with leptospires was demonstrated to simulate leptospiral infection in pigs. Leptospires strain UP-MMC of serovar Manilae were inoculated to four miniature pigs intraperitoneally or intranasally. It was found that pigs were susceptible to leptospiral infection by both injection routes. The pigs did not show any clinical signs or pathologic changes in the tissues of their kidneys and livers. Leptospiral genes were detected in tissues and blood of pigs inoculated with leptospires. In addition, after inoculation of dexamethazone, those were detected in their urine. The present results indicate that leptospires invade to hosts via mucosa and that leptospires chronically infect and grow during the impairment of host's immunity in their kidney. For the control of leptospirosis in pig, the continual surveillance of that is needed.

Molecular basis of the pathogenicity of classical swine fever virus —Mechanism of the suppression of innate immunity by viral infection—

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In the cells infected with classical swine fever virus (CSFV), the viral nonstructural protein N^{pro} blocks type-1 interferon (IFN) secretion by downregulation of the expression of IFN regulatory factor (IRF)-3. It may be concerned in the pathogenicity of CSFV. In the present study, the author aims to clarify the mechanism of the suppression of host innate immunity.

Swine IRF-7 and IFN promoter stimulater-1 genes were cloned. In the present study, using antibodies to recombinant IFN-β, IRF-7 and IPS-1 and anti-swine IRF-3 mAb 34/1 (Igarashi, 2004), the expressions and the localizations of the cellular proteins in the cells infected with CSFVs were analyzed. IFN-β was not detected in the cells during infection of the virulent CSFV strain. In the cells, IRF-3 was degraded at the proteasome and the expression of IRF-7 was decreased. On the other hand, in the cells infected with avirulent CSFV strain, IFN-β secretion at 24 hpi, the nuclear localization of IRF-3 at 48hpi and the enhanced expression of IRF-7 with time were observed. The present results indicate that IFN signaling pathway was activated in the cells infected with avirulent strain. For the analysis of the effects of viral proteins against RNA interference (RNAi), viral N^{pro} and E^{rns} were co-expressed with GFP in the leaves of *Nicotiana benthamiana*. The fluorescence of GFP in the expression sites of the E^{rns} of both strains were enhanced because cellular RNAi was downregulated by E^{rns}.

In conclusion, the significant involvements of N^{pro} as an IFN suppressor and E^{rns} as an RNAi suppressor in the suppression of innate immunity were suggested.

Characterization of histamine binding proteins from *Ixodes* persulcatus and their assessment as candidates for anti-tick vaccine

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Tick feeding activities and transmission of pathogens cause great losses not only in the livestock industry, but also in public health. Particularly, *Ixodes* ticks are known as vectors of *Borrelia burgdorferi* sensu lato which cause Lyme borreliosis. Since ticks play important roles as vectors of various pathogens, suppression of their population is the most effective way to control the diseases which they transmit. At present, ticks can be effectively controlled by the use of acaricides, which have many disadvantages. Hence, it is necessary to

develop alternative tick-control methods such as an immunological way, which is currently considered as a major sustainable and practical method.

Previously, it was reported that immunosuppressive factor from *Ixodes persulcatus*, termed Salp15, promoted the infection of *B. burgdorferi* by its adhesion to a bacterial surface protein, OspC. In addition, RNA interference-mediated repression of Salp15 in *I. persulcatus* drastically reduced the capacity of *B. burgdorferi* to infect mice. Thus, the objective of this study was to obtain immunosuppressive factors from *I. persulcatus,* which is a vector for Lyme borreliosis and tick-borne encephalitis in East Asia.

Total of 198 cDNAs were cloned from a cDNA library constructed from salivary glands of fed female ticks. Four cDNA clones, termed as *IpHBP 1, 2, 3* and *4,* have sequences similar to the *Histamine binding protein* genes expressed in other lxodes ticks. RT-PCR analysis showed that *IpHBPs* were expressed specifically in the salivary glands of fed females. Western blot analysis showed that IpHBP 2, 3, and 4 were 'exposed' antigens. Although recombinant IpHBPs (rIpHBPs) expressed in *Escherichia coli* did not show the binding activities to histamine, vaccination of mice with the rIpHBPs delayed engorgement of ticks and reduced their engorged body weight. These results suggested that IpHBPs suppressed the inflammation during infestation of ticks and could be potential candidate antigens for anti-tick vaccine.

Molecular epidemiology of Marek's disease virus in wild waterfowls in Japan and Russia

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Marek's disease (MD) has been controlled by the vaccination with nonpathogenic strains of Marek's disease virus (MDV). However, due to the selective pressure by the use of more effective vaccines, the virulence of MDV isolates has been increasing in the field. In 2001, an MD case was reported in a white-fronted goose (*Anser albifrons*), a migratory bird to the Hokkaido area, for the first time in the world. This implied that virulent MDV carried by wild birds could potentially spread over poultry farms. In addition, Newcastle disease virus (NDV) and avian influenza virus have caused serious economic losses in the poultry industry. These viruses were isolated from various free-living-birds (ducks, pigeons, parrots etc). Therefore, wild birds are considered as reservoirs and vectors of NDV and influenza virus. And other pathogens, such as infectious bronchitis virus, mycoplasmas, can also infect wild birds, suggesting that these pathogens could threaten not only domestic fowls by their transmission from wild birds, but also the conservation of wild birds. Thus, this work aims to investigate the distribution of various pathogens and molecular analysis of viruses isolated from wild waterfowls in Japan and Russia.

The epidemiological survey of MDV confirmed the high rate of MDV infection in waterfowls in Hokkaido. The most of the *meq* and *glycoprotein L* (*gL*) gene sequences of the

MDV genomes detected in these wild birds and domestic chickens in Japan were similar to those of very virulent plus or very virulent MDV strains, but also showed several genetic variations. These results suggest that MDV with more variations in *meq* could be present in the wild birds in the world. In addition, it was also shown that MDV strains from waterfowls and chickens shared several same mutations in *meq* and *gL*, suggesting some relationships between MDV strains which cause MD in chickens and MDV strains which wild birds carry.

In this study, 2 NDV strains and 5 influenza virus strains (3 of H4N6, 2 of H3N8), which have been frequently isolated from wild ducks and geese, were isolated from total of 1,053 fecal and cloacal swab samples of wild ducks and geese collected in Hokkaido in 2005.

The deduced amino acid sequence for the cleavage site of the fusion protein of the 2 NDV strains isolated showed the features of low virulent type, and the phylogenetic analysis indicated that these NDV isolates belong to genotype I, maintained in wild ducks but not in chickens recently. Though other pathogens examined were not detected in this study, it is essential to continue both epidemiological investigation and monitoring of diseases in the wild birds to understand what pathogens can be threat to chickens and wild birds at any given time.

Improvement of Copro-DNA detection in prepatent period of *Echinococcus multilocularis* infection in dogs

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Echinococcus multilocularis is an important zoonotic parasite that causes alveolar echinococcosis in humans. Moreover, since infected animals were found sporadically in the main island of Japan, the expansion of the endemic areas is of great public concern. Several cases of natural infection in pet animal per year have been recognized in recent years. The prevalence of the parasite in wild foxes in Hokkaido has been around 40% in the last decade. Considering this situation, accurate diagnosis in the definitive host, especially in companion animals, which are in intimate contact with humans, is indispensable. At present, the standard diagnostic method in the definitive hosts is detection of parasitic antigen and eggs in the host's feces as screening tests. When these tests are positive, the final diagnosis has been done by the detection of oval DNA. However, in prepatent period or in case of light infection, no eggs can be detected while the coproantigen is positive. Infection cannot be asserted only by the positivity of the host by antigen detection because of the possibility of false positive result. Therefore, it is essential to detect the parasite DNA from the feces even when the eggs cannot be detected. The detection frequency of copro-DNA in prepatent period using previous method has been low thus it is not appropriate to use the method for the final diagnosis.

The detection sensitivity of several primer sets that are specific for the DNA of *E. multilocularis,* and application of commercial

PCR kits are examined. It was found that PCR using the combination of COI primers (Emsp1-A'/Emsp1-B') and HotStarTaq Master Mix Kit (System 1), and PCR using the combination of U1sn RNA primers (UP1/UP 2) and Ampdirect Plus (System 2) are useful for DNA detection from the feces. The parasite DNA in the feces during the prepatent period (11dogs) and the feces that were collected after praziquantel treatment (6 dogs) were examined by these methods.

Eleven dogs were orally given 1,000 to 1,000,000 protoscoleces and the feces of these animals were collected daily. The rate of DNA positive days per number of examined days (20-22 days) in each dog is 26-73% and 5-45% for System1 and System2, respectively. Thus, System1 was superior to System2. The DNA was not constantly detected during the time course, and constant patern of the detection were not observed in the prepatent period except for positive results in most of the dogs on 1st day postinfection.

It was thought that the feces contained dewormed parasites after the anthelmintic treatment. Six dogs were orally given 1,000 to 1,000,000 protoscoleces, and were treated with praziquantel 14 days after infection. From all of these dogs, the parasite DNA was detected within 2 days after praziquantel treatment (15-16 days postinfection) using System 1 or System2. It is suggested that feces, after anthelmintic treatment, would be good material for diagnosis.

In conclusion, copro-DNA of *E. multilocularis* could not be detected throughout the prepatent period, suggesting that the method is not reliable enough for detectiong every prepatent infection. However, when anthelmintic treatment is involved in the diagnostic process, feces collected after anthelmintic treatment provide a high chance of detecting the parasite DNA.

Analysis of *Leishmania* proteins for the serodiagnosis of cutaneous leishmaniasis in Pakistan

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Human cutaneous leishmaniasis (CL) is a serious zoonotic protozoan infectious disease caused by the genus *Leishmania* species. In endemic areas, diagnosis of CL is usually made only by clinical observation, which may cause misdiagnosis for other dermal diseases. The confirmatory diagnosis is based on demonstrating the parasites in the tissue or culture specimen, and detecting the parasite specific DNA in the specimen by PCR. However, it is not easy to obtain the biopsy materials from the lesions of the patients for those examinations. Thus, serodiagnosis is considered to be very useful since blood samples would be more easily obtained. In this study, immunoblot and proteomic methods were used to detect antigens useful for the serodiagnosis of CL.

A recent epidemiological study revealed that *Leishmania* (*Leishmania*) *major* is distributed in the lowlands in southern Pakistan and *L.* (*L.*) *tropica* is distributed in the highlands northwestern Pakistan. Sera collected from CL patients in these different endemic

areas were subjected to Western blot analysis using crude soluble antigens prepared from Pakistan isolates of these two *Leishmania* species. Antigen recognition patterns were different between sera from patients with *L.* (*L.*) *major* infection and those from patients with *L.* (*L.*) *tropica* infection. The fractions of 93, 70, 50, 30, and 14 kDa in both species were frequently (40-60%) recognized by sera from CL patients. These fractions were considered to include possible candidate antigens for serodiagnosis of CL.

For identification of candidate antigens, fractions of interest were excised from the gels and subjected to proteomic analysis by MALDI time of flight mass spectrometry and peptide mass fingerprintings. Pakistan isolates of *L.* (*L.*) *major* and *L.* (*L.*) *tropica* and the genome sequenced-*L.* (*L.*) *major* Friedlin strain were used. As a result, 41 different proteins were identified from these *Leishmania* strains. Some of proteins, such as heat shock proteins, ribosomal proteins, LACK, GP63 and histones have already been reported to be antigenic and considered to be candidate antigens for serodiagnosis of CL. Among them, heat shock protein 70 is the best candidate because a 70-kDa fraction was most reacted by immunoblotting with sera from CL patients. Production of recombinant protein is underway to examine whether these candidate proteins are really antigenic.

In summary, immunoblotting patterns of sera from CL in Pakistan were different between *L.* (*L.*) *major* and *L.* (*L.*) *tropica* infection. Proteomic techiniques were useful to analyze the *Leishmania* proteins and identify candidate proteins for serodiagnosis of CL.

In vitro effects of respiratory chain inhibitors against larval *Echinococcus multilocularis*

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Alveolar echinococcosis (AE) is a life-threatening zoonosis caused by larval *Echinococcus multilocularis*. Presently, benzimidazole derivatives including albendazole are commonly used for chemotherapy of AE. However, they have only parasitostatic rather than parasitocidal effects.

Parasites use various energy metabolic pathways that differ from those of their hosts, and therefore could be promising targets for chemotherapy. Recently it was reported that *E. multilocularis* protoscoleces use anaerobic respiratory chain, which is absent in mammalian host species living on aerobic respiratory system. In addition, quinazoline 8-OH derivative was found as a relatively selective inhibitor against NADH-fumarate reductase activity, the main activity of the anaerobic respiratory system of the parasite. In the present study, the anti-parasitic effects of quinazoline 8-OH and other compounds were evaluated under *in vitro* culture system.

First, protoscolicidal effects of quinazoline 8-OH, rotenone, and albendazole were tested. Each chemical was added into the parasite culture at final concentrations of 5 μM and 50 μM. As a result, 50 μM quinazoline 8-OH and 50 μM rotenone showed strong protoscolicidal effects: the percent viability of the protoscoleces started to decrease on day 3,

and then reached 0% on day 5 or 8, respectively.

Subsequently, the effects of quinazoline 8-OH, rotenone, nitazoxanide, and albendazole were evaluated using *in vitro*-generated *E. multilocularis* metacestode cysts. The culture medium of the metacestode cysts was supplemented with each chemical at final concentrations of 5 μM and 50 μM. In the first approach, the activity of alkaline phosphatase (EmAP), which derived from the damaged cyst into the culture medium, was measured as a marker of damages in the parasite cysts caused by chemical treatment. The results of this assay suggested that each chemical causes damages in the parasite cysts in different manner, showing gradual increases of EmAP activity in the culture supernatant. However, this approach was inappropriate for the evaluation of quinazoline 8-OH because this compound inhibited EmAP activity in this assay system.

As the next approach, the proportion of floating cysts in the culture medium was determined as a parameter of damages. Live and active cysts float on the surface of the culture medium, whereas damaged and dead cysts sink onto the bottom of the culture flask. The proportion of floating cysts during treatment with 50 μM quinazoline 8-OH, 50 μM rotenone, and 5 μM/50 μM nitazoxanide reached 0% as early as on day 6-8.

Finally, a modified MTT assay was used was examined to determine whether the parasite cysts were completely dead at this time point of treatment. In this assay NADH/NADPH was detected to indicate the existence of live and metabolically active cells. As a result, the parasite cysts treated with 5 μM/50 μM quinazoline 8-OH, 50 μM rotenone, and 50 μM nitazoxanide were suggested to be dead.

The present study suggests that respiratory chain of larval *E. multilocularis* is a promising target for the chemotherapy of AE and that quinazoline derivatives are hopeful compounds in the development of novel drugs targeting the mitochondrial respiratory system of the parasite.

Production of congenic mice carrying antviral loci, *Mx* and *Oas*

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The role of the heat shock protein 70 in canine babesiosis

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Diagnosis of feline Sandhoff disease in Japan

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Anti-tumor effects of photodynamic therapy by local administration of Benzoporphyrin derivertive monoacid ring A (BPD-MA) to tumorous tissues in mice

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Photodynamic therapy (PDT) consists of applying a photosensitizer followed by irradiation of selective visible light with appropriate wave length to activate the agent. The drug tends to be distributed into the tumor tissue due to the tissue affinity after its intravenous administration. Subsequent irradiation leads to selective destruction of neoplastic tissue, in which the drug was accumulated. Local injection of the agent directly to targeting tissues may be raised as an alternative route of drug administration, which is expected to lead to less systemic adverse reactions, its high local tissue concentration with lower dose of the drug and shorter treatment time. The purpose of the present study was to evaluate *in vivo* photodynamic effects using intratumor injection of BPD-MA in two different murine tumor models, KLN205 (squamous cell carcinoma) and LM8 (osteosarcoma with high metastatic potential to the lung).

Cytotoxity of PDT after 10 minute-exposure of BPD-MA was evaluated in cultured tumor cells *in vitro*. Phototoxicity was parallel to the drug concentration and was attenuated if the drug was removed from culture media after certain period of exposure. This transcribes that photodynamic effects would be principally induced from extracellular drug-light reaction in case of local administration of the drug for PDT *in vivo*.

In tumor-implanted mice, 10 or 30 minutes of drug-light interval PDT by direct injection of BPD-MA to tumor tissues was performed. In two different kinds of tumor models KLN205 and LM8, definite phototoxicities were seen parallel to the drug concentration injected. In KLN205, 10 minutes interval PDT was more effective in cytotoxity than 30 minutes interval PDT ($p<0.05$). Histologically, the region of tumor destruction was seen in the central area of tumor tissue. The shape of necrotic region of KLN205 was more centered with clear margins, while pleomorphism and dissemination of necrotic region were found in LM8. This different presentation of lesions may reflect the structure of parenchyma and vascularization in each tumor mass.

In conclusion, the results suggested that local PDT with topical administration of photosensitizers could effectively lead cytotoxity in the area with less unexpected events. As

the maximal rate of necrotic tissue was 72% only in tumor models used in this study, more detailed investigation deciding requirements and conditions of the procedure should be necessary before application to clinical cases of indication.

Evaluation of non-steroidal anti-inflammatory drugs towards proapoptotic effects on canine cultured synovial cells

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Since arthrosis is estimated to affect as much as 20% of canine population, a new therapeutic approach and understanding of it are expected to be necessarily improved. Non-steroidal anti-inflammatory drugs (NSAIDs) are commonly used as a symptomatic treatment for joint disorders such as osteoarthritis in small animal practice. NSAIDs are employed to alleviate discomfort and minimize pain with the purpose of restoring affected joints to its normal condition. However, it is still unclear how exactly NSAIDs produce anti inflammatory effects. The purpose of this study was to elucidate mechanisms, which through NSAIDs contribute to diminish inflammatory effects using canine cultured synovial cells model according to their apoptotic status.

Canine synovial fibroblasts were cultured from synovial membrane samples harvested from normal joints and joints affected by cranial cruciate ligament rupture or rheumatoid arthritis. In this study it was investigated whether meloxicam, piroxicam, tepoxalin, MK 886 and AA861 produce a cytotoxic effect on cultured synovial fibroblasts by means of MTT-assay along with its morphological aspects evaluated by nuclear staining with the use of hoechst33342. In addition, it was applied the usage of caspase 3 inhibitor in order to determine whether caspase 3 was intrinsically involved in the cellular apoptotic event. In a separate trial, the synovial cells in culture were submitted to interleukin-1 β (IL-1 β) treatment 24 hours before the drug administration was started.

As a result, a distinguished effect between COX and LOX administration to such cultured synovial cells was observed. COX inhibitors did not appear to induce proapoptotic effects to cells opposing to the LOX performance, which it was actually to provoke the proapoptotic effect. For the pre-usage of caspase 3 inhibitor during administration of tepoxalin, it was observed a strong inhibition of the proapoptotic cascade, as for the MK886 and AA861 administration it was shown to act as an inhibitor but not so intensively compared to the tepoxalin treated cells. Based on this results it is concluded that the cultured synovial cells undergo some order cell death mechanism besides the one mediated by caspase 3. The evaluation of the IL-1 β on normal and osteoarthritic derived cells showed a slight difference between pretreated cells with IL-1 β and non pretreated ones. On the other hand, it showed an improved viability result with the rheumatoid derived cells.

In conclusion, these results of this study suggested COX-2 inhibitors, commonly used

for the purpose of reducing inflammation, did not produce proapoptotic events, which means it would not have an overall reasonable effect. Once the healing of inflammation and restoration of the synovial membrane to its normal condition are expected, it would be proper to induce somehow a limited frequency of apoptotic events. It is therefore suggested that the usage of COX/LOX and LOX inhibitors would have proapoptotic effects, which would be inhibited by IL-1β.

Analysis of mechanism of inhibitory effects *in vitro* with non-steroidal anti-inflammatory drugs against canine transitional cell carcinoma

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Transitional cell carcinoma (TCC) is the most common malignancy of canine urinary bladder, with an extremely poor prognosis. An effective treatment for TCC has not been established yet. A previous report showed that piroxicam, which was a kind of non-steroidal anti-inflammatory drugs (NSAIDs), had antitumor-activity against canine TCC. This drug might be a usefull treatment, however the precise mechanisms of antitumor effects of NSAIDs have not been elucidated.

In the present study, in order to understand the mechanism of antitumor effect induced by NSAIDs, a new clonal TCC cell line (cLTCC) was established by using a limiting dilution-culture method. The cytotoxic activity against cLTCC and an established TCC cell line CUBT-3 were studied by using MTT assay, and apoptosis induced by NSAIDs was examined by using giemsa stain, Hoechst dye 33342 fluorescent stain and analyzing the reaction to caspase-3 inhibitor. Additionally, to study associations between NSAIDs and another factors related to cytotoxic activity *in vitro,* Tumor Necrosis Factor (TNF)-α and Interleukin (IL)-4 were analyzed. Prostaglandin (PG) E_2, one of the metabolites of cyclooxygenase (COX)-2 produced by degrading arachidonic acid, was evaluated according to its influence on cytotoxic activity and the apoptosis induced by NSAIDs. Additionally, tumor associated hydroquinone (NADH) oxidase (tNOX) was also examined, with the purpose on checking the association with an independent pathway different from the COX-2 intermediate reaction.

As a result, it was shown that 1 mM piroxicam and meloxicam exhibited cytotoxic reaction by inducing apoptosis in TCC, and 20 ng/ml TNF-α increased the cytotoxic activity. However, effective concentration of NSAIDs that expressed apoptotic effect in the present study was much higher than that in peripheral blood. Hence, it was considered that further study on antitumor activity correlated with direct and consequential effect of NSAIDs and other factors would be necessary. In the present study, PGE_2 and tNOX did not exhibit association with apoptosis induced by NSAIDs. It was, therefore, suggested the indication of subsequent analysis of whether antitumor effects by NSAIDs are dependent on or independent from the COX activity.

A basic study on clinical application of antitumor vaccine therapy of dendritic cells plused with allogeneic tumor-lysate

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Dendritic cells (DC) are considered to be one of promising natural adjuvants for therapeutic cancer vaccines because of their unique potential to induce tumor-specific cytotoxic T lymphocytes (CTL). In immunological approaches for the treatment of human cancer patients, the DC-based antitumor vaccine, which could be a replacement for non specific immunotherapy, has emerged as a hopeful cancer immunotherapy with proven clinical efficacy. In veterinary clinical trials, the DC-based antitumor vaccine therapy is believed as a new antitumor therapy, which elicits tumor-specific immune responses with less side-effect. The information of this therapeutic approach is however very limited in the leteriture of veterinary oncology.

In this study, DCs were derived from canine peripheral blood mononuclear cells (PBMC) by the treatment of recombinant canine (rc) GM-CSF, rcIL-4 and rcTNF-α. These cells are inoculated with two kinds of lysates originated from different tumor cell lines, canine malignant melanoma (CMeC) and canine osteosarcoma (HMPOS). Intra-and extracellular distribution of fluorochrome-conjugated tumor lysate revealed the recognition of these lysetes by DCs *in vitro*. Six healthy beagles were assigned in two groups and vaccinated intradermally for four times with respective allogeneic tumor lysate-pulsed DCs every 7 days. Before each injection of the vaccine and 3 weeks after last vaccine, corrected PBMC were regularly examined its proliferative and functional properties including proliferation of lymphocytes stimulated by lysate-pulsed DC *ex vivo* (DSL) assessed by incorporation of [^{3}H] thymidine and lymphocyte surface marker analysis using flow cytometry, as well as interferon (IFN)-γ and IL-10 production by DSL using enzyme-linked immunosorbent assay.

After vaccination, 67% of dogs (three out of four in CMeC lysate-pulsed DC vaccination, one out of two in HMPOS lysate-pulsed DC vaccination) showed significant proliferation of DSL and IFN-γ production by DSL *ex vivo*. Ratios of tumor-spcific CD8+ and CD4+ T cells in DSL were also increased after vaccination. These results reported here suggest that autologous DC pulsed with tumor lysate may be applicable to promote necessary immunological reaction against tumorous tissue as an aduvant and be considered as a specific vaccine for tumor patients in dogs.

Pathological and Epidemiologial Study on So-called Fowl Glioma in White Leghorn Egg Layer Chickens

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So-called fowl glioma is caused by fowl glioma-inducing virus (FGV), which belongs to an avian leukosis virus subgroup A (ALV-A), and characterized by multiple nodular growths of astrocytes associated with disseminated nonsuppurative encephalitis. FGV infection was suggested to be prevalent in Japanese fowls in Japan. On the other hand, some outbreaks of the ALV-A infection, which caused subcutaneous tumor at the cephalocerbical region in commercial White Leghorn egg layers, have been observed in our country from 2003. So-called fowl glioma was also observed in these affected chickens. In this study, hitopathological and molecular biological examinations on the affected chickens and birds kept with them were carried out to clarify the pathomorphologic characteristics of the glioma and the pathogen. Microscopically, gliomas were observed in 11 of 18 chickens with subcutaneous neoplasm and 4 of 222 birds kept with the affected chickens. Brain lesions were noted in 121 chickens. Of these chickens, 104 birds had nonsuppurative encephalitis, 2 birds had diffuse proliferations of astrocytes or small nodules of atypical astrocytes, and 13 birds had nodular growths of astrocytes (gliomas). The gliomatous lesions frequently occurred in optic tectum as well as in cerebrum. In addition, 2 affected chickens had diffuse proliferations of small-round cells, which have not seen in the previous cases induced by FGV. The FGV genome was not detected in the affected brains by nested polymerase chain reaction. ALVs were isolated from the affected brains and the sequence analyses of the SU region of *env* were performed for the identification of causal virus. Sequence homology among 7 isolated ALVs ranged 98～100%, and they were most closely related to avian myeloblastosis associated virus type 1 (MAV-1) and the sequence homology among them ranged 97～98%. The histology of brain neoplasms were almost consistent with the lesions of so-called fowl glioma, but the brain lesions were occasionally associated with proliferation of small-round cells and had a tendency to occur in optic tectum as well as in cerebrum. From these results, this disease in egg layer flocks in Japan is suggested to be related to MAV-1 infection and ALV is considered to be able to induce subcutaneous neoplasm and glioma. The results show that the cause of fowl glioma is not just FGV, and that several ALV strains have oncogenicity in the central nervous systems.

Cryopreservation of zebrafish (*Danio rerio*) embryos : effect of cryoprotectants and preparation of vitrification solution for 14- to 20-somite stage embryos

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During the last decade, cryopreservation of oocytes and embryos has become an important technology in the field of animal reproduction and conservation. However, this technology has not been well developed in fishes. Therefore, the solution for vitrification of zebrafish embryos was developed. Embryos were collected at the 1- to 4-cell stage, some embryos were dechorionated, and cultured for 16 to 19 hours to the 14- to 20-somite stage. Some of dechorionated embryos were microinjected with the GFP-*nos* 13' UTR mRNA to visualize primordial germ cells (PGC). First, the toxicity and glass forming property of six cryoprotectants : dimethylsulphoxide (DMSO), ethylene glycol (EG), methanol (MeOH), propylene glycol (PG), 1,3-butylene glycol (1,3-BG) and 2,3-butylene glycol (2,3-BG) were investigated. EG and MeOH had high toxicity and low glass forming property, 1,3-BG and PG had low toxicity and high glass forming property and DMSO had intermediate toxicity and high glass forming property. Then, 1,3-BG or DMSO were mixed with PG at different concentrations and examined their toxicity and glass forming property. Mixture of 2 M1,3-BG + 3 M PG (BP23) showed a low toxicity but formed ice (devitrification) at warming. Mixture of 3 M 1,3-BG + 4 M PG (BP34) showed a high glass forming property with a relativery low toxicity. Finally, dechorionated embryos were exposed to BP23 and BP 34 in step-wise manner, then, immersed in liquid nitrogen. After warming, about 40% of embryos remained morphologically intact and some PGC were alive; although no live embryo was obtained.

Identification of bovine glycophorin A, a major transmembrane sialoglycoprotein in red cells

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Glycophorin A (GPA), a 36-kDa monotopic transmembrane protein, is the most abundant sialoglycoprotein in human red cells and plays important roles as blood group antigens and receptors for malaria and some viruses. In contrast, bovine red cells possess major sialoglycoproteins with Mr > 250 kDa (GP>250) in SDS-PAGE, which has been supposed to be the bovine GPA (bGPA) and to carry blood group antigens V2 and/or F. However, no information about the polypeptide backbone for bGPA has yet been available.

The purpose of the present study is to identify the gene and translation products for bGPA. Partial cDNA sequences of GPA-like proteins were obtained by BLAST search for the bovine genome database and the full-length cDNA clones were isolated to show that the polypeptide consists of 99 amino acid residues including a N-terminal signal sequence, possible *O*-glycosylation sites of 5 Ser/Thr residues in the extracelluler and one transmemrane span followed by C-terminal cytoplasmic tail. RT-PCR analysis showed expression of this GPA-like gene in hematopoietic tissues. When transiently expressed in 293 cells, GPA-like polypeptides were localized to the plasma membranes and showed Mr of 18 kDa, similar to that of the polypeptide backbone of >250-kDa glycoproteins determined by deglycosylation analyses. Human GPA is well known to colocalize with red cell anion exchanger 1 (AE1) as a molecular chaperone. The GPA-like proteins, co-expressed with AE1 in 293 cells, were colocalized with erythroid-type AE1 (at plasma membranes) or kidney-type AE1 (at endoplasmic reticulum). In addition, the >250-kDa proteins were extremely decreased in $AE1^{-/-}$ bovine red cells, as obseved in $AE1^{-/-}$ mouse red cells. Thus it is strongly suggested that the cDNA obtained in this study was the bGPA gene encoding major sialoglycoproteins in bovine red cells, and >250-kDa glycoproteins represent the high molecular weight complex containing bGPA.

Molecular pathogenesis for combined deficiency of ankyrin in band $3^{Bov.Yamagata}$: intracellular retention and ER-associated degradation of ankyrin through hetero-oligomer formation with R664X AE1

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Band 3 (AE1) deficiency in cattle due to nonsense mutation R664X of the *AE1* gene (band $3^{Bov.Yamagata}$) leads to dominant hereditary spherocytosis with a combined deficiency of membrane skeletal proteins, ankyrin and spectrin. Previous studies demonstrated that the R664X AE1 is retained intracellularly in the ER followed by ER-associated degradation through the proteasomal pathway and has a dominant-negative effect on the expression of wild-type AE1. However, precise mechanisms for reduction in ankyrin and spectrin contents remained to be clarified. The purpose of the present study is to verify a hypothetical role of the R664X mutant AE1 in pathogenesis for combined deficiency of ankyrin. Enhanced green fluorescent protein (EGFP)-tagged AnkN90, the N-terminal AE1-binding domain of ankyrin, predominantly exhibited localization at the plasma membrane when it was co-transfected to HEK293 cells with the wild-type AE1, indicating that a population of EGFP-AnkN90 associated with wild-type AE1 and targeted the plasma membrane. However, EGFP-AnkN90 was preferentially retained in the ER in the presence of R664X AE1. Immunoprecipitation and sedimentation analysis of the sucrose density gradient centrifu-

gation demonstrated specific and hetero-oligomeric association of EGFP-AnkN90 with wild-type and/or R664X AE1. While lactacystin, a proteasome inhibitor, caused accumulation of EGFP-AnkN90 with formation of aggresome-like structures when transfected alone into HEK293 cells, it increased retention and accumulation of EGFP-AnkN90 in the ER without apparent formation of aggresomes. These findings indicate that R664X AE1 has a dominant-negative effect on expression of ankyrin in the plasma membrane, as demonstrated for the wild-type AE1, through the ER-associated degradation, leading to combined deficiency of ankyrin, and possibly of spectrin, in band 3[Bov.Yamagata]

A novel sequence in the N-terminal cytoplasmic domain required for effective trafficking of bovine AE1 to the plasma membrane in non-polarized cells

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Anion exchanger 1 (AE1, band 3) is expressed in the plasma membrane of the red cell and the distal nephron. Various mutations of the human *AE1* gene cause dominant hereditary spherocytosis or distal renal tubular acidosis. Recent findings on affected vesicular transport of AE1 mutants suggested that some characteristic sequences within the AE1 polypeptide would regulate the membrane traffic of AE1. The purpose of the present study is to clarify intramolecular motifs or specific sequences essential to proper trafficking to the plasma membrane in non-polarized cells of bovine erythroid AE1.

While the deletion mutant of C-terminal 11 amino acid residues (ΔCt11) of human AE1 has impaired trafficking to plasma membrane, bovine AE1 ΔCt11 was preferably targeted to the plasma membrane, suggesting that AE1 would possess multiple structures that affect its adequate targeting to the cell surface. Fluorescence microscopy and cell-surface biotinylation studies on a series of human/bovine chimeric AE1 tagged with EGFP demonstrated that ~20 amino acid sequence located adjacent to the N-terminus of the cytoplasmic domain of bovine AE1 facilitated the trafficking efficiency of AE1. Further studies on several AE1 constructs containing substitution mutations in the N-terminal region finally demonstrated that a short sequence Ser^{25}-Val-Ser-Ile-Pro^{29} from bovine AE1 plays key roles in effective trafficking of AE1 proteins to the plasma membrane in non-polarized HEK293 cells.

Identification of genes for glycophorin A and C, major glycophorins in canine erythrocytes

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Glycophorins are sialoglycoproteins in red cell membranes and play various physiological roles in maintenance of cell shape, expression of blood group antigens and binding with for pathogens. In the present study, we analyzed membrane glycoproteins in canine red cells and cloned cDNAs of canine glycophorin A (GPA) and C (GPC) from bone marrow in order to determine the molecular structures of these glycophorins. Partial cDNA sequences of canine GPA (cGPA) and GPC (cGPC) were obtained by BLAST search, and full-length cDNAs were amplified by rapid amplification cDNA ends method. The amino acid sequences of GPA were not conserved between dogs and other species except transmembrane regions. Extracellular region of cGPA possessed several potential *O*-glycosylation sites, suggesting that this cDNA codes a glycoprotein. Unexpectedly, we isolated several splicing variants of cGPA, in contrast to cGPC, suggesting that the molecular variations affect the functions and antigenicity of cGPA. The primary structures of GPC were well conserved between species, especially in the transmenbrane and intracellular domains. There are several potential *N*-and *O*-glycosylation sites at the extracellular domain of cGPC. We, therefore, identified the genes of major canine red cell glycophorins, GPA and GPC. Moreover, we investigated the tissue distributions of cGPA and cGPC mRNA in dogs. We observed the most abundant expression in bone marrow and reticulocytes, and weak expression in liver and kidney, suggesting the possible roles of glycophorin in several tissues other than red cells.

Application of the recombinant particles of tick-borne encephalitis virus to serological diagnosis and vaccine development

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In October 1993, a human case of encephalitis was diagnosed as tick-borne encephalitis (TBE) in Kamiiso, Hokkaido. Far-Eastern subtype TBE virus strain Oshima 5-10 was isolated from a sentinel dog in the same area. Since the susceptible vector ticks and reservoir rodents are commonly found in Japan, it is possible that TBE virus is endemic in other parts of Japan. To prevent the endemic of TBE virus, developing the diagnosis for an epidemiological survey, effective vaccine and specific treatment are needed. However TBE virus is highly pathogenic and the handling of live TBE virus must be car-

ried out under biosafety level 3 conditions (BSL-3). Therefore in this study, I made recombinant particles of TBE virus and applied to serological diagnosis and vaccine development.

In section Ⅰ, for the serological survey, we established enzyme-linked immunosorbent assay (ELISA) system by using TBE virus subviral particles (SPs) as its antigen. SPs are secreted from the cell expressing viral prM and E protein, and have the same antigenicity and immunogenicity with native virus. Compared to the results of neutralization test of wild rodents' sera in Kamiiso, the ELISA showed high sensitivity (91.4%) and specificity (100%). Furthermore, in the epidemiological research of wild rodents of Khabarovsk, endemic area of TBE, the ELISA could identify positive samples of TBE virus infection. Therefore, the ELISA can be applied to epidemiological survey to estimate the distribution of TBE virus in Japan.

In section Ⅱ, SPs and the plasmid expressing SPs were applied to the vaccine by a needle-free jet injection. The vaccination of the plasmid induced high levels of neutralizing antibody in most mice. Compared to the normal needle-syringe injection, fewer amount of the plasmid DNA, even 1 μg, were able to induce high immune response. Therefore a needle-free jet injection can be more useful than a needle-syringe injection in terms of the safety, the quick and painless injection, and the efficient induction of neutralizing antibody. The plasmid expressing SPs can be a useful vaccine for protection against the endemic of TBE virus.

In section Ⅲ, the replicon RNA of TBE virus was applied to the expression of heterologous gene and serological diagnosis. In our previous study, a replicon RNA of TBE virus strain Oshima 5-10 was constructed. The replicon RNAs contain the genes for genome replication, but not for the viral structural proteins. In this study, green fluorescent protein (GFP) gene was inserted into the replicon RNA, and this led to the expression of GFP in the replicon-transfected cell. Then, this replicon RNAs were co-expressed with viral structural C-prM-E protein, and this result in the secretion of single round infectious virus-like particles (VLPs) packaging the replicon RNA. GFP was expressed in the VLPs-infected cells, indicating that the VLPs could be a vector for heterologous gene expression. Furthermore, VLPs were applied to neutralization test. There was a highly significant correlation between the results of VLPs and native TBE virus, indicating that VLPs can substitute for native virus in serological diagnosis. These data suggest that this TBE virus replicon RNA and packaging system can be a useful tool for the development of gene delivery vectors, and further application.

Construction and characterization of virus-like particles with envelope membrane of West Nile virus

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West Nile virus (WNV) is a member of the *Flavivirus* genus, the family *Flaviviridae*. In

nature, virus transmission occurs between avian hosts and mosquito vectors. Humans and horses are infected by virus-infected mosquito bite, and in some cases, it develops conditions such as fatal encephalitis. WNV appeared in New York City for the first time in American continent in 1999, and rapidly expanded to the whole country of the United States. It is anxious that WNV may invade Japan due to the frequent trade and traveling between U.S. and Japan. For the prevention against the epidemic of WNV in Japan, it is important to detect the viral invasion, and simple and highly specific diagnosis is required. Neutralizing test (NT) is the most specific in serological diagnosis. However, the handling of live WNV in NT must be carried out under biosafety level (BSL)-3 conditions.

Previously, a subgenomic replicon of tick-borne encephalitis virus (TBEV) was constructed and applied to virus-like particles (VLPs) system in our laboratory. The subgenomic replicon is a genomic RNA lacking of structural proteins region and cannot produce progeny infectious viruses. The expression of virus structural proteins in replicon-transfected cells leads to the secretion of VLPs, which contain subgenomic replicon RNA. In this study, we constructed chimeric VLPs with envelope membrane proteins derived from WNV by the expression of TBEV C protein and WNV prM/E proteins in replicon-transfected cells. In the cells infected with chimeric VLPs, the TBEV replicon replicated but there was no secretion of infectious particles extracellularly. This shows that VLPs have single-round infectivity. Furthermore, the RNA extracted from the chimeric VLPs showed no recombination between TBEV replicon RNA and mRNA of virus structural protein. This single-round infectivity and genetic stability of VLPs enable safe handling under BSL-2 conditions. The viral structural proteins of chimeric VLPs showed same antigenecity with native WNV, and the infection of chimeric VLPs was blocked by WNV immune serum. These data indicate that the chimeric VLPs can substitute for native WNV in NT. Therefore, WN-TBE chimeric VLPs are useful tools for the NT of WNV infection, with the additional safety aspect that these chimeric VLPs can be used instead of full-length native viruses.

Seroepidemiological study of hantavirus in wild rodents in Mexico

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Hantavirus causes two forms of severe human diseases, which are hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS). HPS is respiratory syndrome caused by Sin Nombre virus (SNV) and Andes virus (ANDV) distributed over American Continent countries by specific natural rodent hosts. Mexico has no surveillance system of HPS outbreak and the epidemiology of hantavirus infection among wild rodents in Mexico has not been studied, though the southwestern states of U.S.A. which are epidemic for HPS is adjacent to Mexico. Because the economic relationship be-

tween Japan and Mexico is expanding year by year, it is necessary to know the epidemiological situation of HPS. In this paper, the development of a serodiagnostic method of hantavirus infection using the recombinant nucleocapsid protein of SNV (SNV-rNP) and epidemiological investigation of wild rodents in Mexico were conducted.

A total of 211 wild rodents were captured in Mexico and 28 sera showed positive by the ELISA. A seroprevalence rate of *Peromyscus* was 16.9% (15/89) and that of *Reithrodontomys* was 20.0% (8/40) by the ELISA. The seroprevalence was comparable with other HPS -epidemic countries. Therefore, it is suggested that HPS is epidemic in Mexico.

In University of Mexico, the species of the seropositive rodents were identified. It was revealed that 7 species had anti-hantavirus antibodies ; *Peromysus aztecus, Peromyscus beatae, Reithrodontomys sumichrasti, Reithrodontomys magalotis, Neotoma mexicana, Megadontomys thomasi, Hodomys alleni*. Since these rodents without *R. magalotis* have not been known to be infected with hantavirus, new hantaviruses may be identified in these species.

Western blotting was performed for the confirmation of antibody positive in ELISA-positive rodents. Sera from 11 ELISA-positive rodents including *P. aztecus, P. beatae, R. sumichrasti, N. mexicana and M. thomasi* showed positive signals in Western blotting. Therefore, it was confirmed that hantavirus is prevalent among these rodents.

Characterization of Vitamin K epoxide reductase in warfarin resistant roof rats (*Rattus rattus*)

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Warfarin is a commonly used rodenticide worldwide. It inhibits coagulation of blood by inhibiting vitamin K epoxide reductase (VKOR) activity. An inadequate supply of vitamin K blocks the production of prothrombin and cause hemorrhaging.

Recently, warfarin-resistant roof rats have been found around Tokyo areas in Japan. However, the mechanism which causes warfarin resistance in roof rat was still unclear. In this study, I focused on the VKOR in roof rats to figure out the mechanisms of warfarin-resistance.

The liver was obtained from warfarin-sensitive and-resistant roof rats. I cloned *VKORC1* gene from roof rats and sequenced. I found novel substitution of leucine to proline at 76 position of *VKORC1* amino acid sequence.

Then, I determined the kinetic differences between VKOR mutant and wild type. I prepared liver microsomes from-sensitive and -resistant rats. The VKOR-dependent activity was measured over a range of vitamin K epoxide concentration from 12.5 to 100 μM by HPLC in absence and presence of warfarin. Lineweaver-Burk and Hanes plots were used to determine *Vmax* and *Km* values, and a constant of inhibition by warfarin (*Ki*) was calculated according to Dixon plot and Secondary plot.

The values of *Vmax* was significantly

lower in resistant rats (9.58±4.35 pmol/min/mg) than those of-sensitive rats (22.06±7.56 pmol/min/mg). I could not find any significant differences in *Km* values between-resistant (67.59±64.87 μM) and -sensitive (30.36 ±18.94 μM) rats. Low *Vmax* values lead to lower enzymatic efficiency (*Vmax* / *Km*) in-resistant rats (0.31±0.33) than those of-sensitive rats (0.95±0.53). However, since I found large individual differences in *Km* values in-resistant rats, further study may be need to clarify the VKOR catalytic efficiency at physiological concentration of vitamin K epoxide.

In this study, result from warfarin inhibition assay revealed a good exploration for rodents, which acquired the warfarin resistance.

The value of *Ki* was 50-to 100-fold higher in warfarin-resistant than those of-sensitive rats.

Finally, I concluded that one of mechanisms which cause warfarin resistance in Japanese roof rat might be low inhibiting effect of warfarin on VKOR mutant.

Residue of Persistent Organic Pollutants (POPs) in wild Norway rats (*Rattus norvegicus*) and their effects on liver and testis

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Persistent Organic Pollutants (POPs) were ubiquitous contaminants in the environment, such as dioxin, PCBs, organohalogen pesticides and polybrominated diphenylether (PBDE). Due to the high lipophilicity and resistance to biological degradation, wildlife and human accumulate POPs through the food chain. In this study, to assess the risk of POPs for humans and wildlife, I investigated the residue levels of POPs in livers of wild Norway rats (*Rattus norvegicus*), and measured the mRNA expression levels in livers and testes using GeneChip techniques. From urban areas (Shinjyuku, Ikebukuro and Umeda, Nanba), landfill (Sapporo) and isolated island (Teuri island) in Japan, I collected wild Norway rats. Laboratory rats (Wistar) were used as non-contaminated controls in this study.

In present study, I found highest TEQ value (21,000pg-TEQ/g lipid, coplanar PCBs >70%) in the liver of Tokyo rat, and there was a tendency to accumulate the highly chlorinated-substances in livers of wild rats. I also found DDTs dominated in residue of organohalogen pesticides in wild rats. In addition, I detected the much amount of PBDEs in rats from the landfill areas and urban areas ; DeBDE were major components of PBDEs in their livers.

The gene expression levels of CYP1A1, 1A2, NQO1, Heme oxygenase-I and metallothioneins in liver were elevated in rat from Landfill, Shinjyuku and Nanba, indicating the possibility of induction of genes due to exposure to dioxin-like compounds and heavy metals. In screening assay of the mRNA expression profiles in wild rat testes, I found the levels of testosterone synthetic genes such as StAR (steroidogenic acute regulAatory protein), cytochrome P450(CYP)11A and CYP17 were suppressed in wild rats from Shinjyuku.

The plasma testosterone levels in Shinjuku rat was also reduced, showing the possibility of coplanar PCBs and DDTs might have some effects on testis. In addition, we found induction of mRNA expression of CYP17 in wild rats from Landfill. I detected the correlation between high residue levels of PBDEs and expression levels of steroid syntheses genes.

In summary, I was able to identify the residue of POPs in wild rodent, and to show the possibility to detect the alteration of biomarkers caused by environmental pollutants using the screening with Gene chip techniques.

Genetic variation and characteristic of Chinese Wolf (*Canis lupus chanco*)

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Previous genetic studies have shown that few haplotypes have been observed in the grey wolf (*Canis lupus*). While these haplotypes were observed to have a markedly wide distribution, recent studies have demonstrated the existence of unique haplotypes that diverged from those of the widespread wolf lineage. These haplotypes were found in wolves with ranges extending from the southern foothills of the Himalayas to the Tibetan Plateau and lowland peninsular India. We therefore conducted a genetic analysis of the Chinese wolf (*C. l. chanco*) using hair and pelt specimens collected from several locations in China to elucidate the genetic variation and characteristics of the Chinese wolf.

A total of 18 haplotypes were obtained in the present analysis. Four haplotypes from the Tibetan Plateau diverged from the widespread wolf lineage as reported previously. However, the remaining 14 haplotypes fell within the widespread wolf lineage. Considering the genetic distance between groups, the Tibetan population diverged from other canids in the early stages of the adaptive radiation that occurred within canids.

Since, hair and pelt samples generally contained very small amounts of DNA, we confirmed the effects of deffating by ethanol and acetone, and relieved PCR inhibition using T4 gene 32 protein for hair and pelt tissue specimens.

Population structure and origin of Hokkaido Sika deer (*Cervus nippon yesoensis* Heude, 1884) on Shiretoko Peninsula assayed using mitochondrial control region sequences

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Recent overabundance of Sika deer, *Cervus nippon,* in numerous regions of Japan has

resulted in extensive damage to the natural vegetation and ecosystems in these areas. The population of Sika deer on the Shiretoko Peninsula in Hokkaido has been increasing since the 1980s. This increased pressure has had detrimental effects on the ecosystem, including a decrease in rare species and the alteration of forest physiognomy, which prompted the Ministry of the Environment to launch the "Conservation and Management Plan for Sika Deer (*Cervus nippon*) in Shiretoko Peninsula" to deal with this problem. The ecological data required for developing this Sika deer management plan have been obtained from the populations on the peninsula. However, insufficient information related to the population structure of this species has been elucidated to date. In this study, mitochondrial DNA control region sequences from 76 female Sika deer were collected from three wintering areas on the peninsula and genetic variability was assessed.

Results revealed the existence of one new haplotype (j-type) and four previously reported haplotypes (a-, b-, c-and d-types). In addition, analysis revealed a significant difference between a group that used a particular wintering area during the snowfall season and a group that used same area during non-snowfall seasons. This suggests the existence of a seasonal migrant subpopulation, and a non-migrant subpopulation. Analyses between the wintering area at the base of the peninsula and in middle of the peninsula were significantly different. Insufficient samples from the northernmost region of the peninsula meant that these data were excluded from the analysis. 79% (60/76) hinds investigated in this study had the a-haplotype, supporting the traditional assumption that Sika deer on the peninsula originated from a population that inhabited the Lake Akan region. However, the existence of a j-haplotype that was distinct from Shiretoko, and a c-haplotype that has not yet been observed in modern Sika deer from Eastern Hokkaido, indicates that a genetically distinct population may have remained in Shiretoko.

Comprehensive gene expression analysis of Neuro 2a mouse neuroblastoma cells stimulated with anti-PrP antibodies

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Cellular form of prion protein (PrP^C) is a membrane glycoprotein anchored to the cell surface via glycosyl-phospatidyl inositol moiety. Although PrP-deficient mice do not show apparent phenotypic changes, PrP^C is thought to be involved in neurogenesis and differentiation, synaptic function, and anti-oxidative activities. In addition, cross-link of cell surface PrP^C by anti-PrP antibodies activated Fyn kinase, suggesting that PrP^C plays a certain role in intracellular signaling pathways. In this study, the author attempted to anticipate possible roles of PrP^C from alterations of gene expression in Neuro2a (N2a) mouse neuroblastoma cells stimulated with anti-PrP mAbs. N2a-5, a subclone of N2a, was stimulated with anti-PrP mAb in the presence of 10% FBS and DNA microarray analysis was

carried out. Among the genes whose expressions were changed by antibody stimulation, the author selected 5 genes and tired to confirm the alteration of gene expression. However, the changes in expression of these genes could not be confirmed by quantitative RT-PCR analysis. To eliminate the effect of growth factors in serum, N2a-5 cells were also stimulated with anti-PrP mAb in the presence of 1% FBS. The results of DNA micorarray analysis showed that expressions of Stat3 and Socs3, which are related to JAK/Stat signaling pathway, were decreased, and this change could be confirmed by quantitative RT-PCR analysis. However, the decrease was not due to the specific interaction between PrP^C and anti-PrP mAb, but due to the result of unexpected interaction between N2a-5 and negative control mAb, P1-284, mAb against feline panleukopenia virus. Next, the author stimulated authentic N2a cells with anti-PrP mAbs and analyzed gene expression. Twelve genes were identified as those expressions were commonly changed by stimulation with 3 different anti-PrP mAbs 106, 31C6, and 44B1. Among them, Numbl, which is associated with Notch signaling and related to arborization of sensory axon. In addition, two other genes, Rbpush and Rfng, whose expressions were changed by mAb 44B1 and mAb 31C6 stimulation, respectively, were also related to Notch signaling. Hence, alteration in the gene expression of molecules associated with Notch signaling by anti-PrP mAb stimulation is of interest. Detailed analysis of the relation between PrP^C and Notch signaling will elucidate the role of PrP^C in neurogenesis and differentiation at molecular level.

Studies on culture conditions and fluctuation of PrP in cells persistently infected with prion

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Cells persistently infected with prion synthesize the abnormal isoform of the prion protein, designated PrP^{Sc}, and they are thus useful in vitro system for studying prion diseases. However, PrP^{Sc} in prion-infected cells sometimes decreases or disappears, possibly by minute changes in culture conditions. This impedes to perform accurate and reliable experiments. In this study, the author investigated culture conditions and fluctuation of PrP in prion-infected cells in detail. After seeding the cells, time-dependent increases in the levels of PrP^{Sc} were observed in prion-infected mouse neuroblastoma (ScN2a) sublines, especially when cells grew from subconfluent to confluent and then to overgrowth. Similar time-dependent increase in PrP^{Sc} level was observed in prion-infected GT1-7 hypothalamic neuronal cells. Compared with the PrP^{Sc} level immediately before passage, those in ScN2a sublines were extremely low at 24 hours after passage, and this decrease was not caused by the digestion of PrP^{Sc} with trypsin during the passage. Moreover, the lower level of PrP^{Sc} was detected at 24 hours after passage when cells had been plated at lower dilution rate. Further investigation revealed that the level of PrP^{Sc} decreased rap-

idly within 24 hours after seeding the cells. These results indicate that biosynthesis of PrP^{Sc} in prion-infected cells is affected by physiological conditions determined both by cell density and cell growth. In addition, when the ScN2a sublines were cultured with Opti-MEM, PrP^{Sc} level increased nearly 4-fold compared to cells cultured with DMEM. Although the growth of ScN2aII9-4, PrP overexpressing N2a subline, slowed down with medium containing low serum concentration, the levels of PrP^{Sc} in ScN2aII9-4 increased approximately 10-fold. This phenomenon was specific to ScN2aII9-4 sublines. Thus, medium compositions and serum concentrations influenced on the levels of PrP^{Sc} in ScN2a sublines but these effects varied among N2a sublines. These results provide useful information to improve the quality of experiments using prion-infected cells.

Interference of PrP^{Sc} formation by EGFP-tagged PrP and binding between EGFP-tagged PrP and PrP^{Sc}

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Since immunological discrimination of PrP^{Sc} from PrP^{C} has not been well established so far, a molecular probe that specifically binds PrP^{Sc} will be useful for analyzing the process of the biosynthesis of PrP^{Sc}. PrP^{C} binds PrP^{Sc} in the process of the biosynthesis of PrP^{Sc}, hence a fusion protein consisting of PrP^{C} and a reporter protein will act as a PrP^{Sc}-specific molecular probe. To examine whether the EGFP-PrP fusion protein (EGFP-PrP) is useful as PrP^{Sc}-specific probe, in this study, the author analyzed the influence of the fusion protein consisting of EGFP and mAb3F4-epitope-tagged PrP on PrP^{Sc} formation in prion-infected cells. When EGFP-PrP28 and EGFP-PrP76 were expressed in ScN2a-5, a subclone of Neuro 2a (N2a) mouse neuroblastoma cells persistently infected with Chandler strain, mAb3F4 positive PrP^{Sc} ($3F4PrP^{Sc}$) derived from exogenous PrP^{C}, was generated but only low level. Part of EGFP-PrP28 and 76 were truncated in ScNa-5 and the truncated PrP products lacking EGFP were possibly converted into $3F4PrP^{Sc}$, suggesting that EGFP-PrP28and EGFP-PrP76 were inefficiently converted into $3F4PrP^{Sc}$ or not. Expression of EGFP-PrP in ScN2a-5 resulted in the decrease in $MoPrP^{Sc}$ formation with the increase of EGFP-PrP expression. This suggested that EGFP-PrP bound PrP^{Sc} but were not converted into PrP^{Sc}, as a consequence, EGFP-PrP interfered PrP^{Sc} formation. In addition, the interference of PrP^{Sc} formation was not observed in ScN2aII9-4, Chandler strain-infected N2a overexpressing PrP^{C}, indicating that EGFP-PrP competes with endogenous mouse PrP^{C} in binding to $MoPrP^{Sc}$. Furthermore, EGFP-PrP lacking glycosyl-phosphatidyl inositol anchor did not inhibit PrPSc formation in ScN2a-5. This demonstrated that membrane anchored form of EGFP-PrP is essential in binding to PrP^{Sc} in the cell. EGFP-PrP did not bind PrP^{Sc} pre-denatured with 8M GdnHCl, implied that EGFP-PrP could recognize PrP^{Sc}-specific conformation. Taken together, these results suggest that EGFP-PrP may act as a PrP^{Sc}-specific molecular probe.