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INFORMATION

Hokkaido University confered the degree of Bachelor of Veterinary Medicine to the following 42 graduates of the School of Veterinary Medicine on March 25, 2010. The authors summaries of their theses are as follows:

Molecular dynamics of the blood-testis barrier components in murine spermatogenesis

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The blood-testis barrier (BTB) separates the seminiferous epithelium into the adluminal and basal compartments. During murine spermatogenesis, preleptotene/leptotene spermatocytes migrate from the basal to the adluminal compartment through the BTB during stages VIII-IX for further development. However, the molecular dynamics of the BTB in migration of those cells remains largely unknown. In the present study, the author focused on the tight junction (TJ) molecules, the principal components of the BTB, e. g., claudin-3, claudin-11, occludin, and zonula occludens-1 (ZO-1), and analyzed their spatiotemporal expression over the cycle of the seminiferous epithelium in the adult mouse testis. Immunohistochemistry and immunoelectron microscopy revealed that claudin-3, claudin-11, occludin, and ZO-1 were localized at the BTB. Although claudin-11, occludin, and ZO-1 were detected during all stages, claudin-3 was only detected during stages VI-IX. TJ fibrils consisted of claudin-3, claudin-11, occludin, and ZO-1 were observed at both the basal and luminal sides of the preleptotene/leptotene spermatocytes during stages VIII-IX. Using quantitative real-time PCR, the author clarified the changes in mRNA levels during spermatogenesis for those BTB component molecules and the genes associated with occludin degradation (*Itch*) and endocytic recycling (*Rab13*). Therefore, it was suggested that degradation and recycling of the BTB component proteins were stage-dependently regulated. The tubulobulbar complexes, structures considered to be involved in the internalization of junctional complexes, were observed at the BTB site. These findings indicated that the integrity of the BTB is maintained during all stages, and stage-specific localization of claudin-3 plays a crucial role in regulating the BTB permeability during preleptotene/leptotene spermatocytes migration.

Proliferation and destiny of oocytes in newborn mouse ovary

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Observation of the morphological processes of oogenesis and oocyte death is important to unravel the normal functions of the ovary. MRL/MpJ strain, a useful model for autoimmune disease, is known to have several unique characteristics, such as meiotic apoptosis in spermatocytes, heat stress-resistant spermatocytes, testicular oocytes in newborn mice, and ovarian cysts originating from rete ovarii. After the onset of autoimmune diseases, MRL mice show low fertility associated with increased abortion and reduced litters. Research on the biological characteristics of newborn MRL mice would be helpful to clarify the influences of autoimmune diseases on pregnancy and newborns. In particular, understanding the behavior of immune cells during the perinatal period is thought to be useful to establish a method to predict the age at disease onset. Furthermore, evidence of germ cell proliferation in postnatal ovaries has been under discussion recently.

In the present study, the author investigated the fate of postnatal oocytes in the ovaries of mice aged 0–7 days, by immunohistochemistry using several oocyte-related markers (Stella, SYCP3 and NOBOX), by toluidine blue staining for mast cells, and by electron microscopy. As a result, there was no difference in the developmental stage of oocytes between MRL and C57BL/6; however, on Day 0, many mast cells and macrophages were observed in the surface epithelial region in the MRL ovary, but not in C57BL/6, a control strain. Although a BrdU labeling experiment was performed to verify the existence of proliferative germ cells in postnatal ovaries, BrdU was not colocalized with NOBOX, an oogenesis marker, in any cells.

These findings demonstrated that unique organ- and tissue-specific activation of immune cells is already initiated in newborn MRL mice without clinical signs of autoimmune diseases. Furthermore, it was suggested that mast cells and macrophages act as early screening systems of oocytes. This idea would be interesting when discussing the developmental processes of follicles, including prediction of the oocyte fate under atresia. Further investigation would be expected to provide new concepts of the pregnancy risks in autoimmune disease patients.

Organ-specific increase in noradrenaline turnover induced by three different stressors in mice: Comparison of cold exposure, immobilization, and LPS treatment

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When animals are exposed to various stressors, the central nervous system evokes physiological responses that ultimately result in the activation of efferent pathways to maintain whole body energy homeostasis. In this context, the hypothalamic-pituitary-adrenal system and the sympathetic nervous system play important roles. In this study, the effects of three stressors from different categories, namely, cold exposure, immobilization, and lipopolysaccharide (LPS) treatment, were compared in the sympathetic nervous system of C57BL/6J mice. In order to evaluate changes in the tonus of sympathetic nervous activity in various peripheral organs (heart, brown adipose tissue, spleen, liver, pancreas, and mesenteric white adipose tissue), noradrenaline turnover was assessed from the decline in noradrenaline concentration after the inhibition of catecholamine biosynthesis with α-methyl-*p*-tyrosine. Plasma levels of corticosterone, insulin, glucose, and non-esterified fatty acids (NEFA) were also measured to evaluate metabolic changes.

Cold exposure stress study: Noradrenaline turnover was assessed from mice kept at 4°C. Mice kept at room temperature (23°C) served as controls. Noradrenaline turnover was significantly increased in the heart, but not in brown adipose tissue. Assuming that high noradrenaline turnover in the control brown adipose tissue group reflects activated sympathetic nerve tonus at room temperature, in the next cold exposure study, control mice were kept at thermoneutral ambient temperature (30°C). In this experiment, noradrenaline turnover was significantly accelerated by cold exposure not only in the heart, but also in brown adipose tissue and the pancreas. Noradrenaline turnover in the spleen, liver and mesenteric white adipose tissue was unchanged. Plasma levels of corticosterone, insulin, glucose, and NEFA were also unaffected. To maintain the control condition, control mice were consistently kept at 30°C in the following experiments.

Immobilization stress study: Noradrenaline turnover was assessed from immobilized mice. Free-moving mice kept at 30°C served as controls. Noradrenaline turnover in the spleen, pancreas and mesenteric white adipose tissue was accelerated by immobilization. Noradrenaline turnover in the heart, liver and brown adipose tissue was unchanged. Plasma levels of corticosterone and glucose were significantly elevated, NEFA was significantly decreased, but insulin was unaffected.

LPS treatment stress study: Noradrenaline turnover was assessed from LPS-injected mice (3 mg/kg, i.p.). Phosphate-buffered saline (PBS)-injected mice kept at 30°C served as controls. Noradrenaline turnover in all six organs assessed was not significantly affected by LPS treatment. The plasma level of corticosterone was significantly elevated, glucose was significantly decreased, but insulin and NEFA were unaffected.

In summary, sympathetic nervous system responses to three stressors differed greatly, as indicated by organ-specific noradrenaline

turnover acceleration patterns; therefore, the difference in activation patterns of the sympathetic nerve system is dependent on stress types. This important feature may lead to multiple biological responses.

Identifying phenotypic differences in sleep using wild-derived inbred mice

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Sleep is a complex behavior that is regulated by many factors and their interactions. In fact, hundreds of factors are known to determine the timing, amount, and incidence of rapid eye movement (REM) sleep, non-rapid eye movement (NREM) sleep, and wakefulness (W). Among them, neurotransmitters, including noradrenaline (NA), 5-hydroxytryptamine (5-HT), and dopamine (DA), are well-studied factors involved in sleep/wake regulation. Since known sleep factors alone are not able to fully explain the complexity of sleep, identifying new genetic factors involved in sleep/wake regulation is important.

As a first step in identifying the genetic factors underlying sleep/wake regulation, in the present study, electroencephalograms (EEG) were analyzed to obtain various sleep phenotypes in seven inbred mouse strains. Since inbred mouse strains have far surpassed 20 generations of inbreeding, they are homozygous in virtually all gene loci. Therefore, the strain differences in sleep phenotypes identified from this study can be attributed to differences in genotypes. The inbred mice used in this study were C57BL/6J (B6), a commonly used laboratory mouse strain, and six wild-derived mice strains, namely, PGN2/Ms (PGN2), NJL/Ms (NJL), BLG2/Ms (BLG2), KJR/Ms (KJR), MSM/Ms (MSM), and HMI/Ms (HMI). Wild-derived inbred mice have a far more diverse genetic background than laboratory mice, increasing the opportunity of identifying phenomenal sleep phenotypes. Brain monoamine (NA, 5-HT, DA) contents were also measured in seven inbred mouse strains to evaluate any correlations between brain monoamine contents and the sleep phenotypes identified.

In this study, five phenomenal strain differences were found in sleep phenotypes: 1) A block of 'high levels of W with less than 10% of sleep per hour' was observed immediately after dark onset in PGN2, NJL, BLG2, and KJR. In particular, the duration of this period reached four hours in BLG2 and KJR; 2) The amount of REM sleep during the dark period varied significantly among strains. HMI had the most REM sleep, 5-fold more than B6; 3) HMI had the significantly most REM sleep/NREM sleep during the light period among seven inbred mouse strains. The other five wild-derived mice strains had a similar ratio to B6. There were significant positive correlations between REM sleep/NREM sleep during the light period and brain NA or 5-HT contents; 4) HMI had the significantly lowest delta peak frequency (DPF) during NREM sleep among the seven inbred mouse strains. The other five wild-derived mice strains had similar DPF to B6; 5) KJR had clear alpha oscillations in addition to theta oscillations during REM sleep, which made it unique to this strain.

In summary, five phenomenal sleep

phenotypes were identified, which were potentially under strong genetic control. Since sleep phenotypes assessed by EEG spectral analyses during NREM or REM sleep were the most reproducible traits, strain differences in DPF observed during NREM sleep would be the most important 'candidate' sleep phenotype to pursue the responsible gene by the forward genetic approach.

Diet-induced obesity delays the processes of pregnant-associated mammary gland development in mice

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The mammary gland is one of the unique organs that primarily develop after birth. It is embedded in connective stromal tissue of the subcutaneous fat, and consists of slightly branched ducts with some side branches sprouting laterally in the inactive postpubertal state. Additional development of the mammary gland begins following the onset of pregnancy; that is, an increased number of side branches from the main ducts and the emergence of acinous structures at the tips of side branches, which will become the milk-secreting acini.

The events are highly regulated by circulating reproductive hormones and also local factors produced by the stroma. Disturbance in stromal environments by diet-induced obesity results in impaired lobuloalveolar development during pregnancy and reduced lactation in mice. Similarly, reduction of lactation yield and shortening of breastfeeding periods are found in obese cows and women, respectively; however, the mechanisms behind such observations remain to be elucidated.

We have recently demonstrated that diet-induced obesity disrupts ductal development in the mammary glands of nonpregnant mice. Obese mice had enlarged mammary glands, reflecting fat pad size, whereas the ducts in obese mice showed less dense distribution with less frequent branching. Additionally, the ducts were surrounded by thick collagen layers, and were incompletely lined with myoepithelium. Therefore, it was hypothesized that the disruption of fundamental duct development might lead to obesity-induced lactation failure. To test this hypothesis, the mammary glands of pregnant obese mice fed a high-fat diet were compared with those of lean mice fed a normal diet.

Obese mice showed a similar conception rate, litter size and fetal growth to lean mice. Obese mice also showed similar plasma estrogen levels during pregnancy, but slightly higher progesterone concentrations than the controls.

Stereoscopic examination revealed that mammary glands time-dependently developed in lean mice as follows: increased number of side branches occurred during early pregnancy, emergence of acinous structures increased mid-pregnancy, and milk-producing acini appeared during late pregnancy. In contrast, obese mice had fewer side branches and acinous structures in early and mid-pregnancy, respectively. In addition, obese mice had fewer acini with lipid droplets even in late pregnancy.

Histological examination revealed no difference in the cellular composition of side branches and acinous structures between lean

and obese mammary glands, while the main ducts in obese mice were surrounded by thick collagen layers, and luminal epithelia of the duct were incompletely lined with myoepithelium, as mentioned. Side branches and acinous structures were also composed of luminal epithelia and myoepithelia surrounded by the basement membrane, but not by the collagen layer.

Biochemical examination exhibited a decrease in the expression of pregnancy-associated genes in the mammary glands of obese mice. Expression of *Wap* mRNA was suppressed mid- and late pregnancy, while the expression of *Occludin* mRNA was decreased mid-pregnancy.

The expression of leptin and leptin receptor was then examined in the mammary gland, because it is a representative stromal factor and we have demonstrated that leptin inhibits mammary epithelial cell proliferation and differentiation *in vitro*. Leptin was highly expressed in the obese mammary gland and hyperleptinemia was produced, while leptin receptors were localized in the adjacent region of main ducts and side branches but rarely in the surrounding region of acini, in both lean and obese mammary glands.

In summary, it was demonstrated that diet-induced obesity delays the morphogenic and functional processes of pregnant-associated mammary gland development in mice, especially increased side branch formation in the early pregnancy period. Since leptin receptors were localized in the region adjacent to side branches and leptin inhibits mammary epithelial cell proliferation and differentiation, it is suggested that highly induced hyperleptinemia and local leptin are involved, at least in part, in the disruption of side branch development that proceeds to acinus formation. Thus, disruption of fundamental duct development and sprouting of side branches might lead to obesity-induced lactation failure.

Effects of dopamine on monosynaptic and nociceptive reflex potential in rat spinal cord

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The function of the spinal cord is strongly modulated by monoamines, such as noradrenaline (NA), serotonin (5-HT) and dopamine (DA). It is well-known that descending NA and 5-HT fibers originating from the brainstem project to dorsal horn neurons and that these descending fibers produce inhibitory and/or excitatory effects on synaptic transmission in the spinal cord. For example, NA produces antinociception by inhibiting the release of glutamate and substance P from the primary afferent terminals through the activation of α_2 adrenoceptors. There is a wide body of evidence that descending DA fibers project to the dorsal horn neurons. However, the effect of DA on the spinal cord is not understood. In the present study, the effect of exogenous and endogenous DA on synaptic transmission in the spinal cord was examined.

In the isolated spinal cord from neonatal rats, monosynaptic reflex potential (MSR) and nociceptive reflex potential (slow ventral root potential; sVRP) were recorded from the lumbar ventral root by electrical stimulation of the corresponding dorsal root. DA (0.01–3 μM) inhibited sVRP in a concentration-dependent manner, but did not affect MSR. The inhibition

of sVRP by DA was reversed by D1-like-receptor (D1-like-R) antagonists, SCH23390 and LE300, but not by a D2-like-receptor (D2-like-R) antagonist, haloperidol. In the presence of raclopride, a more selective D2-like-R antagonist, sVRP inhibition by DA was partly attenuated, but the inhibitory effect was largely remained. The effect of DA was mimicked by a selective D1-like-R agonist, SKF83959. Furthermore, methamphetamine (MAP), an endogenous DA releaser, inhibited sVRP. Similar to the effect of DA, sVRP inhibition by MAP was abolished by D1-like-R antagonists (SCH23390 and LE300), but not by D2-like-R (haloperidol and raclopride), α_2-adrenoceptor (atipamezole) and 5-HT_{2A}-R (ketanserin) antagonists. On the other hand, MAP inhibited MSR, which was reversed by haloperidol but not by raclopride. Similar to haloperidol, LE300 attenuated the inhibition of MSR by MAP, but SKF83959 did not inhibit MSR. It is reported that haloperidol and LE300 are effective in inhibiting 5-HT_{2A}-R. Ketanserin, a 5-HT_{2A}-R antagonist, reduced MSR inhibition by MAP.

In conclusion, exogenous and endogenous DA selectively inhibits sVRP via D1-like-R, and MSR inhibition by MAP is due to endogenous 5-HT through the activation of 5-HT_{2A}-R. Our data suggest that endogenous DA modulates spinal nociceptive transmission because sVRP is considered to reflect nociception.

Study of the pathogenicity of influenza virus in chickens

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Highly pathogenic avian influenza viruses (HPAIVs) cause lethal systemic infection in chickens. The cause of death of chickens infected with HPAIVs is poorly understood. Sakabe found that, in chickens, the 50% lethal dose of A/turkey/Italy/4580/1999 (H7N1) (Ty/Italy/99) was 10^{-4} of that of A/chicken/Netherlands/2586/2003 (H7N7) (Ck/NL/03). In the present study, each strain was inoculated intranasally into four-week-old chickens, and viral growth and pathological findings were examined to elucidate the pathogenesis of HPAIVs.

Ty/Italy/99 replicated efficiently in each organ, including the brains and hearts of chickens, causing sudden death within four days' post-inoculation. On the other hand, Ck/NL/03 replicated more slowly than Ty/Italy/99 and killed chickens within six or seven days' post-inoculation. Viruses in the blood of chickens infected with Ty/Italy/99 increased rapidly and were maintained until they died, while viruses detected in the blood of chickens infected with Ck/NL/03 increased slower and were more transient. It is suggested that the efficiency of viral replication in endothelial cells differed between strains. In histopathological analysis, severe inflammatory lesions were observed in the brains and hearts of chickens infected with Ck/NL/03, indicating that the cause of death of chickens infected with Ty/Italy/99 may be different from that with Ck/NL/03, such as multiple organ disorder induced by cytokine storm.

Future studies are needed on viral replication in endothelial cells and innate immune response to infection with these viruses in order to determine the factors responsible for the pathogenicity of influenza viruses in chickens.

Antigenic analysis of envelope glycoprotein E2 of bovine viral diarrhea virus

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Bovine viral diarrhea virus (BVDV) has been classified into two genotypes, BVDV-1 and BVDV-2, which are antigenically correlated. BVDV-1 is genetically subdivided into 1a to 1o subgroups and BVDV-2 into 2a and 2b. In this study, the antigenicity of viral glycoprotein E2 was analyzed among BVDV-1a, BVDV-1b and BVDV-2a subgroup viruses, which recently became prevalent in Japan. Antigenic comparison of antigenicity was carried out using monoclonal antibodies (MAbs) recognizing different epitopes on the E2 molecule, which is responsible for attachment and entry of the virus into host cells.

Seventeen anti-E2 MAbs were produced from hybridoma cells fused with the lymphocytes of mice immunized with *E. coli*-expressed recombinant E2 proteins of BVDV-1a strain Nose, 1b IS27CP/01 and 2a KZ-91-CP. Six of those MAbs with neutralizing activity were used for the analysis of cross-reactivity among the different subgroup viruses isolated in Japan by the cross-neutralization test. It was found that three MAbs recognized subgroup-common neutralizing epitopes on the E2 protein of BVDV-1, two MAbs recognized the epitopes varying between viruses of BVDV-1 subgroups, and the other recognized a BVDV-2-specific epitope.

In conclusion, the present anti-E2 MAbs are useful for differentiating genotypes 1 and 2 of BVDV and antigenic analysis of the virus. It was also suggested that antigenicity of the E2 protein varied in BVDV-1 viruses. Further studies are required using MAbs to analyze the viral infection cycle in host cells, and the structure and function of BVDV E2 protein.

Molecular characterization of immunoinhibitory factors PD-1/PD-L1 in bovine leukemia virus-infected cattle

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An immunoinhibitory receptor, programmed death-1 (PD-1), and its ligand, programmed death-ligand 1 (PD-L1), are involved in immune evasion mechanisms for several pathogens causing chronic infections. Blockade of the PD-1/PD-L1 pathway by antibodies specific to either PD-1 or PD-L1 resulted in the re-activation of immune reactions, and is expected to be applied to new therapies for chronic infectious diseases; however, no functional analysis of these molecules has been reported for domestic animals. Thus, in this study, cDNA encoding for

bovine PD-1 and PD-L1 was cloned and sequenced, and then its expression and roles were analyzed in bovine leukemia virus (BLV)-infected cattle.

Full-length cDNA sequences encoding for bovine PD-1 and PD-L1 were cloned, and the deduced amino acid sequences of bovine PD-1 and PD-L1 showed high homology with those of human and mouse PD-1 and PD-L1, respectively. Functional domains, including an immunoreceptor tyrosine-based inhibitory motif in the intracellular domain of PD-1, were well conserved among cattle and other species. *PD-1* mRNA was predominantly expressed in T cells while *PD-L1* mRNA was detected in monocyte-lineage cells, and both *PD-1 and PD-L1* mRNAs were upregulated in T cells by stimulation with Concanvalin A or anti-CD3 antibody.

Next, the expression of PD-L1 was analyzed on peripheral blood mononuclear cells (PBMC) from BLV-infected cattle in asymptomatic (AL), persistent lymphocytosis (PL), and enzootic bovine leukemia (EBL) stages by real-time PCR and flow cytometry. PD-L1 expression on PBMC, especially B cells in cattle in PL and EBL stages, were higher than in cattle in the AL stage or in uninfected cattle. In these cattle, PD-L1 was expressed on BLV-infected cells. The level of PD-L1 expression correlated with the degree of disease progression, although real-time PCR showed that *PD-1* and *PD-L1* mRNA expressions in PBMC from BLV-infected cattle, especially in the PL stage, were lower than those from uninfected cattle. Blockade of the PD-1/PD-L1 pathway in PBMC by either PD-L1-specific antibodies or recombinant PD-L1 upregulated the production of IL-2 and IFN-γ in both BLV-infected and uninfected cattle, as well as inhibiting the proliferation of BLV in PBMC from BLV-infected cattle.

The immunoinhibitory functions of bovine PD-1 and PD-L1 were shown in this study, and PD-L1-induced inhibition may play a key role in the disease progression of chronic BLV infection. Thus, immune reactivation, such as blockade of the PD-1/PD-L1 pathway, would be essential to protect cattle from BLV infection and to inhibit disease progression. Further investigations are necessary to develop new vaccination and therapy methods against BLV infection using inhibition of the PD-1/PD-L1 pathway.

Identification of Salp16 Iper, an immunosuppressant molecule, in *Ixodes persucatus*

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Ixodid ticks are harmful vectors of several pathogens causing infectious diseases and afflicting both human public health and the livestock industry. Particular emphasis is placed on tick control using anti-tick vaccine since this could also prevent the transmission of multiple pathogens. Amongst numerous pathogens, *Anaplasma phagocytophilum* has a remarkably broad host range from humans to reptiles, and induces granulocytic anaplasmosis, causing influenza-like symptoms and abortion in humans and livestock, respectively.

Salp16 is a 16-kDa tick salivary gland protein, derived from *Ixodes scapularis*, which is known as a vector of *A. phagocytophilum* in Northern America, and has been reported in the

transmission of *A. phagocytophilum*. Infection with *A. phagocytophilum* enhances the expression of the *Salp16* gene in the tick salivary gland, and silencing of *Salp16* by RNA interference inhibits primary infection with *A. phagocytophilum* in ticks. However, how Salp16 facilitates infection with *A. phagocytophilum* in ticks and contributes to tick blood feeding have not yet been elucidated in detail. Recent studies have revealed that *A. phagocytophilum* is present in Japan, and that *I. persulcatus* acts as a vector, raising the possibility that *A. phagocytophilum* also utilizes specific molecules present in *I. persulcatus* for its transmission in Japan. Thus, in this research, Salp16 homologues were identified in *I. persulcatus*, and their functions in blood feeding by ticks were studied.

Two cDNA clones, which show high similarity to *Salp16* cDNA of *I. scapularis*, were isolated from salivary glands of fed female *I. persulcatus* ticks, and designated as *Salp16 Iper-1* and *-2*. Expression analyses of *Salp16 Iper* by semi-quantitative and real-time RT-PCR showed that its expression was salivary gland-specific and was upregulated by blood feeding. In addition, the expression of *Salp16 Iper* was highly upregulated in *A. phagocytophilum*-infected ticks. To investigate the function of Salp16 Iper, recombinant Salp16 Iper (rSalp16 Iper) was prepared in both bacterial and insect cells. rSalp16 Iper was recognized by tick-immunized hamster sera, indicating that it is a secretory protein when exposed to host animals. Chemiluminescence assay showed that oxidative burst of activated bovine neutrophils, target cells for the infection with *A. phagocytophilum*, was attenuated in the presence of rSalp16 Iper. In a vaccination trial of rSalp16 Iper to examine its role in tick blood-feeding, ticks that fed on rSalp16 Iper-immunized animals died during feeding, or died without laying eggs even after becoming engorged. In addition, the number of neutrophils sucked into ticks was markedly increased in those that fed on rSalp16 Iper-immunized animals compared to unimmunized control animals. These results imply that Salp16 Iper molecules contribute to the establishment of blood feeding as an immunosuppressant of neutrophils, which play a pivotal role in host innate immunity. Moreover, inhibition of the oxidative burst of neutrophils by ticks seems to be significant in helping the transmission of *A. phagocytophilum*, which has been confirmed to be vulnerable to reactive oxygen species. Thus, further characterization of tick molecules, including Salp16 Iper, which ticks use to modulate host immune responses, could lead to the development of new strategies to prevent both the transmission of tick-borne diseases and blood-feeding by ticks.

Comparative studies of the regulatory mechanisms of immune responses of wild waterfowls and chickens

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Marek's disease (MD), caused by Marek's disease virus (MDV), is T cell lymphoma in its natural host, chickens. MD has been controlled by the administration of live vaccines of attenuated or nonpathogenic strains of MDV. Recently, increases in the virulence of several MDV field isolates and several MD cases, even in vaccinated chickens, have been reported. In

addition, although the natural hosts of MD are thought to be chickens and quails, wild waterfowl, including white-fronted geese and mallards, have also been found to be infected with MDV, while very few clinical MD cases have been reported thus far. These observations suggest the risk of future outbreaks in both chickens and wild birds. For this reason, it will be necessary to clarify the molecular mechanism of the transformation by MDV, and subsequently to develop new strategies to control virulent MDV in the field. Previous epidemiological surveys and studies using experimental birds also showed the possibility that immune responses in wild waterfowl are different from those in chickens, and thus wild waterfowl acquire resistance to MD. In this study, comparative analysis was performed on the regulatory mechanisms of immune responses of wild waterfowl and chickens.

Several host factors as well as viral factors are involved in the disease progression of MD and resistance to MD. One is interferon γ (IFNγ), which plays an important role in cell-mediated immunity against MDV and the maintenance of latent MDV infection. Promoter regions of the *IFN*γ gene were cloned from white-fronted geese, MD-susceptible B19 chickens, MD-resistant B21 chickens, and commercial chickens, and their promoter activities were measured by luciferase reporter assay. No notable difference in activities was observed among them; however, promoter activity was more strongly repressed in white-fronted geese than chickens when Meq, an MDV oncoprotein of a transactivator, was co-expressed. In addition, polymorphisms found in the 5' region of 1–90 are responsible for this difference. No difference in promoter activities was observed among B19, B21, and commercial chickens. These results suggest that, in white-fronted geese, reduced expression of IFNγ by Meq early after MDV infection could result in the lower degree of T cell activation, and subsequently decrease the infection rate of activated T cells, the target of MDV transformation, to reduce the chances for the birds to develop MD. Moreover, the nucleotide sequence of the *IFN*γ gene promoter region of white-fronted geese showed very high homology with that of mallards, suggesting that mallards could have a similar immune response to white-fronted geese against MDV.

Next, the promoter activities of the regulatory region of the genes for BNK and B-lec, known as natural killer (NK) receptors of chickens and candidates for a resistant-determining factor, were compared between B19 and B21 chickens since no difference was shown in the regulation of IFNγ expression between them. B21 chickens showed lower activities of *BNK and B-lec* promoter regions than B19 chickens. This result coincides with reports that BNK can contribute to resistance to MD because BNK is thought to be an inhibitory NK receptor, and that B21 chickens show higher NK cell activity than B19 chickens.

This study showed that a lower expression of IFNγ in white-fronted geese could be an important factor contributing to resistance to MD, while BNK may be an important determinant of the differences in resistance to MD between B19 and B21 chickens. However, further detailed investigation will be required to confirm these findings by using birds experimentally infected with virulent MDV. In addition, other factors involved should be examined in the future.

Application of "EKITTO®", a new immunochromatography kit for the detection of canine *Echinococcus multilocularis* coproantigen in vulpine feces

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Echinococcosis, caused by *Echinococcus multilocularis*, is a serious parasitic zoonosis in Hokkaido, Japan. The parasite is primarily maintained in the sylvatic cycle, and foxes serve as the principal definitive hosts. For an epidemiological survey of *E. multilocularis,* examination of *E. multilocularis* coproantigen in vulpine feces collected in the field is an important tool. In this study, a detection kit for canine *E. multilocularis* coproantigen using an immunochromatography method,"EKITTO®, was applied to vulpine feces to improve its accuracy.

Results of EKITTO® using manufacturer-provided buffering solution and the prescribed instructions for vulpine feces were compared with the results of ELISA (standard method). The sensitivity, specificity and accuracy of EKITTO® based on ELISA were 71.4%, 74.3% and 73.9%, respectively; therefore indicating that EKITTO® with the existing buffer and conditions would be difficult to use for vulpine feces.

To apply EKITTO® to vulpine feces, the composition of the buffering solution and the procedures were investigated. It was suggested that 0.2 M phosphate buffer with 0.87% NaCl, 2.5% BSA, 5% amino acid X, 1% formalin, adjusted to pH 6.4, was suitable for vulpine feces. When using EKITTO® for canine feces, the fecal suspension is immediately applied to the reaction sheet and the results can be visualized in 30 min. However, for antigen elution from vulpine feces and sufficient buffering, the suspension should be maintained at room temperature for over 20 hr and the results will be available after 2 hr. With these improvements, the sensitivity of EKITTO® was markedly increased to 100% (based on ELISA and egg examination).

For the practical use of EKKITO® for vulpine feces in the field, this improved buffering solution was examined using a feces-collection container specialized for EKITTO®. As a result, the non-specific reaction increased because development on the reaction sheet was inhibited by impurities passing through the filter in the container.

Furthermore, feces treated by heating (70°C, 12hr) showed more non-specific reaction than those treated by freezing (−80°C, over 2wks). Freezing treatment was recommended as an ovicidal method when using EKITTO® for vulpine feces.

Although further improvements are required, EKKITO® with the proposed buffering solution and procedures in this study could be applied for vulpine feces as a detection kit for *E. multilocularis* coproantigen.

Epidemiological survey of hemoprotozoan infections in domestic ruminants in Myanmar

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In Asia and Africa, hemoprotozoan diseases, such as piroplasmosis and trypanosomosis, are responsible for serious economic losses in the livestock industry. In Myanmar, although there have been case reports of hemoprotozoan diseases in domestic ruminants, a nationwide epidemiological survey has never been carried out. In the present study, the current situation of *Babesia bovis*, benign *Theileria* parasites (*T. sergenti*/*buffeli*/*orientalis*) and *Trypanosoma evansi* infections in cattle and goats in Myanmar was examined using molecular diagnostic tools.

In January 2009, 310 blood samples were collected from 191 cattle and 119 goats at six farms and two villages in the suburbs of three cities, Nay Pyi Daw, Mandalay and Pyin-Oo-Lwin, in Myanmar. Blood samples were applied to FTA® Elute cards (Whatman®) and total DNA was extracted from the cards. Target genes for PCR analysis were the 18S rRNA gene of *B. bovis,* major piroplasm surface protein (MPSP) genes of benign *Theileria* parasites, and RoTat 1.2 variable-surface glycoprotein gene of *T. evansi*, respectively.

As a result of the PCR assay, in cattle, *B. bovis* and benign *Theileria* parasites, but not *T. evansi* were detected. In goats, none of the parasites was detected. The prevalence of *B. bovis* was 27.7% (52/191) in cattle in three regions. According to cattle breeds, the prevalence in Zebu, indicus cattle, which is known to be resistant to piroplasmosis, and other breeds was 8.5 and 38.8%, respectively. According to feeding conditions, *B. bovis* was detected in both grazing and no-grazing cattle. According to age, *B. bovis* was detected in only young cattle in Zebu, but in both young and adult cattle in other breeds. The prevalence of benign *Theileria* parasites was 2.6% (5/191) in two regions, Mandalay and Pyin-Oo-Lwin, and all of these animals were non-Zebu breeds. Phylogenetic analysis of MPSP genes of benign *Theileria* parasites in Myanmar revealed at least four genetic types, one of which appears to be closely related to a virulent type, which occasionally causes economic losses in Japan and Korea.

In conclusion, this epidemiological survey showed that tick-borne hemoprotozoan parasites are prevalent in Myanmar. Further studies, including a survey of piroplasmosis in different regions and seasons, and detection of parasites from ticks are required.

Analysis of genes responsible for T-helper immunodeficiency (*thid*) in the LEC rat

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Expression patterns of the tensin family in the mouse kidney

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Effect of ascidian powder on immunoregulation in mouse inbred strains

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Study on clinical significance of serum Tenascin-C levels in dogs with cardiac diseases

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Tenascin-C (TNC), is an extracellular matrix hexamer glycoprotein that plays an important role in tissue remodeling. TNC is highly expressed during embryonic development and processes involving extracellular matrix remodeling and cell migration, such as in cancer invasion, fibrosis, and wound healing. TNC is not detected in the adult human heart; however, it is re-expressed under pathologic conditions, such as acute myocardial infarction and myocarditis, and dilated cardiomyopathy (DCM). In the heart, TNC plays important roles in inhibiting the strong linkage between cardiomyocytes and connective tissues, and

promotes myocardial remodeling. TNC also has the potential to promote fibrosis of the myocardium by recruiting myofibroblasts. Furthermore, while TNC molecules are deposited in local extracellular spaces, soluble forms are also released into the bloodstream. As a result, serum TNC levels are significantly elevated, corresponding to the local expression of TNC. Therefore, TNC might be useful as a novel biomarker, and it is currently known that serum TNC levels are significantly elevated in patients with acute myocardial infarction, myocarditis, and DCM. In contrast, there are no reports on the expression of TNC in dogs with cardiac diseases. Consequently, the aim of this study was to establish serum TNC levels in normal dogs and to clarify the clinical implications of serum TNC levels in various canine cardiac diseases.

Serum TNC levels were measured using an enzyme immunoassay (EIA) kit in 67 dogs with various cardiac diseases and 62 normal controls. Clinical, radiographic, and echocardiographic findings were analyzed using Student's *t*-test, Tukey-Kramer's HSD test, and Pearson's correlation analysis to estimate correlations between variables. The mean serum TNC level in normal controls was 299.5 ng/ml (standard error: $\pm$ 14.7 ng/ml). Serum TNC levels showed a significantly negative correlation with age in normal controls ($P < 0.01$, $r = -0.38$); therefore, the mean serum TNC level in dogs with MR occurring with TR ($n = 15$) was significantly higher than in normal controls of more than 6 years old ($n = 30$) ($P < 0.01$). The mean serum TNC levels in the International Small Animal Cardiac Health Council class III were significantly higher than in other classes and normal controls ($P < 0.001$). In addition, serum TNC levels showed a significantly positive correlation with the Vertebral Heart Score (VHS) ($P < 0.05$, $r = 0.41$) and left atrial diameter/aortic diameter ratio (LA/Ao) ($P < 0.05$, $r = 0.35$); however, serum TNC levels showed no correlation with cardiac murmur, ejection fraction (EF), early diastolic filling wave (E wave), and regurgitated blood flow velocity.

In conclusion, it was demonstrated that serum TNC levels may be a useful biomarker for assessing the severity of cardiac diseases and for myocardial remodeling. Further study on the disease specificity and prognostic value of serum TNC levels in dogs with cardiac diseases is necessary for its clinical application.

Molecular cloning of canine interleukin-17A and quantitative analysis of its gene expression in peripheral blood mononuclear cells

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Interleukin(IL)-17A is a recently discovered proinflammatory cytokine produced by activated $CD4^+$ and $CD8^+$ T cells, and thought to play an important role in various human and murine inflammatory, immune-mediated and allergic disorders. Th17 cells are a distinct lineage of $CD4^+$T cells that produce IL-17A, and their differentiation from naïve T cells is under the control of transforming growth factor (TGF)-β and IL-6 in humans and mice. However, little is

known about the roles of IL-17A in various disorders of dogs and the mechanism of canine Th17 cell differentiation. In the present study, canine IL-17A were cloned, and the mechanism of canine Th17 cell differentiation investigated to quantify canine IL-17A gene expression in peripheral blood mononuclear cells (PBMC) from patients with various inflammatory and immune-mediated disorders.

At first, the entire open reading frame of canine IL-17A, encoding a 155 amino acid sequence, was cloned from mitogen-activated PBMC by reverse transcription-polymerase chain reaction (RT-PCR) and rapid amplification of cDNA end methods. The expression of canine IL-17A in PBMC and various tissues was examined by quantitative RT-PCR (qRT-PCR), and canine IL-17A mRNA expression was restricted to activated PBMC.

Next, to elucidate the mechanism of canine Th17 cell differentiation, the effect of TGF-β and IL-6 on the canine IL-17A expression level in activated PBMC was analyzed by qRT-PCR. Co-stimulation of activated PBMC with TGF-β and IL-6 showed a tendency toward an increased canine IL-17A gene expression level, but this change of the gene expression level was not statistically significant. Therefore, the effects of TGF-β and IL-6 on PBMC without mitogen activation were analyzed by qRT-PCR, and a significant increase in the canine IL-17A gene expression level was observed in co-stimulated PBMC.

Lastly, canine IL-17A gene expression levels in PBMC of patients with several inflammatory (n=8) and immune-mediated (n=1) disorders were measured by qRT-PCR. Canine IL-17A gene expression levels in PBMC of these patients were significantly lower than in normal controls (n=8).

In conclusion, the present study demonstrates that canine IL-17A is produced by activated PBMC, and that co-stimulation of canine PBMC with TGF-β and IL-6 induces IL-17A-producing mononuclear cells, which correspond to Th17 cells. Further studies are necessary to clarify the role of canine IL-17A in the pathogenesis of inflammatory and immune-mediated disorders in dogs.

Babesia gibsoni: A study on its invasion into peripheral blood mononuclear cells and its proliferation in those cells *in vitro*

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In the present study, to investigate the role of canine leukocytes in the lifecycle of *Babesia gibsoni*, the possible invasion of parasites into leukocytes, their proliferation in those cells, and the multiplication of *B. gibsoni*-infected leukocytes were observed *in vitro*.

Canine leukocytes remaining in the culture of *B. gibsoni* had a large and foamy shape. *B. gibsoni* was detected in those leukocytes by Hoechst staining and immunofluorescent staining using the rabbit anti recombinant *B. gibsoni* heat shock protein 70 (BgHsp70) antibody. Since those cells were positive for CD21, CD5 and CD14, they were mononuclear

cells.

Subsequently, when *B. gibsoni* removed from erythrocytes was cultured with fresh erythrocytes, a change in the number of parasites was observed by counting copy numbers of the BgHsp70 gene using quantitative real-time PCR (qRT-PCR). As a result, the number of parasites was not increased before the appearance of infected erythrocytes. Moreover, when free parasites were cultured with peripheral blood mononuclear cells (PBMC), the number of parasites was also not increased. These results suggested that *B. gibsoni* proliferated neither in PBMC nor outside of erythrocytes. On the other hand, a change in the number of leukocytes or PBMC was also observed by counting copy numbers of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene using qRT-PCR in the experiments described above. As a result, the numbers of leukocytes and PBMC decreased. These results indicated that *B. gibsoni*-infected leukocytes would not multiply.

Additionally, free *B. gibsoni* was cultured with PBMCs, and fresh erythrocytes were added to the culture on day 3. Although the parasites were cultured for 7 days, infected erythrocytes were not observed during the culture period. This result showed that *B. gibsoni* did not go through PBMCs before invading erythrocytes.

Furthermore, when PBMCs isolated from an experimentally chronic *B. gibsoni*-infected dog were cultured for 4 months, both the BgHsp70 gene and GAPDH gene disappeared within one month, indicating that *B. gibsoni* could not survive for long periods within leukocytes. Therefore, it was supposed that *B. gibsoni* would not invade mononuclear cells as part of its lifecycle although parasites were detected in those cells.

Finally, free *B. gibsoni* was cultured without host cells, and fresh erythrocytes were added to the culture on day 3. Although the parasites were cultured for 7 days, infected erythrocytes were not observed, indicating that *B. gibsoni* could survive for less than 3 days without erythrocytes.

In conclusion, although *B. gibsoni* was detected in mononuclear cells, it did not proliferate in those cells and leukocytes did not multiply. Accordingly, it was considered that leukocytes would not be necessary for the lifecycle of parasites. In other words, *B. gibsoni* needed only erythrocytes for its lifecycle and could grow and proliferate.

Study on the change of matrix metalloproteinase (MMP)-2 and -9 activities in synovial fluid from thoroughbred horses with osteochondrosis dissecans or intra-articular fracture

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In thoroughbred racehorses, joint disease is the main cause of lameness and is a common and important problem that influences their athletic performance. In order to determine suitable treatment and prognosis, clinical and laboratory assessment of intra-articular pathologies is essential in the early stage of joint disease.

Recently, biochemical markers have been under investigation to identify physiological and pathological tissue remodeling in joint cartilage. Matrix metalloproteinases (MMPs) are enzymes with a key role in cartilage homeostasis and it has been reported that their activities are increased in several joint diseases. In racehorses, there have been studies on the activities of MMPs and their relationship with joint pathology; however, the details of their role are unclear and very limited. The object of this study was to discuss the significance of the change of MMP-2 and -9 in synovial fluid from thoroughbred racehorses with osteochondrosis dissecans (OCD) and intra-articular fracture.

First, MMP-2 and -9 activities in equine synovial fluid were examined by gelatin zymography. Synovial fluid samples were collected from diseased and contralateral joints at the time of arthroscopic surgery for OCD or intra-articular fracture. Second, procollagen II c-propeptide (CPII) concentrations and collagen type II cleavage (C2C) concentrations were examined by inhibition enzyme-linked immunosorbent assay (ELISA). Synovial fluid concentrations of total protein (TP) and lactate dehydrogenase (LDH) were also measured. The correlation among MMP activity, biomarkers, and the value of biochemical examinations was evaluated.

Zymographic analysis revealed that MMP-2 and -9 activities of synovial fluid from affected joints were significantly increased compared to contralateral joints. Concentrations of CPII were significantly elevated in both OCD and intra-articular fracture. In intra-articular fracture, concentrations of C2C in diseased joints were significantly elevated compared to contralateral joints. Furthermore, C2C concentrations in fracture joints were significantly increased, compared with OCD joints. TP concentrations were also significantly higher in affected joints than contralateral joints in both OCD and fracture. LDH in intra-articular fracture is significantly elevated compared with contralateral joints. There is some correlation between MMPs and biochemical markers.

In conclusion, the results indicated that gelatin zymography would be simple and easy to analyze MMP-2 and -9 activities in synovial fluid from horses. Both synovial MMP-2 and -9 activities seemed to be useful markers to indicate the pathological status of joint degeneration, as well as remodeling in OCD and intra-articular fracture of thoroughbred racehorses.

Mechanism of anti-inflammatory effect of polysulfated glycosaminnoglycan in cultured equine synoviocytes and chondrocytes

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Osteoarthritis is one of the most common performance-limiting diseases of race and competition horses. Recently, glycosaminoglycans, including glucosamine and chondroitin sulfate, and hyaluronic acid have been used to improve osteoarthrosis; however, their mechanisms of action are not known in detail. The purpose of this study was to investigate the underlying

mechanisms of the clinical effect of polysulfated glycosaminoglycan (PSGAG), using cultured equine synoviocytes and chondrocytes.

Cultured equine synoviocytes and chondrocytes were stimulated by 10 ng/ml interleukin (IL)-1β or 10 ng/ml tumor necrosis factor (TNF)-α with or without pretreatment with 10 or 100 μg/ml PSGAG for 24 hr. Subsequently, phosphorylation of two mitogen-activated protein kinases (MAPKs) (JNK and p38) and the expression of matrix metalloproteinase (MMP)-3 were analyzed by Western blotting, and nuclear translocation of nuclear factor (NF)-κB was evaluated by the immunofluorescence technique.

In both synoviocytes and chondrocytes, both IL-1β and TNF-α induced the phosphorylation of JNK and p38, production of MMP-3, and nuclear translocation of NF-κB, all of which were reduced with PSGAG pretreatment compared to the controls.

These results suggest that PSGAG has a series of anti-inflammatory effects, which inhibit the activation of certain MAPKs, nuclear translocation of NF-κB, and, subsequently, the production of MMP-3 in equine synoviocytes and chondrocytes in an inflammatory environment.

Isolation and characterization of canine adipose-derived stromal cells

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The therapeutic potential of pluripotent stem cells for applications such as tissue engineering and gene therapy is enormous. Adipose tissue would be an attractive source of mesenchymal stem cells (MSCs) because of its abundance and ease of access to donor sites with minimal morbidity. The purpose of this study was to provide basic information about the isolation technique and characterization of canine adipose-derived stromal cells (ADSCs) to take them into consideration for clinical application.

First, 10 clinically healthy dogs were used as sources of adipose tissue, from which ADSCs were isolated and characterized by their cell numbers. In four dogs, mRNA expression of three stemness markers, Sox2, Nanog and Oct3/4, was positive using reverse transcription-polymerase chain reaction (RT-PCR) for their ADSCs. In three dogs, which were positive for all three stemness markers on their ADSCs, further investigation of multilineage differentiation, including adipogenic, osteogenic and chondrogenic lineages, was performed using the respective lineage-specific induction factors. In these three ADSCs, their differentiation was confirmed by staining or the detection of lineage-specific markers using RT-PCR, although the degree of differentiation varied in each ADSC.

Second, the neovascularizing capacity of ADSC was investigated by injecting ADSCs into the hindlimb of Balb/c nude mice with surgically created acute ischemia, and the degrees of tissue vascular distribution and perfusion were examined by laser Doppler. The laser Doppler perfusion index of ADSC-treated animals was significantly higher than those of negative control animals.

In conclusion, it was suggested that ADSCs possess adipogenic, osteogenic and chondrogenic

potential *in vitro* and angiogenic ability *in vivo*. Further studies are necessary to optimize the preparation conditions of ADSCs from clinical animals and to provide complete information to fully use their ability in the clinical field of reconstruction and regeneration veterinary medicine.

Approach to identify host-derived transcripts binding to rabies virus nucleoprotein

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Rabies is a lethal viral zoonosis occurring in many mammals, including humans. After the appearance of neurological signs due to rabies, almost all patients invariably die. The development of therapeutic measures for rabid animals and humans is therefore required. Nucleoprotein (N) is a rabies virus-derived protein most abundantly expressed in infected cells. Transcription and replication of the viral genome is regulated by N through binding with its RNA. It has been reported that N is related with the pathogenicity of rabies virus because recombinant low-pathogenic virus that exchanged its *N* gene from the low-pathogenic to high-pathogenic type killed mice. In this study, it was hypothesized that N expression in neuronal cells might induce cell dysfunction by interacting with host-derived transcripts, and I attempted to identify the genes by performing cross-linking and immunoprecipitation (CLIP) methods. After constructing N-expressing plasmid, N expression was confirmed by Western blotting and immunocytochemistry in a mouse neuroblastoma cell line, NA. By isolation and sequence analysis of the N-RNA-interacting fraction, seven host-derived genes were identified (*Ngg1 interacting factor 3-like 1* (*Nif3l1*), *ADP-ribosylhydrolase like 1* (*Adprl1*), *POU domain class1 transcription factor 1* (*Pou1f1*), *RIKEN cDNA 4930564C03 gene* (*4930564C03Rik*), *Phosphoinositide-3-kinase interacting protein 1* (*Pik3ip1*), *Spire homolog 1* (*Spire 1*), *Ubiquitin-conjugating enzyme E2, J1* (*Ube2j1*)). *Nif3l1* was detected from several clones, and it was strongly suggested that *Nif3l1* interacts with N. Because Nif3l1 is a molecule related with intracellular transport of proteins, virus trafficking or expression levels of host-derived genes in rabies virus-infected cells might be affected by N. While other genes are related with apoptosis, it was also suggested that the viability of cells infected with rabies virus might be affected by N expression.

Role of COX-2 in suckling mice with type A influenza virus infection

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Influenza encephalopathy is a central nervous system disease of humans induced by influenza virus infection, and most frequently occurs in infants. It is well-known that the prognosis of influenza encephalopathy worsens by treatment of cyclooxygenase-2 (COX-2) -selective inhibitors, such as diclofenac sodium and mefenamin acid. On the other hand, it has been reported that COX-2-deficient adult mice infected with type A influenza virus show higher survival rates than wild-type mice, indicating that COX-2 expression deteriorates the clinical condition. In the present study, the role of COX-2 in sucking mice infected with type A influenza virus was examined. Type A influenza virus was inoculated into 1-week-old COX-2-deficient mice and wild-type C57BL/6 mice, and the histopathological changes, viral titers and the induction of inflammatory cytokines were analyzed in both mice. Cyclooxygenase-2-deficient mice showed a higher survival rate than wild-type mice. Viral titers isolated from the lungs were almost the same in wild-type and COX-2-deficient mice. Histopathologically, pulmonary lesions in wild-type mice were diffuse bronchointerstitial pneumonia, whereas those in COX-2-deficient mice were localized and accompanied marked infiltration of neutrophils, macrophages and mature T lymphocytes. Viral antigens were confined and diffusely observed within pulmonary lesions of both wild-type and COX-2-deficient mice. Moreover, higher production of MCP-1, TNF and IL-12p70 was seen in the lungs and plasma of COX-2-deficient mice. These results indicate that the pneumonia in COX-2-deficient mice induced by influenza virus infection was milder and more localized than that in wild-type mice due to the increased production of cytokines and infiltration of inflammatory cells, and this resulted in increased survival rates of COX-2-deficient mice. In previous reports, COX-2-deficient adult mice also showed an increased survival rate with influenza virus infection, but this was the result of decreased cytokine production. Further, histopathological lesion of the brain was not seen in either wild-type or COX-2-deficient mice. Thus, it was suggested that the mechanism of improving the clinical condition by COX-2 deficiency might be different between suckling and adult mice. Moreover, inoculation of type A influenza virus into COX-2-deficient mice is not sufficient to form a similar lesion with influenza encephalopathy.

Comparison of neuropathogenicity and *env* sequences in Fowl glioma-inducing virus mutants

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Fowl glioma-inducing virus (FGV) belongs to avian leukosis virus subgroup A (ALV-A), and causes so-called fowl glioma, perineurioma and cerebellar hypoplasia in chickens. A long terminal repeat (LTR) of the ALV provirus is mainly responsible for oncogenicity, whereas it was recently suggested that the viral envelope is the major determinant of the induction of lymphoid and myeloid tumors by ALV. In order to examine whether the *env* gene mutation in FGV influences fowl glioma-related nervous lesions, the pathogenicity was compared among 4 FGV mutants. C/O specific-pathogen-free (SPF) chickens were inoculated *in ovo* with FGV mutants (Tym-43, U-1, Sp-40 or Sp-53) or a chimeric virus constructed by substituting the *env* SU gene of FGV into the retroviral vector RCAS (A). Similarly, the pathogenicity of the CTS_5371 strain isolated from neurofibroma and MAV-1-like virus from mesenchymal neoplasm in the United States was also examined. Phylogenetic analysis of 3'LTR showed that all mutants grouped together in a cluster; however, the *env* SU sequences of 4 FGV mutants showed 85–96% identity with the corresponding region of FGV, and phylogenetic analysis of *env* SU revealed that Tym-43, U-1 and FGV grouped together in a cluster, while Sp-40 and Sp-53 formed a completely separate cluster. Brain lesions, including nonsuppurative encephalitis, a remnant of the external granular layer of the cerebellum, disorganization of the Purkinje cell layer and fowl glioma, were noted in these inoculated chickens with different frequencies and degrees. Among 4 mutants, Sp-53 had a tendency to induce more severe CNS lesions, such as multifocal severe fowl glioma and diffuse cerebellar hypoplasia. In contrast, chickens inoculated with chimeric virus RCAS-(FGV*env*SU) had only mild non-suppurative encephalitis. CTS_5371 induced mild to severe perivascular lymphocytic infiltration, and mild to moderate proliferation of perineurial cells and Schwann cells in spinal ganglions and sciatic nerves. MAV-1-like virus caused mild perivascular lymphocytic infiltration, a mild microglial reaction and a mild remnant of external granular layer of the cerebellum with no astrocytic proliferation. These results suggested that Sp-53 has earlier and more severe pathogenicity than FGV, and revealed that the *env* SU gene with 85–96% identity had little effect on the neuropathogenicity of FGV, but could affect the frequency and severity of CNS lesions. It was also demonstrated that CTS_5371 could cause the proliferation of perineurial cells and Schwann cells in the peripheral nerve system. In addition, it is speculated that MAV-1-like virus has no ability to induce fowl glioma-related lesions.

Analysis of seminal plasma proteins that stimulate endometrium epidermal growth factor (EGF) production in cattle

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It was reported that intra-vaginal injection of seminal plasma (SP) increased the concentration of endometrial epidermal growth factor (EGF) in cows. It is suggested that the stimulation of endometrial EGF was highest in an SP protein fraction ranging 16–29 kDa. To evaluate proteins that increase the concentration of endometrial EGF, in the present study, proteins in cattle SP were analyzed using two separation methods; SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and two-dimensional electrophoresis.

In the first experiment, SP obtained from 5 sires were injected into the vaginae of estrous cows. The concentrations of endometrial EGF 4 hr after SP injection were different among bulls. Proteins in SP were separated by SDS-PAGE, and the protein amount in each protein band injected into the vagina was calculated based on the total amount of proteins in SP. A positive correlation was observed between the amount of injected 25.4 kDa protein band and the concentration of endometrial EGF 4 hr after injection into the vagina.

In experiment 2, proteins of seminal plasma of molecular weight 16–29 kDa were separated into 5 fractions by two-dimensional electrophoresis based on the isoelectric point (pI). Proteins isolated from 5 fractions were injected into the vaginae of cows, and the concentration of endometrial EGF was measured after 4 hr. As a result, increases in endometrial EGF concentrations were observed when injecting 3 fractions (pI 5.8–6.2, 6.2–6.5 and 6.5–7.0). The concentration of EGF was highest when injecting the fraction of pI 6.2–6.5.

In conclusion, SP proteins within the molecular weight range 16–29 kDa and pI 5.8–7.0 stimulated the production of endometrial EGF in cows.

Identification of glycophorin A and CD58 as constituents of the high molecular weight sialoglycoprotein complex in bovine red cell membranes

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Bovine red cells possess periodic acid Schiff (PAS)-positive high molecular weight glycoprotein of $>$ 250 kDa (GP $>$ 250) as the major component of their membrane proteins. The present study describes the identification of glycoproteins of GP $>$ 250 and their molecular

diversity. PAS staining of red cell membrane proteins separated on SDS-polyacrylamide gels exhibited variations of GP > 250 in size and band intensity among individuals. These various GP > 250 were all positive for immunoblotting with the anti-bovine glycophorin A (GPA) antibody, which also recognized the 18-kDa monomer and 32-kDa dimer of GPA, indicating that GP > 250 contained GPA as a component. A crude glycophorin fraction consisting of GP > 250 and some minor PAS-positive glycoproteins was then extracted from red cell membranes and deglycosylated with trifluoromethanesulfonic acid, resulting in the generation of > 100-kDa and 28-kDa polypeptides. Mass spectrometric analysis of the tryptic fragments demonstrated that the 28-kDa polypeptide was a transmembrane glycoprotein, CD58; however, no high molecular weight complex formation between GPA and CD58 was observed when these proteins were co-expressed in CHO cells. These findings demonstrate that GP > 250 in bovine red cell membranes is a hetero-oligomeric complex consisting of GPA and CD58, suggesting that genetic variations of these proteins or some unidentified components generate the molecular diversity of GP > 250.

Epidemiological analyses of bovine viral diarrhea virus (BVDV) infection in a dairy area of Hokkaido

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Bovine viral diarrhea virus (BVDV) is a ubiquitous pathogen of cattle, but the clinical appearance of BVDV-infected cattle varies. BVDV causes profound economic damage to dairy farms. In Japan, no BVDV control program has been developed until now at the national level, but in Betsukai town in Hokkaido, the largest dairy town in Japan, a BVDV control program was established in 2006. In the present study, epidemiological analyses were performed based on the genetic variety of the E2 gene in 67 field isolates of BVDV. These isolates were obtained from persistently infected (PI) cattle in 37 farms in Betsukai town between 2006 and 2009.

In 2 areas of the town, Naka-syunbetsu and Nishi-syunbetsu, more PI cattle were detected than in other areas. There was no other distinctive tendency regarding the detection year, birth year and feeding area of PI cattle.

In Betsukai town, BVDV1a, 1b, 1c and BVDV2 were identified. Forty-eight of 67 isolates (71%) were BVDV1b, and were closely related genetically. Many BVDV1b were isolated every year, whereas the frequency of BVDV1a and 1c isolation decreased gradually. These results indicated that BVDV1b was the dominant genotype in Betsukai town.

Phylogenetic analyses revealed that closely related BVDV1b have been continuously transmitted among cattle within the town.

Two or more PI cattle were detected at 16 farms, and closely related virus strains were isolated. Moreover, viruses from individual farms, except for one farm, were identical. At these farms, PI cattle were the source of infection, and were raised by horizontal infection of pregnant cattle and subsequent vertical infection to fetuses. It was suggested that the virus could transmit to neighboring farms, and

become endemic within a narrow area.

Based on the present results, it is concluded that continuous efforts to detect PI cattle by surveillance and diagnosis followed by the elimination of PI cattle from herds are needed for BVDV eradication in Betsukai town in Hokkaido.

Relationship between dairy milk yield and expression of molecules inducing neutrophil activation

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During the peripartum period, dairy cows tendency to become ill more frequently than in other periods, and this is a serious problem because of decreasing dairy productivity. The frequent occurrence of peripartum diseases is associated with decreased immune reactivity and diseases such as mastitis, endometritis and retained placenta are particularly related to neutrophil dysfunction. Poor nutritional condition, various hormonal balance and oxidative stress are known as causes of neutrophil dysfunction, and milk secretion might be included as a cause; however, the relationship between lactation and neutrophil function is not clear. In the present study, the expressions of L-selectin and interleukin (IL)-8 receptor β, which are molecules inducing neutrophil activation, were examined, and the relationship between their expression levels and milk yield was discussed.

Initially, the variation of each gene expression level during the peripartum period was examined using the difference in milk yield from the last dairy period, average-producing dairy cows and high-producing dairy cows. Each gene expression level was measured by reverse transcription quantitative polymerase chain reaction and was estimated by relative quantification using the comparative threshold cycle (Ct) method. As a result, each gene expression level decreased immediately after parturition and recovered within a few days. After this recovery, each gene expression level varied widely in cows with average milk production, whereas in cows with high milk production, the gene expression level was almost constant.

Then, the variation of each gene expression level was estimated using the difference in milk yield in the present dairy period classified by the lactation period. As a result, L-selectin expression level in cows with high milk production was suppressed with the progress of lactation, whereas IL-8 receptor β expression level increased with the progress of lactation. This variation did not depend on the different milk yield and was associated with improvement of the nutritional condition.

These results showed that the L-selectin expression level was suppressed with the progress of lactation in cows with high milk production, and IL-8 receptor β expression level increased with an improved nutritional condition.

Seroepidemiological survey of tick-borne encephalitis in Japan

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Tick-borne encephalitis virus (TBEV), a zoonotic disease agent, causes severe viral encephalitis named tick-borne encephalitis (TBE) in humans. In October 1993, a human case of encephalitis was diagnosed as TBE in Hokkaido. TBEV Oshima strains were isolated from sentinel dogs, wild rodents and ticks in the same area, and the endemic focus was demonstrated in Hokkaido. There was no evidence of TBEV distribution in other parts of Japan; however, the susceptible vector ticks and reservoir rodents are widely distributed in Japan; therefore, it is possible that TBEV is endemic in other parts of Japan. In this study, we conducted a seroepidemiological survey of wild rodents in 8 areas of Japan to investigate the distribution of TBEV. We also attempted to isolate TBEV from wild rodents to analyze the characteristics of TBEV, which is now endemic in this area.

Nine hundred and thirty-one rodent sera were collected: 224 from Hokkaido, 14 from Aomori, 381 from Toyama, 89 from Gifu, 92 from Aichi, 58 from Shimane, 28 from Tokushima and 45 from Tsushima. TBEV-specific antibodies in serum samples were first examined by screening ELISA using subviral particles as antigens. Positive sera for ELISA were subsequently examined by a neutralizing test for definite diagnosis. Seventeen serum samples from Hokkaido and two from Shimane were diagnosed as positive with TBEV-specific antibodies, indicating that TBEV is endemic in these areas.

TBEV was isolated from wild rodents by intracerebral inoculation of the spleen suspension to suckling mice. Virus was successfully isolated from *Apodemus speciosus* in Kamiiso, and the antigen of tick-borne flavivirus was detected in cells infected with the virus by immunofluorescence assay. The sequence of the isolated virus was determined and the virus was identified as TBEV, which is closely related to Oshima strains isolated in Kamiiso, Hokkaido. In addition, an unidentified pathogen that killed suckling mice was isolated from *A. argenteus* in Kamiiso.

In conclusion, our results demonstrated that the TBEV endemic focus in Hokkaido has been maintained for more than 10 years and suggested that there is an endemic focus in Shimane in mainland Japan. Further epidemiological surveillance is required to identify endemic foci in Japan. The isolation of endemic TBEV is also an important issue to analyze the characteristics of the virus, such as virulence and antigenicity. This information will be useful for risk analysis and the prevention of TBE.

Isolation of hantaviruses from wild rodents in Far East Russia and investigation of the epidemiological situation in humans

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Hemorrhagic fever with renal syndrome (HFRS) is a rodent-borne zoonosis, which is caused by viruses of the genus *Hantavirus*. In Far East Russia, multiple species of hantavirus causing HFRS are prevalent with a case fatality rate reaching 5%. Since this region is geographically proximate to Japan and the number of visitors from Japan to this area has been increasing in recent years, it is important to identify the epidemiological situation in humans in Far East Russia. In this study, hantaviruses were isolated from wild rodents captured in Far East Russia and the serotypes of hantavirus infection in HFRS patients were determined by a cross neutralization test using the isolates. In addition, the development of a serological diagnostic ELISA was attempted to facilitate an epidemiological survey in rodents.

In Section I, five viruses were successfully isolated from wild rodents captured in Khabarovsk City, Far East Russia. Two isolates derived from Korean field mice (*Apodemus penisulae*) #209 and striped field mice (*Apodemus agrarius*) #57 were designated as Khekhtsir/AP209/2005 and Galkino/AA57/2002, respectively. Genetic analysis revealed that the virus from *Apodemus* (*A.*) *peninsulae* was Amur virus (AMRV) and that from *A. agrarirus* was Far East virus (FEV), a subtype of Hantaan virus (HTNV). In addition, a cross neutralization test using infected mouse serum revealed that the two isolates and Seoul virus (SEOV) SR-11 were antigenically different. Furthermore, differential serodiagnosis of HFRS patients in Prymorsky Region and Khabarovsk Region was carried out by a cross neutralization test using these three viruses (AMRV Khekhtsir/AP209/2005, HTNV(FEV) Galkino/AA57/2002, and SEOV SR-11). The results show that SEOV is the most prevalent in Vladivostok City, Prymorsky Region and AMRV in Prymorsky Region, except Vladivostok City. HTNV is the most prevalent in Khabarovsk Region. These data indicate that the epidemiological situation of HFRS is different in each region of Far East Russia.

In Section II, an antibody detection ELISA using recombinant nucleocapsid protein derived from AMRV Khekhtsir/AP209/2005 isolated from *A. peninsulae* has been established. This ELISA enables the detection of anti-hantavirus antibodies not only in *A. peninsulae*, but also in *A. agrarius*, and is a useful serodiagnostic method with high specificity and sensitivity compared with the conventional immunofluorescent assay.

X-irradiation induces the activation of mitochondrial function and late generation of reactive oxygen species (ROS) in A549 cells

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One target of radiation therapy is to cause tumor cells to enter apoptosis. Recently, several studies have shown that reactive oxygen species (ROS) play an important role in the signaling pathway of apoptosis. It was reported that mitochondrial ROS generation increased several hours after irradiation and participated in cytochrome *c* release from mitochondria in A549 cells (Ogura *et al.*, *Cancer Lett.*, 277:54, 2009). In this study, to reveal the mechanisms of radiation-induced mitochondrial ROS generation, various parameters of mitochondrial functions were analyzed with or without X-irradiation. Human lung carcinoma A549 cells were irradiated with 10 Gy X-rays. Intracellular ROS level was significantly increased from 6 hr after irradiation. Superoxide production from isolated mitochondria was assessed by the ESR spin-trapping method and was also elevated at 6 hr after irradiation. To evaluate mitochondrial functions, mitochondrial membrane potential, the cellular oxygen consumption rate, intracellular ATP level and complex I activity were examined. Mitochondrial membrane potential elevated time-dependently up to 24 hr after irradiation and was suppressed by an uncoupling agent, CCCP. The oxygen consumption rate was increased 12 hr after irradiation and was inhibited by a complex I inhibitor rotenone. Intracellular ATP level increased over time up to 24 hr after irradiation and was suppressed by a F_0/F_1-ATPase inhibitor, oligomycin. Complex I activity was upregulated 12 hr after irradiation. These results suggested that 1) X-irradiation activated the mitochondrial electron transport chain, leading to increased cellular ATP content; 2) while activation of the mitochondrial electron transport chain was accompanied by the increase of oxygen consumption by complex IV, and X-irradiation-induced extra electron flow caused superoxide production due to insufficient complex IV activity.

To verify radiosensitization by modulating cellular energy metabolic status in mouse floor of the mouth squamous carcinoma SCCVII cells

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The upregulation of aerobic glycolysis, known as the Warburg effect, is one of the metabolic changes prevalent in solid tumors compared with normal cells. The Warburg effect is characterized by reliance on glycolysis, which is mitochondrial-independent, regardless of oxygen availability. In this study, we examined whether the modulation of cellular energy metabolism could enhance radiation-induced cell death. SCC VII cells derived from mouse floor of the mouth squamous carcinoma were used in this study. Two pharmacological inhibitors, 2-deoxy-D-glucose (2DG) as a glycolysis inhibitor and dichroloacetate (DCA) as a pyruvate dehydrogenase kinase (PDK) inhibitor, were used. In this study, we evaluated that 1) the effect of DCA on mitochondrial function by measuring mitochondrial membrane potential or reactive oxygen species (ROS) derived from mitochondria, and 2) the effect of 2DG and DCA on radiation-induced reproductive cell death by clonogenic assay. Furthermore, in an in vivo experiment, the effect of 2DG combined with X-irradiation on tumor growth was examined in SCC VII cells transplanted into C3H/HeJ mice. DCA induced elevated mitochondrial membrane potential and mitochondrial ROS. Moreover, radiation-induced reproductive cell death was significantly enhanced by 2DG and DCA, respectively. Significant suppression of tumor growth by 2DG was also observed combined with X-irradiation in an in vivo experiment. Since DCA was known to activate pyruvate dehydrogenase by inhibiting PDK and promoting the subsequent production of acetyl-CoA for the activation of mitochondrial function, it was suggested that the modification of cellular energy metabolism, i.e., inhibition of glycolysis and activation of mitochondrial energy metabolism, induced the enhancement of radiation-induced cell death.

Radiosensitization by nanogel reagent containing gold nanoparticles

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High atomic number molecules, such as gold and platinum, are known to enhance the biological effect of X-irradiation. This is generally thought to be due to the increase of

DNA damage, mainly by photoelectric and Compton effects when cells are irradiated by X-ray in the kilo-voltage range. Herold *et al*. (*Int. J. Radiat. Biol.*, 76:1357, 2000) demonstrated radiosensitization by gold particles (3 μm diameter) in mouse tumor EMT-6 cells. Although non-functionalized gold nanoparticles have shown some level of accumulation in tumor cells, gold nanoparticles still have some potential for improvement to achieve higher efficiency for application to tumor cells. To modify the properties of gold nanoparticles, Nagasaki *et al.* (*Colloid Polym. Sci.*, 285:1055–1060) newly synthesized gold-nanogel (GNG) consisting of polyamine, polyethylene glycol, and gold nanoparticles (6 nm diameter). Average diameter of GNG was 106–148 nm by transmission electron microscopy (TEM). In this study, we investigated whether GNG could enhance cell death induced by X-irradiation and studied the potential mechanism of radiosensitization. Mouse squamous carcinoma SCCVII cells, human lung adenocarcinoma A549 cells, and Chinese hamster V79 cells were incubated with or without various concentrations of GNG for 14 hr. After X-irradiation, cytotoxicity and reproductive cell death were evaluated by colony formation assay. In all cell lines, GNG treatment increased reproductive cell death after irradiation without marked cytotoxicity. PI staining also revealed that GNG treatment enhanced apoptosis in SCCVII cells induced by X-irradiation. Furthermore, the results obtained by immunofluorescence analysis, Western blotting and pulsed-field gel electrophoresis indicated that GNG treatment decreased DNA double-strand breaks in irradiated cells. TEM images revealed that, after uptake by endocytosis, GNGs were distributed in the cytoplasm, but not in the nucleus. In addition, Western blotting showed that GNG treatment led to the activation of BiP/IRE1/JNK, indicating the activation of endoplasmic reticulum (ER) stress responses.

These results suggested that GNG induced radiosensitization by increasing the damage to cytoplasmic organelles, including ER, but not by increasing the damage to nuclear DNA.

Expression of drug-metabolizing enzymes and the mechanism of metabolic activation / detoxification of mutagens in tongue

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The metabolism of xenobiotics in oral tissues, especially in the tongue, has never been reported. In the present study, the metabolic activation / detoxification ability of Promutagens in the tongue, and the expression levels of related drug-metabolizing enzymes were investigated in this organ.

RT-PCR analysis of rat tongue demonstrated the constitutive expression of numerous drug-metabolizing enzymes. In particular, not only mRNA expression but also protein expression and enzymatic activity of cytochrome P450 (CYP) 1A1 were detected in tongue tissue. The ability of metabolic activation of promutagens in the tongue was estimated using benzo[a]pyrene or heterocyclic amines (HCAs), included in cooked

meat and tobacco products, as substrates. It was found that the metabolic activation level of HCAs in the tongue was comparable to those in the liver. In contrast, the expression levels of glutathione S-transferase (GST) and UDP-glucuronosyltransferase (UGT) in the tongue were considerably low compared to in the liver and, as a result, mutagenic activity in the tongue was not decreased by GST- or UGT-dependent conjugation.

Treatment of rats with Sudan III, a typical inducer of CYP1A1, resulted in markedly increased expression of CYP1A1 mRNA and protein expressions, CYP1A-dependent enzymatic and mutagenic activities. In addition, it was shown that CYP1A1 mRNA expression in carcinoma cells (SAS) was induced by Sudan III exposure.

In conclusion, I identified the ability of mutagenic activation of xenobiotics and the risk of cancer in the tongue. Furthermore, ingestion of inducers of drug-metabolizing enzymes has the potential to increase metabolic activation in the tongue and the risk of biomolecule attack by promutagens.

Comparison of warfarin sensitivity between rat and bird species

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Coumarin-derived anticoagulants, such as warfarin, are commonly used for rodent control worldwide; however, scattering of coumarin-derivative rodenticide in wide areas has caused primary and secondary poisoning incidents in non-target wild birds. Unexpectedly, a laboratory study of LD_{50} showed that chicken and other bird species seemed markedly resistant to warfarin. One possibility, which may cause a discrepancy between the frequent accidents in wild birds and the high LD_{50} of laboratory birds, is large inter-species differences in the factors determining sensitivity to warfarin. In this study, bird species were compared based on the aspects of vitamin-K epoxide reductase (VKOR) kinetics, VKOR inhibition by warfarin, and warfarin metabolism assay in rats.

In VKOR characterization, chickens and ostriches showed significantly lower V_{max} / K_m than rats (one-sixth and one-third, respectively), suggesting that bird species depend more on a non-VKOR vitamin K source. On the other hand, the inhibitor constants (K_i) of VKOR toward warfarin were significantly different between chickens and ostriches (11.3 ± 2.5 μM and 0.64 ± 0.39 μM). Interestingly, ostrich K_i was similar to the value in rats (0.28 ± 0.09 μM), indicating sensitivity to warfarin. The result of K_i reveals the surprising possibility that VKOR in some bird species is easily inhibited by warfarin: there is a very wide inter-species difference in K_i.

Warfarin metabolism assay also showed a large inter-species difference in bird species as follows: chickens and ostriches showed quite higher metabolic activity than rats, while mallards and raptor species showed very little ability to metabolize warfarin. In addition, unique dominant metabolites were found in bird species, and these specific metabolites indicated the possibility of a different metabolic pathway for warfarin in birds than mammals.

In this study, a wide inter-species difference was identified in birds in xenobiotic metabolism and sensitivity to rodenticide.

Seasonal testicular changes and assessment of sexual maturity in feral male raccoons (*Procyon lotor*) in Hokkaido

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The raccoon (*Procyon lotor*) is a mammal indigenous to North America. In Hokkaido, feral raccoons have been increasing since 1979 and are currently under nuisance control. One reason for this increase is thought to be their high reproductive potential, but little is known about their reproductive physiology. The aim of this study was to clarify seasonal changes in the testes and the timing of sexual maturation in feral male raccoons in Hokkaido.

We investigated external characteristics, histology of the testes, and plasma testosterone concentration in two captive raccoons (0-year-old and 5-year-old males) from December 2008 to September 2009, and in 210 feral male raccoons captured from May 2008 to September 2009. The captive 0-year-old male became able to produce spermatozoa in April 2009. The captive 5-year-old male produced spermatozoa actively from autumn to spring. From May to October in almost all feral 1-year-old males, the weight of the testes and the diameter of the seminiferous tubules increased rapidly, and the penis became extrusible. In October, spermatozoa could be observed. Some feral males produced spermatozoa before they were 1 year old. Spermatogenesis in feral males over 2 years old was active from September or October. Spermatozoa were observed in the cauda epididymis throughout the year, but the amount of spermatozoa decreased in the summer.

In the present study, it was clarified that many male raccoons entered puberty (differentiation of spermatogonia) at 1 year old in the spring, and started to produce spermatozoa during the following winter mating season; however, some males with good nutrition can produce spermatozoa earlier. Moreover, after they reach sexual maturity, they show seasonal changes in the testes; although spermatozoa are observed in the cauda epididymis throughout the year, spermatogenesis does not occur actively during the summer season of June to September. In order to further understand the reasons for the increasing numbers and distribution, studies of social and behavioral characteristics of reproduction are necessary.

Monitoring of behavior and body temperature during hibernation in captive Japanese black bear (*Ursus thibetanus japonicus*)

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Hibernation of bears is different from that of small hibernators in that bears maintain near-normal body temperature and females give birth to cubs and nurse them during hibernation. In addition, it has been reported that bears maintain some behavioral activity during hibernation, suggesting that these activities contribute to the maintenance of body temperature; however, little is known about the behavior of bears during hibernation. In this study, we monitored the behavior and skin temperature (Ts) of pregnant and non-pregnant captive bears during hibernation. In non-pregnant bears, behavioral activity (e.g., standing behavior) decreased at the beginning of December and remained stable until March, followed by an increase in April. In contrast, the pregnant female suddenly changed behavioral patterns after delivery, as shown by a decrease in standing behavior and an increase in dindle. As circadian change, behavioral activity was elevated during the daytime, indicating that bears have a diurnal behavioral pattern during hibernation. Ts of both females dropped to the lowest level in the middle of the hibernation period (non-pregnant: 25.9°C, pregnant: 20.8°C). Mean Ts of pregnant females remained higher (33.8°C) than that of non-pregnant female (32.2°C) during pregnancy, but became lower (21°C) correlating with the decrease in behavioral activity after delivery. This change in Ts may reflect changes in behavioral activity and the physiological state in the postpartum bear. On the other hand, Ts varied during the day and showed a spike pattern; however, the Ts spike did not correspond to changes in behavioral activity. Taken together, these results suggest that behavioral activity during hibernation may affect the long-term change of basal Ts, but does not directly regulate short-term Ts variation.

Tracking of spotted seals (*Phoca largha*) in Hokkaido using satellite transmitters

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Recently, the number of spotted seals (*Phoca largha*) coming to Hokkaido on the Sea of Japan side in winter has been increasing. The reason for this increase is not well known, but might be due to environmental change in their summer habitats. In this study, in order to establish

efficient methods of capturing and handling seals, and to survey where they come from to the Sea of Japan side of Hokkaido, we tracked their movements using satellite transmitters. We captured 6 seals using box traps, and attached transmitters to them under anesthesia with a medetomidine hydrochloride-ketamine hydrochloride mixture. Location data acquired from 4 seals (1 seal from Yagishiri Island, 2 seals from Bakkai Port, and 1 seal from Rebun Island) showed that their movements varied among individuals, although no data were obtained from the other 2 seals. The seal captured at Yagishiri Island started moving north soon after attachment of the transmitter in late May, and stayed in Aniva Bay, south of Sakhalin, between June and September. One adult male captured at Bakkai Port started moving north in mid-March, and stayed at Tatarskii Proliv, known as a breeding site of spotted seals, until mid-April. Another subadult seal captured at Bakkai Port stayed along the coast of Hokkaido until mid-April, and started moving north. As the signals from the transmitters of these two seals captured at Bakkai Port stopped while they were moving north between late April and early May, it was suggested that they were on the way to their summer habitats. On the other hand, the seal captured at Rebun Island stayed in its vicinity for almost the entire tracking period, suggesting that spotted seals might be settling there. Taken together, these results suggest that spotted seals on the Sea of Japan side of Hokkaido consisted of those coming from around Sakhalin and those settled on the coast of Hokkaido.

Identification of the viral and host cellular factors associated with West Nile virus replication

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West Nile virus (WNV) belongs to the genus *Flavivirus*, family *Flaviviridae*. In nature, virus transmission occurs between avian hosts and mosquito vectors. The virus infects humans and horses as dead-end hosts. An outbreak of West Nile fever occurred in 1999 in New York City. Since then, WNV has been noted as an etiological agent of emerging disease. Although WNV has never been identified in Japan, one imported infection case was reported in 2005; therefore, there is some concern in Japan about invasion. WNV infection ranges from a mild febrile illness to severe neuroinvasive disease and death. Although there is currently no vaccine or therapy for WNV infection in humans, several strategies are being pursued to develop effective prophylaxis and treatments.

Identification of viral and host cellular factors, associated with viral replication, have an important role in developing effective prophylaxis and therapies. In this study, it was attempted to establish a system to identify viral and host cellular factors involved in viral replication. In the first part of the study, a method for the generation of a mutant recombinant WNV was established, and it was shown that viral envelope protein glycosylation plays an important role in viral release from the host cell. In the second part, to establish a system for identifying cellular factors for viral replication, a random mutation was introduced into host cell genome DNA by transfection of the

sleeping beauty (SB) transposon. By infecting isolated cell clones with VLP and WNV, the SB transposon of which was introduced into their genomic DNA, cell clones were isolated with different characteristics of viral infection. SB transposon insertion sites of cellular genome DNA were determined by Splinkerette-PCR. Finally, several candidates of host cellular factors were identified that were associated with viral replication.

Further study might lead to the identification of new viral or cellular factors that play an important role in viral replication, and these discoveries might promote the development of an effective vaccine and treatments.

Therapeutic effect of peripheral administration of anti-PrP antibody and bone marrow-derived mesenchymal stem cells on mice infected with prions.

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Inhibitory effect against prion propagation, protective effect for neuronal tissues and functional recovery of degenerated nerve tissues are required to establish effective treatments for prion diseases. In addition, treatments should be effective even when they are given after the clinical onset of the disease and through peripheral route. In this study, I used anti-prion protein (PrP) monoclonal antibodies (mAbs) as an inhibitor for prion propagation and bone marrow-derived mesenchymal stem cells (MSCs) as a tool for neuronal protection and regeneration of degenerated nerve tissues, respectively.

In Part I, I administered mAb 31C6 via tail vain of prion-infected mice at the time of clinical onset (120 days post inoculation), and examined the distribution of mAb in the brain parenchyma and the effect of mAb 31C6 on survival of mice. The mAb 31C6 was distributed to the cerebellum and thalamus of mice infected with the Chandler prion strain, and more than half of mice survived longer than mice administered with negative control mAb. The level of PrP^{Sc} in mAb 31C6-administered mice was lower than that in mice administered with negative control mAb, and the progression of neuropathological lesions appeared to be delayed in the cerebellum, where mAbs were well distributed. These results suggest that administration of anti-PrP mAb through peripheral route is a candidate for the treatment of prion diseases.

In Part II, I examined the effect of intravenous transplantation of immortalized human MSCs (hMSCs) on treatment of prion diseases. When hMSCs were administered from the tail vain of mice infected with the Chandler strain at 120 dpi, the survival time of mice was significantly prolonged compared with mock-treated mice. Although transplantation of hMSCs did not appear to antagonize prion propagation, spongiform changes appeared milder in mice transplanted with hMSCs than in mock-treated mice. Thus, these results show the possibility that transplantation of hMSCs through peripheral route can be applied for the treatment of prion diseases. I also examined the mechanism of the hMSCs migration to brain parenchyma via blood-brain-barrier with an *in vitro* migration

assay using human umbilical vein endothelial cells culture as a model of endothelial cells. The results suggest that cytokines and chemokines, including CCL3, CCL5, IL-1β, and TNF-α, produced in the brains of prion-infected mice, induced expression of adhesion molecules such as ICAM-1 on endothelial cells, and that hMSCs pass through endothelial cells via their interaction with ICAM-1.

The results of this study provide important information on applying antibody-therapy and regenerative medicine/cell therapy for the therapeutics of prion diseases.

Analysis of pathobiology of prion infection in CD14 gene deficient mice

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Prion diseases are fatal neurodegenerative disorders characterized by the vacuolation of neurons and neuropil, reactive astrocytosis, microglial activation, and accumulation of an abnormal isoform of prion protein (PrP^{Sc}) in the central nervous system. The activation of glial cells as well as prion propagation are involved in their pathobiology. Gene expression analysis of prion-infected animals revealed that many factors associated with immune system, i.e., cytokines and ckemokines, are up-regulated in the brains of prion-infected animals. Some of them have been demonstrated to be involved in the disease progression by experimental infection using gene-abrogated mice, however, molecular mechanism of pathobiology has not been fully understood. In this study, I carried out experimental prion infection using CD14 gene-deficient ($CD14^{-/-}$) mice to analyze the involvement of CD14 molecule, which is known as receptor for lipopolysaccharide and to be involved in innate immunity, in the pathobiology of prion diseases. $CD14^{-/-}$ mice inoculated with the Chandler or Obihiro prion strain survived longer than wild-type (WT) mice inoculated with the corresponding prion strain, indicating that CD14 molecule influences the acceleration of disease progression. Although there was no difference in PrP^{Sc} level between brains of $CD14^{-/-}$ and WT mice at the terminal stage of disease, $CD14^{-/-}$ mice had less PrP^{Sc} than WT mice in the middle stage (90 days post inoculation, dpi) and late stage (120 dpi) of disease. Immunohistochemical analysis using anti-Iba1 antibodies revealed that microglial activation was more obvious in prion-infected $CD14^{-/-}$ mice than prion-infected WT mice. In addition, the expressions of markers for activated microglia, such as CD11b and CD45, were also up-regulated in prion-infected $CD14^{-/-}$ mice compared with WT mice. However, no difference was observed in the expression of Mac-2, another marker for activated microglia, between prion-infected $CD14^{-/-}$ and WT mice. These results suggest that the activation state may be different, at least in some portion of microglia between $CD14^{-/-}$ and WT mice. Furthermore, compared with WT mice, the elevated expression of anti-inflammatory cytokines, such as transforming growth factor beta and interleukin 10, was observed from an earlier stage of prion-infection in $CD14^{-/-}$ mice. This implies the involvement of these cytokines in pathobiology of the disease. Further investigation of the relation between CD14

molecule and the function of microglia will help to elucidate some part of molecular basis of the pathobiological mechanism on prion diseases.