HOKKAIDO UNIVERSITY

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INFORMATION

Hokkaido University conferred the degree of Bachelor of Veterinary Medicine to the following 44 graduates of the School of Veterinary Medicine on March 22, 2012. The authors summaries of their theses are as follows:

Pathological analysis of dacryoadenitis model mice using lacrimal fluid

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Sjögren's syndrome is a chronic autoimmune disease characterized by exocrinopathy, resulting in decreased lacrimal fluid and saliva. Prior to exocrinopathy, lymphocyte infiltration in lacrimal and salivary glands is observed in Sjögren's patients. Infiltration of these glands is detectable by biopsy; however, lacrimal gland biopsy is not a general diagnostic method due to its invasiveness; thus, the establishment of a noninvasive diagnostic method is needed for detecting lymphocytic infiltration prior to exocrinopathy in lacrimal glands. In the present study, the pathogenesis of dacryoadenitis in autoimmune disease model mice was analyzed to find a biomarker for early-stage dacryoadenitis.

BXSB/MpJ and C57BL/6 mice were analyzed as an autoimmune disease model and healthy control, respectively. Although there was no significant difference in the amount of lacrimal fluid between BXSB/MpJ and C57BL/6 mice as a result of Schilmer's test, partial destruction of acini and lymphocytic infiltrations, mainly composed of B-lymphocytes, around the ducts were observed in the lacrimal glands of BXSB/MpJ mice. In addition, the cell-infiltrating score in the lacrimal glands of BXSB/MpJ mice was significantly higher than in C57BL/6 mice. By quantifying *Aqp3*, *4*, and *5* mRNA expressions as a marker of cellular water exchange in the lacrimal glands, *Aqp3* mRNA expression in BXSB/MpJ mice tended to be higher than in C57BL/6 mice, while *Aqp4* and *5* mRNAs showed the opposite expressions. Furthermore, *Aqp3* and *Aqp4* expressions of BXSB/MpJ significantly increased and decreased with the cell-infiltrating score, respectively. As a result of detecting inflammatory cell markers (*Cd68, Ptprc,* and *Cd3e*) in the lacrimal fluid using Schilmer's measuring strips by RT-PCR, *Cd68* was detected more frequently in BXSB/MpJ mice than in C57BL/6 mice. *Cd68* was detected in all mice in which *Ptprc* or *Cd3e* was detected. In addition, inflammatory cell marker mRNAs were detected in the lacrimal fluid of MRL/MpJ-*lpr*/*lpr* and NZB/NZW F1, also known as autoimmune dacryoadenitis model mice. In particular, *Cd68* was detected frequently in the lacrimal fluid of MRL/MpJ-*lpr*/*lpr* and NZB/NZW F1 mice as well as BXSB/MpJ mice.

These findings suggest that BXSB/MpJ mice develop a relatively early stage of autoimmune dacryoadenitis characterized by lymphocytic infiltrations around ducts without exocrinopathy, and that the amount of lacrimal fluid is maintained by the compensatory action of water channels during this disease stage. It was concluded that lacrimal fluid can be useful in a noninvasive diagnostic method for dacryoadenitis.

Analysis of TUNEL-positive cells associated with cardiomyocyte differentiation in mouse embryo

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Programmed cell death is an essential event during mammalian morphogenesis, which eliminates unnecessary cells to accomplish histogenesis and organogenesis. Recent study clarified that activation of the molecular cascade mediating cell death through caspase families is closely associated with skeletal muscle differentiation in mouse embryos. In this study, it was verified that cell death and its molecular cascade also regulate the differentiation of cardiomyocytes, striated muscles similar to skeletal muscle.

The occurrence of cell death and activation of caspase in cardiomyocytes were analyzed by TdT-mediated dUTP nick end labeling (TUNEL) assay and immunohistochemistry for ssDNA and active caspase-3 (aCasp3) using C57BL/6 mice on embryonic days (E) 11.5–15.5. TUNEL-, ssDNA-, and aCasp3-positive cells were detected in the cardiomyocytes through E11.5–15.5. In particular, the number of TUNEL-positive cells increased with fetal age, and was much higher than ssDNA- and aCasp3-positive cells. As a result of TUNEL and aCasp3 double staining, three types of positive cells, TUNEL+/aCasp3+, TUNEL+/aCasp3−, and TUNEL−/aCasp3+, were observed. The number of TUNEL+/aCasp3− cells was the highest of the three cell types and increased during E11.5–15.5. TUNEL+/aCasp3− cells did not show morphological features of cell death such as nucleic condensation or cell shrinkage. Real-time PCR analysis revealed that mRNA expressions of anti-apoptotic proteins (Bcl-2 family and IAP family) and cardiac markers (*Tnnc1*, *Myh6*, and *Myh7*) increased during E11.5–15.5. In addition, using rat fetal heart cell line H9C2, the appearance of TUNEL-positive cells was examined during *in vitro* cardiomyocyte differentiation. H9C2 cells increased the expression of a cardiac marker, *Myl2*, on days 0, 4, and 7 in differentiation medium, and formed myotubes that expressed myosin heavy chain proteins. TUNEL-positive H9C2 cells were observed at all time points examined; however, a few cells without nucleic condensation were detected only in myotubes on days 4 and 7.

Although the number of TUNEL-positive cells increased, their dynamics did not coincide with those of ssDNA immunoreaction or caspase activation during cardiomyocyte differentiation in mice. Since the caspase cascade was suppressed, unlike skeletal muscle, it was suggested that the TUNEL-positive reaction indicated DNA damage, but not cell death in embryonic cardiomyocytes. Recently, it has been reported that the DNA damage response promotes terminal differentiation in various tissues; therefore, DNA damage during embryogenesis is also considered to be associated with cardiomyocyte differentiation in mice.

Physiological role of melatonin in the chicken intestine: Effect on intestinal contractility and the expressions of melatonin-related genes

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In birds and mammals, the main tissue of melatonin synthesis is the pineal gland, but the intestine is also strongly suggested to produce melatonin. Especially in the intestine of birds, the specific binding site of melatonin is present with higher density than in mammals; however, the functions of intestinal melatonin have hardly been examined. In the present study, melatonin-induced intestinal relaxation, which is reported in rats, and the involvement of melatonin receptors was investigated in chickens. In addition, mRNA expressions of three subtypes of melatonin receptors (Mel_{1a}, Mel_{1b}, Mel_{1c}) and two melatonin-synthesizing enzymes (AANAT, HIOMT) were quantified in five intestinal parts, respectively. From these results, the physiological role of melatonin in the chicken intestine was discussed.

Changes in the tension of isolated intestines were measured utilizing Magnus apparatus and relaxation by melatonin was evaluated. The chicken duodenum was relaxed by melatonin in a dose-dependent manner. ED_{50} was 83.5 μM, which was higher than that reported in the rat duodenum previously. Relaxation by 100 μM melatonin was compared among the duodenum, jejunum, ileum, cecum and colon. No significant difference was found in the relaxing effect among five segments. After induction of contraction of the isolated duodenum by 0.1 μM acetylcholine, application of 100 μM melatonin evoked relaxation. Five to 50 μM luzindole, a melatonin receptor antagonist, induced relaxation in the duodenum. Inhibition of relaxation by 100 μM melatonin was not observed with luzindole pretreatment. mRNA expressions of five melatonin-related genes were determined by real-time-PCR. Mel_{1a}, Mel_{1b} and HIOMT mRNA were highly expressed in the cecum, while AANAT mRNA expression was high in the duodenum.

In rats, the relaxing effect of melatonin was reported to be greatest in the duodenum and spontaneous contraction was inhibited; however, in chicken, these effects were not observed. Therefore, different mechanisms may be involved in relaxation by melatonin in chickens. Although there was no apparent correlation between mRNA expression and the relaxing effect by melatonin in each intestinal segment, this is the first report to characterize the expression of melatonin receptors and melatonin-synthesizing enzyme mRNA in five intestinal segments. Further, the current study suggested that melatonin plays a role in regulating contraction throughout the intestine in chickens.

A method for classifying equine behavior patterns at pasture using an acceleration data logger

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Acceleration data loggers as a tool to classify livestock behaviors have overcome the limitations of traditional methods. Many studies have been conducted concerning cow behavior, and although there have been studies focused on classifying foraging and non-foraging behavior in horses, detailed monitoring is yet to be performed. In the present study, we aimed at establishing a simple method using a single axial accelerometer to classify a variety of behaviors (stand, forage, walk, trot, and canter) seen in thoroughbred horses at pasture, evaluating the "count per unit" (cpu), a figure indicating the relative quantity of body movement.

We obtained 16 hours and 37 minutes of acceleration data representing the head-tail direction movement of 5 thoroughbred pleasure horses, together with a video record. The data were divided into "Training data" (n = 5) and "Validation data" (n = 2), and the cpu of all behaviors was calculated using Training data in accordance with the video record. We examined individual variances using a box plot and concluded that although deviations were high as a result of outliers, the cpu peaks characterizing each behavior among individuals were similar between individuals and would not affect the classification accuracy; therefore, we used all Training data to draw the histogram for threshold setup.

Each unit of Validation data was classified into behavior patterns using the threshold value, and we calculated sensitivity and precision rates to evaluate the validity of this model. The overall sensitivity of the 5 behaviors was 69.0%. Among the 5 behaviors, walk (70.4%), trot (97.1%), and canter (95.0%) had a high degree of correct classification. On the other hand, the overall precision rate was 38.0%, showing a low figure. Incorrect classifications were due to movements not related to behavior patterns (such as the movement of dermal muscles, head-shaking motions, etc.). Although a process to remove these data would be necessary to use this method as a reliable behavior classifier, the results showed that accelerometer data loggers were efficient in classifying locomotor activities, which are of common interest among equine behaviors.

Mouse odor preference of female BALB/c for male C57BL/6 depends on estrogen

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Female mice obtain information about males via the odor of their urine and choose genetically

heterogeneous males as reproductive partners. In addition, the sexual behavior of females varies according to the stage of the estrus cycle, but there are few reports about variations in odor preference of females for genetically heterogeneous males over closely related males, which depends on the stage of the estrus cycle. In this report, we conducted Y-maze preference tests with BALB/c females using male urine of BALB/c and C57BL/6, and assessed the involvement of the estrus cycle in the female preference for genetically heterogeneous males.

BALB/c females stayed for a significantly longer time in the area adjacent to the urine of C57BL/6 males than that of BALB/c males. In metestrus, diestrus and proestrus, judged by inspecting vaginal smears, females showed a preference, but in estrus, females failed to show a preference. It is known that, in the period from metestrus to proestrus, the blood level of estrogen rises gradually, while in estrus the estrogen level drops; therefore, we speculated that estrogen could control the female preference for genetically heterogeneous males.

In order to examine the involvement of estrogen in the female odor preference for genetically heterogeneous males, we conducted additional experiments in which females were ovariectomized and silicon tubes containing estradiol-17β or sesame oil were subcutaneously implanted. On the 4th to 6th days after surgery, females administrated with estradiol-17β stayed longer in the area adjacent to the male urine of C57BL/6 than that of BALB/c, but females treated with sesame oil did not. On the 7th to 10th days after surgery, this preference disappeared in both groups.

These results suggest that the preference by female mice for genetically heterogeneous males varies according to the stage of the estrus cycle and is partially dependent on estrogen.

Role of neuropeptide QRFP in the regulation of sleep and wakefulness in mice

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QRFP (pyroglutamylated arginine-phenylalanine-amide peptide) is a novel neuropeptide that has been identified as an endogenous ligand for orphan G-protein-coupled receptor (GPR) 103. Both QRFP and GPR103 are highly expressed in brain neurons, particularly in the hypothalamus. QRFP has been shown to affect behaviors such as food intake and spontaneous locomotor activity when administered centrally to mice. These reports suggest that QRFP may affect hypothalamic neural circuits involved in the regulation of sleep and wakefulness. Therefore, in the present study, comprehensive phenotypic analysis of sleep and wakefulness was performed in mice treated with QRFP and in *prepro-qrfp*$^{-/-}$ (QRFP-KO) mice.

Intracerebroventricular (i.c.v.) administration of QRFP in C57BL/6J mice increased the amount of wakefulness (W) and decreased that of non-rapid eye movement (NREM) sleep and rapid eye movement (REM) sleep. QRFP i.c.v. decreased the number of W episodes and markedly prolonged the duration of W episodes, whereas the number of NREM and REM sleep episodes decreased and the duration of REM sleep episodes was shortened.

The effects of QRFP deficit were evaluated under three conditions: baseline, somnolent and

stimulant. First, to determine the role of QRFP in the regulation of spontaneous sleep and wakefulness, the phenotype of QRFP-KO mice was compared with their littermate *prepro-qrfp*$^{+/+}$ (WT) mice under baseline conditions. QRFP-KO mice showed no significant difference in terms of rhythm, amount, and the structure of sleep and wakefulness from WT mice. Second, to determine the role of QRFP in the homeostatic regulation of sleep and wakefulness, both QRFP-KO and WT mice were subjected to 6-hr sleep deprivation (SD) and their rebound sleep during the recovery period was compared. The extent and time course of the NREM and REM sleep rebound response of both genotypes were quite similar under somnolent conditions. Third, to evaluate the role of QRFP in stress-induced arousal, both QRFP-KO and WT mice were subjected to intraperitoneal (i.p.) administration of saline. QRFP-KO mice had significantly longer W and sleep latency than WT mice, suggesting that the QRFP deficit enhanced W by changing the tone of stress response pathways under stimulant conditions.

To examine the role of QRFP affecting W via the hypothalamus-pituitary-adrenal (HPA) axis, both QRFP-KO and WT mice were subjected to saline i.p. and their hypothalamic corticotropin-releasing hormone (CRH), serum adrenocorticotropic hormone (ACTH), and corticosterone levels were compared. The mRNA and protein levels of hypothalamic CRH after stimulus in QRFP-KO mice were comparable with those in WT mice. Serum ACTH and corticosterone responses also showed no difference between the genotypes.

In summary, QRFP i.c.v. markedly increased the amount of W by elongating the duration of W episoded. QRFP deficit enhanced arousal under stressful conditions but the possible involvement of the HPA axis was excluded. These results suggest a role of QRFP in the promotion of the arousal system in mice.

Effects of leptin on estrogen and progesterone-induced mammary gland development in mice

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The mammary gland is one of the 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 post-pubertal 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 of stromal environments by diet-induced obesity results in impaired lobuloalveolar development during pregnancy and reduced lactation in mice. We have recently demonstrated that diet-induced obesity disrupts ductal development in the mammary glands of nonpregnant mice, and that it delays the morphogenic and functional processes of pregnant-associated mammary gland development in mice, especially increased side branch formation in the early pregnancy period; however, the mechanisms behind such observations remain to

be elucidated

Stromal adipocytes store energy as triglyceride and produce various biologically active substances called adipocytokines. Leptin is a representative adipocytokine and increases its expression and plasma levels depending on adiposity. We have demonstrated that leptin inhibits the proliferation and tubulogenesis of mammary epithelial cells and enhances type 1 collagen synthesis by fibroblasts *in vitro*. Moreover, it has been shown that leptin receptors are localized in the region adjacent to main ducts and side branches but rarely in the region surrounding acini, in both lean and obese mammary glands. Therefore, it is hypothesized that systemic hyperleptinemia and/or local hyperproduction of leptin in obese mice affects mammary epithelial cell growth and differentiation in the pre-pubertal and early pregnancy periods.

The present study aimed to examine the possible involvement of systemic hyperleptinemia in the retardation of side branch formation in the early pregnancy period. To pursue this under the control of circulating reproductive hormones, it was first examined whether hormonal treatments could induce mammary gland development similar to that in the early pregnancy period. Female C57BL/6J mice (9 weeks old, n = 24) after ovarian resection were divided into four groups, and each group was given daily subcutaneous injection of corn oil as a solvent (C), estrogen (E), progesterone (P) or estrogen and progesterone (EP) for 8 days. There were no changes in body weight, mammary gland weight, or plasma glucose and non-esterified free fatty acid concentrations among the groups. Stereoscopic examination of whole-mount specimens of the glands revealed that only the mammary glands from mice given EP had an increased number of side branches, compared with the glands from C mice. Histological examination revealed that the ducts of C mice consisted of luminal epithelia lined with myoepithelium and a surrounding collagen layer in the adipose tissue, and there were no differences in the cellular composition and size of adipocytes among the groups. However, the ducts in E mice had increased cellular height of luminal epithelia with an increased number of PCNA, a marker of cell proliferation, -positive cells. In contrast, the ducts in P mice had a thickened surrounding collagen layer without affecting epithelial layers, with no PCNA-positive cells. Interestingly, ducts in EP mice showed two phenotypes: small ducts, presumably representing side branches, and increased cellular height of luminal epithelia with many PCNA-positive cells, whereas the large duct had a thickened surrounding collagen layer with few PCNA-positive cells. These results indicate that EP administration to spayed mice for 8 days induced side branch formation, although the rate of side branches sprouting by hormonal induction seemed to be less than in pregnancy.

Next, the effects of systemic leptin on EP-induced mammary gland development were examined. Female C57BL/6J mice (9 weeks old, n = 12) after ovarian resection were divided into two groups, and each group was given either a single intravenous injection of leptin expressing adenovirus (Lep) or control adenovirus (LacZ), and daily subcutaneous EP administration for 8 days. Mice given Lep showed hyperleptinemia, resulting in decreased food intake, mammary gland weight (fat pad size), and plasma glucose and non-esterified free fatty acid concentrations, compared to LacZ mice of the same body weight as Lep by controlling the feeding amount. These changes could be explained by well-documented leptin functions such as the induction of anorexia and increased energy expenditure through activation of the sympathetic nerve system.

Stereoscopic examination showed that there was a tendency for the number of ductal branches in Lep to decrease compared with that of LacZ. Histological examination displayed shrinkage of fat droplets in adipocytes and increased collagen deposition in the stromal area of Lep. As shown in EP mice, the small ducts of LacZ showed increased cellular height of luminal epithelia with many PCNA-positive cells, while the

number of ducts containing PCNA-positive cells decreased in Lep. In addition, although the ducts of LacZ were lined well with myoepithelium, there were many portions missing tight cell attachment between luminal epithelia and myoepithelium in the ducts of Lep. Therefore, these results suggest that systemic hyperleptinemia suppresses mammary epithelial cell growth, at least in part, by anoikis, cell detachment-induced apoptosis.

In summary, it was demonstrated that EP administration to spayed mice for 8 days induced side branch formation as in the early pregnancy period, and that systemic hyperleptinemia prevented EP-induced mammary epithelial cell growth. The present *in vivo* results further support the hypothesis that systemic hyperleptinemia and/or local hyperproduction of leptin in obese mice affects mammary epithelial cell growth and differentiation in the pre-pubertal and early pregnancy periods.

Exploring novel genetic factors involved in sleep/wake regulation using inter-subspecific consomic strains established from MSM/Ms and C57BL/6J mice

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A number of studies from both humans and animals indicate that sleep is genetically regulated. In fact, genetic factors involved in sleep/wake regulation such as *clock* and *hypocretin type 2 receptor* have been identified through linkage analysis and the positional cloning approach. Chromosome 5 is of particular interest because there are reports in mice suggesting that the genes responsible for regulating the amount and quality of sleep/wake are located in this chromosome. Since known genetic factors alone are not able to explain the entire molecular mechanisms of sleep/wake regulation, in this study, novel genetic factors involved in sleep/wake regulation were explored by a forward genetic approach using inter-subspecific consomic strains established from MSM/Ms (MSM) and C57BL/6J (B6) mice.

As a first step of this study, comprehensive phenotypic analysis was performed in B6-Chr5C^{MSM} (5C) and B6-Chr5T^{MSM} (5T), a consomic strain with 1–45cM and 41–86cM segments of chromosome 5 from MSM on a B6 background, respectively. Their parental strains, B6, a commonly used laboratory mouse strain, and MSM, a wild-derived inbred mouse strain whose genetic background is diverse from that of B6, were analyzed in parallel.

MSM chromosome 5 in the context of a B6 genetic background conferred distinct differences in phenotypes from B6 controls. 5C had more consolidated sleep than B6. 5T had three distinct differences from B6 that can be summarized as follows: 1) The longest wake duration of 5T was 2.6 times longer than that of B6; 2) Theta peak frequency (TPF) during rapid eye movement sleep in 5T was significantly lower than that in B6; 3) 5T showed occasional (up to 19 events in 30 min) spontaneous electroencephalogram (EEG) discharges lasting around 1 second, which was not observed in B6. The amplitude of such EEG discharges was twice as high as that of the normal waking EEG level with a peak frequency

of 6–8Hz.

In order to examine the hereditary patterns of these three phenotypes identified between B6 and 5T, sleep/wake in (B6×5T)F_1 was analyzed and the results were compared with that of B6 and 5T. As a result, (B6×5T)F_1 had B6-like phenotypes in terms of TPF and EEG discharge frequency. Therefore, in the next step, linkage analysis was performed using {(B6×5T)F_1×5T}N_2. As a result, the genetic region associated with both the longest wake duration and EEG discharges was suggested to be located around *D5Mit338* (59cM), and that associated with TPF to be located around *D5Mit338* (59cM) and/or *D5Mit141* (74cM).

The EEG discharges observed in 5T and some {(B6×5T)F_1×5T}N_2 had similar characteristics to spike-and-wave discharges (SWDs) reported in the mouse model of absence epilepsy. Simultaneous video and EEG recordings in {(B6×5T)F_1×5T}N_2 mice revealed that EEG discharge events were accompanied by an arrest of locomotor activity. The specific anti-absence epilepsy drug ethosuximide (200 mg/kg) significantly reduced the number of EEG discharges. These results indicate that the EEG discharges in 5T have similar biological properties to SWDs observed in absence epilepsy.

In summary, three distinct phenotypic differences between B6 and 5T were found and the genetic loci responsible for these traits within chromosome 5 were dissected. Further linkage analysis with {(B6×5T)F_1×5T}N_2 may lead to the identification of novel genetic factors involved in sleep/wake and absence epilepsy.

Molecular analysis of viral factors responsible for the acquisition of pathogenicity by a live attenuated classical swine fever vaccine strain by serial passages in pigs

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A live attenuated classical swine fever vaccine strain, GPE^-, acquired pathogenicity by serial passages in pigs. Three amino acid substitutions, T830A on E2, and V2475A and A2563V on NS4B, were found in viruses obtained after 11 passages in pigs (Yoshino, 2010). To investigate the amino acids responsible for the acquisition of pathogenicity, mutant viruses were generated by site-directed mutagenesis of the GPE^- backbone and by reverse genetics. The results of experimental infection of pigs with mutant viruses revealed that these 3 amino acid substitutions are responsible for the acquisition of pathogenicity in pigs. The E2 T830A substitution resulted in enhanced attachment and entry into host cells. To investigate amino acid substitutions on NS4B responsible for the efficiency of viral replication, replicons of GPE^- and mutant viruses were generated to assess the replication efficiency by luciferase assay. Both of the amino substitutions (V2475A and A2563V) on NS4B are responsible for efficient replication of the virus. The present findings indicate that 3 amino acid substitutions, T830A on E2, and V2475A and A2563V on NS4B, are responsible for the acquisition of pathogenicity by affecting virus entry and replication.

Molecular analysis of factors responsible for the replication of duck influenza virus in chickens

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Wild ducks are a natural reservoir of influenza A viruses. Duck influenza viruses are not transmitted to chickens directly, but through domestic water birds and terrestrial poultry; however, little is known about the molecular mechanisms of how influenza viruses of duck origin replicate in chickens. In the present study, virus factors responsible for the replication of influenza viruses of duck origin in chickens were analyzed.

Duck influena virus, A/duck/Mongolia/54/2001 (H5N2) (Dk/Mongolia), chicken influenza virus, A/chicken/Ibaraki/1/2005 (H5N2) (Ck/Ibaraki) and their genetic reassortants were generated by reverse genetics and inoculated into 4-week-old chickens intranasally. Viruses with the hemagglutinin (HA) gene originating from Ck/Ibaraki replicated in chickens, regardless of their other gene segments. The chimeric HA virus of Dk/Mongolia, whose HA1 is substituted by that of Ck/Ibaraki, named rgHA1-Iba HA2-Mon/Mon, also replicated in chickens. The growth of rgHA Mon/Iba in chickens increased slightly after 8 passages in chickens. Amino acid substitutions were found in both HA1 and HA2 of rgHA Mon/Iba after passaging. Sialylglycosaccharide binding specificities of the viruses did not alter during passaging. On the other hand, the passaged viruses showed higher optimal pH for fusion than the original virus.

Thus, it was found that the HA is a determinant of the growth of Ck/Ibaraki in chickens, indicating that receptor binding affinities and fusion activities of the HA are related to virus replication efficiencies in chickens.

Mechanism of acquisition of pathogenicity of an H7 influenza virus isolated from a feral duck in chickens

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Highly pathogenic avian influenza viruses have a pair of di-basic amino acid residues at the cleavage site of the HA, which is recognized as a major factor responsible for the pathogenicity in chickens; however, little is known about other virus factors involved in pathogenicity in chickens. In this study, a pair of di-basic amino acid residues was introduced at the cleavage site of the HA of A/duck/Hokkaido/Vac-2/2004 (H7N7). Consecutive passages of the virus were performed in the air sacs of chicks, and virus factors involved in pathogenicity in chickens were identified.

Vac2sub-P0, which had a pair of di-basic amino acid residues at the cleavage site of the

HA, did not show intravenous pathogenicity in chickens. After three consecutive passages in the air sacs of chicks, strain Vac2sub-P3 acquired intravenous pathogenicity in chickens. Six amino acid substitutions, K123E in PB2, N16D in PB1, E227G and I388T in HA, G228R in M1, and L46P in M2, were found between Vac2sub-P0 and Vac2sub-P3. The results of the intravenous inoculation of recombinant viruses into chickens indicated that E227G and I388T in the HA are essential for the acquisition of intravenous pathogenicity in chickens. Other amino acid substitutions in PB2, PB1, M1, and M2 may also be involved in the enhancement of pathogenicity. On the other hand, Vac2sub-P3 killed only one third of chickens inoculated intranasally. Further studies are ongoing to elucidate the molecular basis of the intravenous and intranasal pathogenicity of H7 influenza viruses in chickens.

Molecular characterization of an immunoinhibitory receptor Tim-3 in bovine leukemia virus-infected cattle

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An immunoinhibitory receptor, T cell immunoglobulin domain and mucin domain-3 (Tim-3), and its ligand, galectin-9 (Gal-9), are involved in the immune evasion mechanisms for several pathogens causing chronic infections. Previous studies showed that blockade of the Tim-3 pathway by anti-Tim-3 antibody enhanced the production of anti-virus cytokines, proliferation of antigen-specific $CD8^+$ T cells and reduction of viral load. Moreover, combined blockade of the Tim-3 and programmed death-1 (PD-1) pathways enhanced these effects. For these reasons, targeting both Tim-3 and PD-1 could be an effective therapeutic strategy against chronic viral infections. Thus, many studies have been performed on human and mouse Tim-3 pathways; however, there are few reports on the Tim-3 pathway in domestic animals. In this study, therefore, cDNAs encoding for bovine *Tim-3* and *Gal-9* were cloned and sequenced, and their expressions and roles on immune reactivation were analyzed in bovine leukemia virus (BLV) -infected cattle.

Full-length cDNA clones of bovine *Tim-3* and *Gal-9* were identified. For Gal-9, two clones, Gal-9 long and short, were obtained. Predicted amino acid sequences of Tim-3, Gal-9 long and Gal-9 short had high homology with human and mouse homologues. Functional domains, including tyrosine kinase phosphorylation motif in the intracellular domain of Tim-3, were highly conserved among cattle and other species. Quantitative real-time PCR analysis showed that bovine *Tim-3* mRNA is mainly expressed in T cells such as $CD4^+$ and $CD8^+$ cells, while *Gal-9* mRNA is mainly expressed in monocyte lineage cells and T cells. *Tim-3* mRNA expression on $CD4^+$ and $CD8^+$ cells was upregulated with disease progression of BLV infection. In addition, the *Tim-3* expression level on these cells correlated with viral load in infected cattle. The expressions of *IFN-*γ and *IL-2* mRNA were upregulated when peripheral mononuclear cells from BLV-infected cattle were cultured with Cos-7 cells expressing Tim-3 to block the Tim-3 pathway. Moreover, combined blockade of the Tim-3 and PD-1 pathways significantly promoted *IFN-*γ mRNA expression compared with blockade of the PD-1

pathway alone.

These results suggest that Tim-3 is involved in the suppression of T cell function during BLV infection. Further investigations are required to develop a novel vaccine and therapeutic method against BLV infection using the anti-Tim-3 antibody or recombinant Tim-3 Fc fusion protein.

Kinetic and functional analyses of regulatory T cells in bovine leukemia virus-infected cattle

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Regulatory T cells (Treg) play a critical role in the maintenance of the host immune system; however, it has been reported that Treg, especially $CD4^+CD25^+Foxp3^+$ T cells, are involved in the immune evasion mechanism by tumors and several pathogens that cause chronic infections. The blockade of cytotoxic T-lymphocyte antigen 4 (CTLA-4) expressed on $CD4^+CD25^+Foxp3^+$ T cells by antibodies specific to CTLA-4 resulted in the re-activation of immune functions. Furthermore, the blockade of a variety of immunosuppressive factors by antibodies could be applied to new anti-tumor therapies; however, the function of Treg in cattle is still unclear, although Treg have been extensively studied in human and rodents. In this study, the kinetics of $Foxp3^+$cells and $CTLA\text{-}4^+$ cells was analyzed in cattle infected with bovine leukemia virus (BLV), which establishes chronic infection and causes tumors in its hosts. In addition, the immunosuppressive function of CTLA-4 was evaluated through blockade of the CTLA-4 pathway in BLV-infected cattle.

Foxp3 and *CTLA-4* mRNA were predominantly expressed in $CD4^+$ T cells, and the proportion of $Foxp3^+$ cells in $CD4^+$ cells was inversely correlated with *IFN-*γ mRNA expression, suggesting that $CD4^+Foxp3^+$ T cells in cattle have a potentially immunosuppressive function. In BLV-infected cattle, the proportion of $Foxp3^+$ cells in $CD4^+$ cells as well as the $Foxp3^+$ cell/$CD8^+$ cell ratio elevated as the disease progressed. Combined blockade of immunosuppressive molecules by anti-CTLA-4, anti-programmed death-ligand 1 (PD-L1) and anti-lymphocyte activation gene-3 (LAG-3) antibodies upregulated the expression of *IL-2* and *IFN-*γ mRNA in peripheral blood mononuclear cells from BLV-infected cattle compared to blockade with individual antibodies.

In conclusion, the results obtained in this study suggest that $CD4^+Foxp3^+$ T cells in cattle have an immunosuppressive function and, consequently, this suppression may play a crucial role in disease progression in chronic BLV infection. Furthermore, re-activation of the immune system by the blockade of multiple immunosuppressive molecules has the potential to protect cattle from BLV infection and inhibit disease progression. Further investigations are necessary to develop novel vaccination and therapy methods against BLV infection by the control of Treg and by the blockade of related immunosuppressive molecules by specific antibodies.

Identification of an immunosuppressant molecule, Ipis, in *Ixodes persulcatus* ticks

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Ixodid ticks are harmful vectors for several pathogens causing infectious diseases and afflicting both human public health and the livestock industry. Recent studies have focused on the development of an anti-tick vaccine that inhibits blood feeding and oviposition of ticks. There have been several reports on the identification and characterization of tick molecules as effective targets of an anti-tick vaccine. *Ixodes ricinus* immunosuppressor (Iris) is a tick salivary gland protein, derived from *I. ricinus*, which is the major vector for the Lyme disease agent in Western Europe. Iris inhibits the proliferation of mouse splenocytes and the production of several cytokines (IFN-γ, IL-6, TNF-α, and IL-8) from human PBMCs. Iris also binds to monocytes/macrophages and inhibits their ability to secrete TNF-α. In addition, Iris is a member of the serpin superfamily and interferes with coagulation. In Japan, *I. persulcatus* acts as a vector for the Lyme disease agent. Thus, in this research, Iris homologues were identified in *I. persulcatus*, and their functions in blood feeding were studied.

Two cDNA clones which show high similarity to *Iris* were isolated from salivary glands of fed female *I. persulcatus* ticks, and designated as *Ipis-1* and *-2*. Both of the genes encoded 377-amino acid proteins. Ipis-1 and -2 showed 94.4% and 96.6% similarity to Iris, respectively. Reverse transcriptase PCR analysis showed that Ipis-1 and -2 were expressed specifically in the salivary glands throughout the life cycle stages of ticks. To investigate the function of Ipis, recombinant Ipis (rIpis) were prepared. rIpis/TF protein was expressed in *Escherichia coli* and rIpis/FLAG protein was expressed in COS-7 cells, respectively. rIpis/TF protein was recognized by tick-infested hamster sera, indicating that they are secretory proteins exposed to host animals. In addition, the cell proliferation assay showed that rIpis/FLAG protein appeared to inhibit the proliferation of bovine PBMCs. Thus, this molecule seems to be involved in tick feeding by suppressing host immune responses. Further analysis, including immunosuppressive functions and anti-hemostatic effects of Ipis, will be required to develop an effective vaccine against *I. persulcatus*.

Phylogenetic analysis of *Theileria orientalis* in Myanmar based on the *MPSP* gene

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Theileria orientalis is a causative agent of bovine benign theileriosis, which occasionally

reduces the productivity of livestock in tropical and subtropical countries. Currently, *T. orientalis* is classified into 11 genotypes by phylogenetic analysis based on sequences of the *major piroplasm surface protein* (*MPSP*) gene. Since MPSP is expected to be a promising vaccine candidate and serodiagnosis antigen, it is important to investigate the genetic diversity of different isolates in endemic countries. In the present study, we determined *MPSP* genotypes of *T. orientalis* in Myanmar.

The *MPSP* gene of *T. orientalis* was detected by PCR from 257 of 521 (49.3%) cattle blood DNA samples. Fifty-four *MPSP* gene fragments were subjected to nucleotide sequencing, followed by phylogenetic analysis. As a result, six allelic *MPSP* genotypes, Type 1, Type 3, Type 4, Type 5, Type 7 and Type N-3, were determined. Type 5 (41.5%) and Type 7 (34.0%) were the most prevalent and widely distributed, suggesting that they are the dominant genotypes in Myanmar. Although Type 2 has been reported to be responsible for an outbreak of benign theileriosis in Australia, and is distributed in Japan, Korea and China, this genotype was not detected in Myanmar; therefore, genotype analysis of *T. orientalis* should be continued in Myanmar for surveillance of the presence and introduction of Type 2 from endemic countries.

Analysis of the response to mitogenic lectin of peripheral lymphocytes in LEC rats

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Evaluation of left atrial function by left atrial tracking method in dogs with heart disease

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The left atrial tracking method is a novel echocardiographic modality for the evaluation of left atrial function. The purpose of this study was to assess the value of this method for the evaluation of canine heart disease.

Firstly, the repeatability of variables of the left atrial tracking method was investigated with six normal beagles. The within-day and between-day coefficients of variation (CV) of %LASpass, %LASact, and %LAStotal were all clinically acceptable ($< 20\%$).

Secondly, 68 clinical dogs with left heart disease (27 dogs in the International Small Animal Cardiac Council classification class Ia, 25 dogs in class Ib, 12 dogs in class II, 4 dogs in class III) were enrolled in this study to evaluate the association between echocardiographic measurements and heart failure symptoms. There were significant differences in LA/Ao, %LASact, and %LAStotal between asymptomatic (class Ia and Ib) and

symptomatic (class II and III) dogs. Among them, LA/Ao was strongly related with the onset of clinical symptoms. On the other hand, there were significant differences in %LASpass, %LASact, and %LAStotal between mildly symptomatic dogs (class II) and severely symptomatic (class III) dogs. In particular, %LASact and %LAStotal were strongly related with disease severity, whereas LA/Ao was not.

In conclusion, these results show that the left atrial tracking method has adequately high repeatability for routine clinical usage in dogs. Moreover, the evaluation of left atrial function by the left atrial tracking method is valuable not only for detecting symptomatic dogs with left heart failure, but also for evaluating disease severity.

Evaluation of the ultracentrifugation-precipitation method for canine lipoprotein analysis, and its clinical significance in hyperadrenocorticism and hepatic diseases

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In the present study, two studies were conducted to apply canine lipoprotein analysis to veterinary practice. Due to the lack of a gold standard method in canine lipoprotein analysis, it is unclear whether canine high-density lipoprotein (HDL) and low-density lipoprotein (LDL) can be accurately evaluated by the lipoprotein analysis methods used in dogs; therefore, the ultracentrifugation-precipitation method was examined for its suitability as a gold standard method for canine lipoprotein analysis. Furthermore, since the clinical significance of canine lipoprotein analysis has not been determined, canine HDL-cholesterol (HDL-C) and LDL-cholesterol (LDL-C) in patients with hyperadrenocorticism (HAC) and hepatocellular carcinoma (HCC) were measured and analyzed by the homogeneous method.

Firstly, in the ultracentrifugation-precipitation method, dog sera were overlaid with 154 mM NaCl solution and centrifuged at 164,000 × g at 4°C for 18 hours. After removal of the very-low-density lipoprotein fraction, 1 U/μl heparin and 101 mM $MnCl_2$ were added to the infranatant containing LDL and HDL. After centrifugation, the resulting infranatant [HDL] fraction and precipitated [LDL] fraction were analyzed by LC-MS/MS. As a result, the [HDL] fraction contained only HDL, but the [LDL] fraction contained both LDL and HDL. In addition, although various concentrations of heparin were examined in the precipitation method, canine HDL and LDL were not accurately separated by heparin. These results suggested that the ultracentrifugation-precipitation method cannot be used as the gold standard method in canine lipoprotein analysis; however, because this method could separate canine HDL and LDL approximately, it could be useful for canine lipoprotein analysis in the clinical setting.

Secondly, the concentrations of canine HDL-C or LDL-C in HAC and HCC were measured by homogeneous methods, which are well correlated with the ultracentrifugation-precipitating method. The concentrations of canine HDL-C and LDL-C were increased in HAC, and the concentrations of canine LDL-C were significantly increased in pituitary dependent hyperadenocorticism (PDH) but not in adrenal tumor (AT). Therefore,

it could be used in diagnostic evaluation to differentiate PDH from AT. Moreover, since canine LDL-C and the LDL-C/HDL-C ratio were significantly decreased after PDH treatment, it could also be used as an indicator of the effect of PDH treatment. Additionally, the concentrations of canine LDL-C and HDL-C were increased in HCC, suggesting that HCC might involve a lipid metabolism abnormality. Meanwhile, canine HDL-C and LDL-C were lower in HCC than in PDH. In particular, canine LDL-C was significantly different between HCC and PDH, indicating that the significant increase of LDL-C in HCC could be used as a positive indicator of concurrent PDH. It is expected that the clinical significance of canine lipoprotein analysis could be determined in various diseases, and that canine lipoprotein analysis could be applied to those diseases.

Interleukin-17A expression in intestinal mucosal biopsies from dogs with lymphocytic-plasmacytic enteritis and inflammatory colorectal polyp

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Interleukin (IL)-17A is a proinflammatory cytokine produced by T helper (Th) 17 cells, and an increase in IL-17A expression in the intestinal mucosa has been reported in human inflammatory bowel disease (IBD). Lymphocytic-plasmacytic enteritis (LPE) is the most common form of canine IBD, and several previous studies have reported mRNA expressions of Th1, Th2, and immunoregulatory cytokines in the intestinal mucosa from dogs with LPE; however, IL-17A expression has not been examined in dogs with LPE. Inflammatory colorectal polyps (ICRPs) in miniature dachshunds were recently recognized in Japan as a major cause of large bowel symptoms in this breed. ICRPs are characterized by the formation of multiple small polyps (small polyps) in the large bowel mucosa, accompanied by a large space-occupying polyp (large polyp), and are thought to be a novel form of IBD. To explore *IL-17A* mRNA expression in the intestinal mucosa of dogs with chronic enteropathy, mRNA expression levels of IL-17A were quantitatively analyzed in the duodenal and colonic mucosa of dogs with LPE, and colorectal mucosa of dogs with ICRP. IL-17A expression and distribution in the colorectal mucosa of dogs with ICRP were analyzed by immunohistochemistry.

Duodenal mucosal tissue specimens from 22 dogs with LPE and colonic mucosal tissue specimens from 4 dogs with LPE were obtained by endoscopic biopsy or surgical biopsy (from duodenum, $n = 1$). Mucosal tissue specimens were obtained from colorectal polyps of 6 dogs with ICRP by endoscopic or surgical biopsy. Duodenal and colonic mucosal tissue specimens were obtained from 8 control dogs by endoscopic biopsy ($n = 5$) or at necropsy ($n = 3$). Total RNA was extracted from the intestinal mucosa and cDNA was synthesized. Quantitative analyses of *IL-17A*, *IFN-*γ (Th1 cytokine), *IL-4* (Th2 cytokine) and *IL-10* (immunoregulatory cytokine) mRNA were carried out by a quantitative polymerase chain reaction technique.

The transcriptional levels of *IL-17A*, *IFN-*γ,

IL-4, and *IL-10* mRNA in the duodenal and colonic mucosa from dogs with LPE were not different from those of control dogs. In the case of ICRP, *IL-17A*, *IFN-γ* and *IL-10* mRNA expressions in large polyps, but not in small polyps, were significantly higher than those in colonic mucosa from control dogs. *IL-4* mRNA expression was not different among groups. *IL-17A* mRNA expression in large polyps was strongly correlated with serum CRP concentration. On the other hand, *IL-10* mRNA expression in large polyps was negatively correlated with serum CRP concentration. By immunohistochemistry, IL-17A-positive cells were significantly increased in areas of large polyps compared to small polyps.

These findings indicated that the expression level of *IL-17A* mRNA is not increased in the duodenal and colonic mucosa in dogs with LPE; therefore, it is unlikely that IL-17A plays a crucial role in the pathogenesis of LPE. On the other hand, in dogs with ICRP, the expression level of *IL-17A* mRNA was increased in the large polyps, which were composed mainly of neutrophils. IL-17A may play an important role in the recruitment of neutrophils and the formation of large polyps in colorectal areas in dogs with ICRP, while IL-10 could block the proinflammatory effect of IL-17A.

Characterization of anticancer drug-resistant canine mast cell tumor cell lines and evaluation of resistance-related factors

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Mast cell tumor (MCT) is one of the most common malignancies encountered in dogs. Surgical excision, radiotherapy, chemotherapy, or a combination of these modalities have been performed for canine MCTs. Among these treatment modalities, chemotherapy plays an important role, especially for local recurrence or distant metastases. One of the most important factors influencing the efficacy of chemotherapy is cellular drug resistance. The aim of this study was to evaluate factors associated with resistance to anticancer drugs.

First, vinblastine-resistant cell lines were established using three canine MCT cell lines, CoMS, TiMC and LuMC, by continuous exposure to vinblastine. The vinblastine-resistant cell lines were significantly more resistant to vinblastine than their parent cell lines when the 50% growth inhibitory concentration (IC_{50}) of vinblastine was determined by MTT assay. The vinblastine-resistant cell lines maintained the characteristics of their parent cell lines in the degree of metachromasy and histamine release. In addition, the vinblastine-resistant CoMS cell line, which is the most resistant to vinblastine of the three, was significantly more resistant to lomustine, prednisolone and imatinib mesilate than its parent CoMS cell line.

Secondly, to evaluate resistance-related factors, expression levels of *ABCB1*, *ABCC1*, *ABCG2*, *Bcl-2*, *Bcl-xL*, *Mcl-1*, *XIAP*, *c-IAP1*, *c-IAP2*, *survivin*, β-*tubulin* and *c-kit* genes were examined by real-time PCR analysis. The results revealed that the vinblastine-resistant cell lines showed significantly higher expression of the *ABCB1* gene than their parent cell lines. *ABCB1* gene encodes P-glycoprotein (P-gp), which acts as a drug efflux pump. Expressions of P-gp were

identified both in the vinblastine-resistant cell lines and in their parent cell lines by immunofluorescent staining, but comparative analyses of the expressions of P-gp could not be performed between the vinblastine-resistant cell lines and their parent cell lines; therefore, further detailed quantitative analyses of the expressions of functional P-gp in these cell lines should be conducted.

The results suggested that these vinblastine-resistant cell lines showed multidrug resistance, and overexpression of the *ABCB1* gene probably contributed to cellular drug resistance in canine MCTs. Further investigation and the clinical application of drugs which are not substrates of P-gp or inhibit the expression of the *ABCB1* gene have the potential to overcome cellular multidrug resistance.

Analysis of gene expression of differentiation markers and cellular etabolism factors in cultured equine tenocytes

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In thoroughbred racehorses, tendon injury, especially superficial digital flexor tendinitis of the forelimb, is one of the most widespread and career-threatening injuries. This injury usually requires a long time for treatment and recovery, and frequently recurs after affected horses return to training and racing. The mechanism of the occurrence of tendinitis has not yet been elucidated, although a number of etiological factors have been implicated. The induction of angiogenesis is a serious problem during the process of healing an injured tendon because tendons are hypovascular connective tissue intrinsically; however, there is little information on the factors which regulate vascularization in the tendon.

The objective of this study was to investigate the response of cultured tenocytes to inflammatory stimuli which induce tendinitis. Recently, bone morphogenetic protein (BMP)-12 has been suggested to have the potential to induce the differentiation of mesenchymal stem cells into tenocytes; therefore, we also investigated the response of tenocytes to stimulation with BMP-12.

Tendons and other tissues were sampled from two skeletally normal thoroughbred horses, from which equine tenocytes cell cultures were obtained. First, we confirmed the tissue specificity of tenomodulin (TeM) and scleraxis (Scx) gene expression in tendons by a real-time PCR-based relative quantification technique. TeM is a type II transmembrane glycoprotein containing a C-terminal anti-angiogenic domain and is predominantly expressed in tendons and ligaments. Scx is a basic helix-loop-helix transcription factor that is predominantly expressed in tendon progenitors and tenocytes, and positively regulates TeM expression in a tenocyte lineage-dependent manner. Second, cells were stimulated by interleukin-1β or over-heating and changes in gene expression of TeM, Scx, vascular endothelial growth factor (VEGF)-A and some matrix metalloproteinases (MMPs) associated with extracellular matrix metabolism and vascularization, were investigated by a real-time PCR-based relative quantification technique in the presence or absence of BMP-12.

As a result, we found that TeM and Scx were highly expressed and were specific differentiation markers in equine tendons and cultured tenocytes. In tenocytes, the expressions of TeM and Scx

were decreased by inflammatory stimuli, indicating that tenocyte transformation was induced by inflammatory stimuli. The expressions of VEGF-A and MMPs were increased by stimulation, but their levels and timings of increased gene expression were different. These expression changes indicate that inflammatory stimulation promotes and up-regulates neovascularization. The expression of TeM and Scx was increased and some MMPs were decreased by stimulation with BMP-12.

In future research, the mechanism of tendinitis and the validity of BMP-12 for improving tendinitis will be clarified by investigating at the protein and tissue level, and searching for cellular interactions.

Basic examination of the mechanism to acquire metastatic capacity in canine osteosarcoma cells

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Tumor metastasis clearly affects prognosis. Recently, various studies have examined the mechanism of tumor metastasis. Osteosarcoma has a very high rate of metastasis. Various researches of osteosarcoma metastasis have been performed in humans and mice, but canine reports are rare. The purpose of this study was to compare POS and HMPOS (highly metastatic cells isolated from POS), canine osteosarcoma cell lines.

A cell adhesion assay, wound healing assay and cell migration and invasion assays were performed. These assays to examine different stages of metastasis suggested that HMPOS have a significantly higher tendency of adhesion, migration and invasion than POS. Six genes that are reported to be involved in these mechanisms were further investigated. CDH11 (osteoblast-cadherin) and β-catenin were predicted to be related with cell adhesion. β-Actin was predicted to be involved in cell migration. PPET-1, ET_A and Bcl-xl were predicted to be related with cell invasion. These gene expressions were examined by RT-PCR and real-time PCR. All genes were expressed in both POS and HMPOS, and the expression levels of genes for CDH11, β-catenin, ET_A and Bcl-xl were significantly higher in HMPOS than POS.

These results are consistent with previous studies of other tumors in their migration and invasion ability and the related gene expressions; however, the findings regarding the ability of adhesion and the related gene expression were contradictory to previous reports. Therefore, regarding osteosarcoma metastasis, it was suggested that adhesion ability has little relationship with metastasis in comparison to other tumors.

This study suggests that the ability of adhesion, migration and invasion and the expressions of CDH11, β-catenin, ET_A and Bcl-xl are related to the high metastasis potential of canine osteosarcoma. It is suggested that further studies, for example, gene transfection, should be performed to elucidate the mechanism of high metastatic potential in canine osteosarcoma.

Expression and role of immunoinhibitory factor PD-L1 in bovine leukemia virus-related lymphoma

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The inhibitory factor, programmed death-ligand 1 (PD-L1), and its receptor, programmed death-1 (PD-1), are involved in the immune evasion mechanism of many pathogens and tumors. Enzootic bovine leukemia (EBL) is caused by chronic infection with bovine leukemia virus (BLV), characterized by systemic lymphoma. In this study, the expression of PD-L1 was investigated by immunohistochemistry in lymphoma from EBL cows. It was revealed that 63% cases of EBL lymphoma had PD-L1-positive neoplastic cells with infiltration of T cells expressing PD-1, and that EBL lymphoma had a lower level of CD69, a lymphocytic activation marker, IL-2 and IFN-γ than control lymph nodes by real-time RT-PCR. These data indicate the possible mechanism of the immune evasion system of EBL lymphoma, in which the reaction of PD-1/PD-L1 between T cells and tumor cells induces T cell inactivation.

Molecular biological analysis of an avian leukosis virus isolated from multiple perineurioma and the infection status in Japanese native fowl

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Intraneurial perineurioma is a rare neoplasm. In the veterinary field, it has been reported only in a dog, a Japanese native fowl and 11 chickens inoculated with fowl glioma-inducing virus (FGV), which belongs to avian leukosis virus subtype A (ALV-A). An additional naturally occurring case was found in a Japanese native fowl. Here, the pathological features and the molecular biological characteristics of an ALV strain isolated from the affected fowl were described. The case was a 4-month-old, male, Japanese bantam (*Gallus gallus domesticus*), which clinically showed gait abnormality. At necropsy, there were moderate to severe bilateral enlargements of the lumbosacral plexuses, vagal nerves, sciatic nerves and brachial plexuses. On histologic examination, the affected pheripheral nerves were distended because of the proliferation of spindle-shaped cells with characteristic onion bulb-like structures around residual axons. These spindle-shaped cells were immunohistochemically negative for S-100 α/β proteins. These histological findings were consistent with those of avian multiple perineuriomas previously reported. The feather pulp, brain and lumbosacral plexus from the affected bird were positive for FGV determined

by nested PCR. An ALV strain, HE_5873, has been isolated from the brain and molecular biological analysis of the isolate was performed. The *pol* region of the isolate showed 97% similarity to that of an endogenous retrovirus *ev*-1 and the SU region in the *env* gene (*env* SU) showed 97% similarity to those of FGV variants, including Tym-43 and U1. On phylogenetic analysis of *env* SU, this isolate was categorized into a cluster distinct from any groups belonging to FGV, CTS_5371 isolated from a Japanese native fowl affected with neurofibrosarcoma, Tym_S 90 from layers with fowl glioma and the DNA fragments detected from the first case of naturally occurring perineurioma in a chicken. Additionally, the infection rate of ALV was surveyed in Japanese native fowl produced in Kochi prefecture, Japan, as rare peripheral nerve tumors have been successively found in these native fowl. Fifteen (79%) of 19 Japanese native fowl were positive for ALV by PCR and 14 (93%) of the ALV-positive birds were also positive for FGV by nested PCR. These results indicate that HE_5873 is a recombinant virus from *ev*-1, FGV and/or other ALVs, and that avian multiple perineuriomas occur associated with the infection of ALVs other than FGV. In addition, ALVs with FGV-specific regions in the viral genome are suggested to be prevalent among Japanese native fowl produced in Kochi prefecture, Japan.

Pathological study of myocardial abnormality associated with avian retrovirus infection

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Fowl glioma-inducing virus (FGV), which belongs to avian leukosis virus subgroup A (ALV-A), causes so-called fowl glioma, nonsuppurative encephalitis and cerebellar hypoplasia in chickens and is associated with avian perineurioma. In this study, unusual cardiac lesions in chickens infected with FGV variants were histopathologically examined and molecular biological features of the ALV strain isolated from an affected case were analyzed. Feather pulp and brains obtained from eighteen Japanese native chickens reared in Kumamoto were positive for FGV or ALV determined by nested PCR. Histologically, 10 of 18 chickens showed fowl glioma and all chickens had nonsuppurative myocarditis with intrasarcoplasmic matrix inclusion bodies. The inclusion bodies were distributed in myocardial fibers and Purkinje fibers, and were divided by their stainability with hematoxylin and eosin stain into basophilic and non-staining. Additionally, hypertrophic myocardial fibers with giant atypical nuclei were focally to multifocally observed in 7 of 18 chickens (39%). These myocardial fibers occasionally had matrix inclusion bodies and mitotic figures. An ALV strain, Km_5892, was isolated from the affected chicken and the SU region in the *env* gene (*env* SU) and the FGV-specific region showed 90% and 82% identity with the corresponding regions of the FGV prototype, respectively. Moreover, the *pol* region of Km_5892 showed high similarity (97%) to endogenous retrovirus *ev*-1. Phylogenetic analysis based on the *env* SU revealed that Km_5892 was classified into the same cluster as Km_5666, Km_5843 and Km_5845, which were isolated from chickens reared in Kumamoto. Although C/O specific-pathogen-free chickens experimentally inoculated with Km_5892 had nonsuppurative myocarditis

with matrix inclusion bodies, the myocardial fibers had no morphological aberrations. These results suggest that morphological abnormalities of myocardial fibers could develop in association with the infection of an FGV variant, which was considered to be a recombinant virus derived from FGV and endogenous retroviruses such as *ev*-1.

Influence of handling and thawing methods on the quality of bovine sperm frozen with egg yolk-based extender and packaged in 0.5-ml straws

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Artificial insemination (AI) with frozen semen is an essential technique in cattle; however, the appropriate method for handling and thawing bovine sperm frozen with an egg yolk-based extender and packaged in 0.5-ml straws is not well defined. The aim of this study was to investigate the influence of the handling and thawing of straws on sperm quality. In this study, viability, motility, acrosomal integrity and active mitochondria in sperm were assessed after thawing.

Firstly, straws were subjected to 30°C static air or a light breeze for a duration of 2–10 sec and placed back into liquid nitrogen. This exposure repeated five times before thawing in a 37°C water bath for 45 sec. When straws were subjected to static air for 5 sec or to a light breeze air for 3 sec, sperm were damaged. On the other hand, the quality of sperm subjected to a light breeze for 2 sec did not change; therefore, the exposure of straws to air must be within 2 sec. Secondly, straws were subjected to 30°C static air or to a light breeze air temperature for 5–60 sec before thawing in a 37°C water bath for 45 sec. Sperm were damaged when straws were subjected to static air for 30 sec and to a light breeze for 3 sec. Since the quality of sperm subjected to a light breeze for 5 sec was not degraded, the time of straw exposure in the air must be within 5 sec before thawing. Lastly, straws were immersed in a 37°C water bath for 15 or 45 sec, and subjected to 4 or 23°C static air for 15 min. Straws immersed for 15 min in 37°C water after thawing were used as a control. Sperm thawed for 45 sec and subjected to 4°C static air had the lowest quality of all experimental groups. In addition, the rate of sperm with intact acrosomes and active mitochondria was lower when straws were exposed to 4°C for 15 and 45 sec after thawing compared to the control.

In conclusion, to prevent sperm damage, straws should be handled in air within 2 sec, and less than 5 sec before thawing. Also, protecting straws from cold shock is necessary after thawing.

Studies on ovarian follicular dynamics of postpartum dairy cattle at an experiment farm at Hokkaido University

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The objective of this study was to investigate postpartum ovarian follicular dynamics in 18 Holstein cows that calved between November 2010 and July 2011 at the experiment farm of Hokkaido University. Ovarian follicular dynamics was monitored using an ultrasound system at least 3 times a week until 70–120 days postpartum (dpp).

The first follicular wave after parturition emerged within 5–8 dpp in 14 out of 16 cows. The first ovulation was observed between 11–30 dpp (7 cows), 39–53 dpp (6 cows) and 61–96 dpp (3 cows). No ovulation was observed in 2 cows during the investigation period. There was a positive correlation between the period from parturition to first ovulation and the numbers of waves that took in first ovulation ($r = 0.94$, $p < 0.001$). The mean length of the first ovulatory cycle of 6 cows that had their first ovulation between 39–61 dpp (11.3 days) was shorter than that of 6 cows with their first ovulation between 11–30 dpp (19.7 days). The mean maximum diameter of ovulatory follicles (15.4 mm) tended to be greater than that of anovulatory dominant follicles (13.8 mm, $p = 0.1$) in 1 to 3 waves before the emergence of ovulatory follicles. Four cows which were restricted from feeding on concentrate diet soon after calving did not show the first ovulation within 85 dpp, and the average size of their dominant follicles was 12.7 mm. Double ovulation was detected in 7 out of 15 cows (47%) and in 10 out of 56 ovulations (18%). The mean diameter of double ovulation follicles (11.5 mm) was smaller than that of single ovulation follicles (15.4 mm, $p < 0.001$). Six cows with a high number (> 25) of small follicles (< 5 mm in diameter) showed early first ovulation (average 25.6 dpp), and 12 cows with a low number of follicles (< 25) showed late ovulation (average 55.8 dpp).

These results suggest that postpartum cows with an energy shortage tend to need a long period until the first ovulation and that the resumption of ovarian function tends to be early in cows with a high number of small follicles.

Foaling rates of thoroughbred mares in Japan: Analysis of foal productivity from 1998 to 2009

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No report has analyzed the foaling rate of thoroughbred mares in detail in Japan. In this study, the foaling rate per season (number of foals/number of mated mares), the foaling rate per cycle (number of foals/number of mated cycles) and the foaling rate per every mated

session (number of foals in each mated cycle/ number of mated mares in each mated cycle) were analyzed using the production result reports from 1998 to 2009.

Firstly, the foaling rate by the mare status (maiden, foaling and barren) was analyzed. The average number of annual mating mare populations was 10,525 mares and the total foaling rate per season was 74%. The foaling rate per season of maiden mares (88%) was the highest in all mare statuses. The foaling rates per season of foaling mares (73%) and barren mares (70%) did not differ significantly and decreased with aging from 80% (4–8 years old) to 50% ($\leq$ 19 years old). The total foaling rate per cycle of all mare statuses was 43%. The foaling rate per cycle of maiden mares (56%) was higher than those of foaling mares and barren mares (43% and 36%, respectively). In foaling mares, the foaling rate of the first mating of the season (42%) was lower than that of the second mating (47%); however, in barren mares, the foaling rate was highest in the first mating of the season (40%) and decreased with an increasing number of mating sessions.

Secondly, the foaling rates of mares first mated at foal heat (FH) or at second estrus (FH skip) were analyzed. There was no significant difference in the foaling rate per season between FH mares (73%) and FH skip mares (75%); however, the foaling rate per cycle of FH mares (41%) was significantly lower than that of FH skip mares (49%). In FH mares, the foaling rate at first mating (38%) was lower than that at second mating (47%). In FH skip mares, the foaling rate at first mating (51%) was higher than that at second mating (47%).

It was clarified that the foaling rates per cycle were significantly different among the mare statuses (maiden $>$ foaling $>$ barren), and the foaling rate of mares mated at foal heat was lower than when mated at second estrus in foaling mares in Japan.

Endoplasmic reticulum (ER)-associated degradation of R664X mutant of bovine anion exchanger 1 (AE1): Demonstration of the localization and interaction with CLPTM1 in the ER

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The ER-associated degradation (ERAD) of various polytopic membrane proteins, including the most common mutant of cystic fibrosis transmembrane-conductance regulator (CFTR), ΔF508-CFTR, involves recognition in the ER and retrotranslocation into the cytosol of the polypeptide, leading its degradation by the proteasome system. Previous studies have demonstrated that the ERAD of an R664X mutant of bovine anion exchanger 1 (AE1), a causative mutant of hereditary spherocytosis in cattle, is largely different from that of ΔF508-CFTR, being *N*-gylcosylation-independent and ubiquitylation-independent. The purpose of the present study was to examine ultrastructural localization of R664X AE1 and differences in the intracellular transport of R664X AE1 and ΔF508-CFTR in living cells, and to identify ER proteins that interact with R664X AE1 during ERAD. Immunofluorescent microscopy showed that R664X AE1 localized in the ER throughout the cytosol of transfected cells even when the proteasome was inhibited, whereas

ΔF508-CFTR, which resided in the ER in the steady state, was concentrated in the aggresome upon proteasomal inhibition. Several different proteins, including cleft lip and palate transmembrane protein 1 (CLPTM1), were isolated and identified to interact with R664X AE1 tagged with glutathione S-transferase (GST) in the ER by GST pull-down analysis followed by liquid chromatography-mass spectrometry. Interaction of R664X AE1 and myc-CLPTM1 was confirmed by co-immunoprecipitation of R664X AE1 with myc-CLPTM1 by the anti-myc antibody from the lysate of cells expressing these proteins. These findings demonstrate that the ERAD of R664X AE1 involves ER proteins and the mechanism of its retention in the ER is different from that of ΔF508-CFTR.

Genetic predisposition which regulates the spectrin content of bovine red cell membrane: Focal structural alteration of *α*-spectrin generated by polymorphism of the spectrin genes

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The erythrocyte membrane is laminated by a protein skeleton involving mainly spectrin and actin, which is involved in deformability and stability of the membrane. Band 3 deficiency in Japanese black cattle is a novel disorder due to R664X mutation of the band 3 (anion exchanger 1, AE1) gene, exhibiting hemolytic anemia associated with hereditary spherocytosis. Our recent studies suggested that E91K mutation in the allele B-K91 (SpαB/K91) of the α-spectrin gene plays a modulatory role in red cell phenotypes in band 3 deficiency. The purpose of the present study was to analyze the relationship between E91K amino acid substitution and red cell membrane stability and to elucidate the focal structural alteration of α-spectrin generated by E91K. We investigated the deformability and the stability of red cell membranes obtained from cattle carrying 91E/91E, E91/K91 and K91/K91 genotypes by ektacytometry. As a result, we found that an increase in deformability and a marked decrease in the stability of E91/K91 and K91/K91. This suggests structural and functional disorders of α-spectrin derived from SpαB/K91. We therefore created recombinant proteins of α-spectrin, including the N-terminus to segment 1, on which the 91st amino acid residue lies, and examined the effect of E91K on the secondary structure of α-spectrin by far-UV CD spectrometry and NMR-HSQC. We found that Spα[0–1] with K91 has a reduced content of α-helix and lower thermostability than Spα[0–1] with E91 because of disordered and unstable structural alteration of segment 1. These findings suggest that E91K amino acid substitution decreases the physical stability of the red cell membrane via structural abnormality of α-spectrin and acts as genetic predisposition that deteriorates the pathological condition of band 3 deficiency.

Identification of the locus control region of bovine β-like globin genes and its role in globin switching

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Hemoglobin switching in the birth period is a physiological response including transition of β-like globin chains from fetal γ-chains to adult β-chains. This process is mainly controlled by a competitive mechanism that involves transcription factors interacting with the γ- and β-globin promoters and the locus control region (LCR). However, the molecular basis of bovine globin switching is not clear, although the difference in the order of γ- and β-promoter placement between humans and cattle implies that novel mechanisms regulate bovine globin switching. The purpose of the present study was to identify bovine LCR and to analyze its role in the transcriptional regulation of bovine β-like globin genes. According to the DNA sequence of caprine globin LCR, we identified two homologous regions in the bovine genome. Among them, we confirmed that the core hypersensitive site (HS) 2 region in the proximal LCR was indeed hypersensitive to DNase I by quantitative PCR. Insertion of this HS2 into 5' upstream of γ- or β-globin promoters upregulated promoter activity, especially that of the γ-promoter. Such activity elicited by HS2 was inhibited by the presence of BCL11A, suggesting that BCL11A can suppress promoter activity through interaction with HS2. When γ- and β-promoters were tandemly arranged downstream of HS2, the promoter located distal to HS2 was specifically activated in erythroid cell lines. Although each of γ- or β-promoter was activated by EKLF, γ-promoter was selectively upregulated when these promoters were arranged in tandem downstream of HS2 in any order. Promoter activity upregulated by EKLF was partly suppressed by BCL11A. These results suggest that EKLF associated with bovine γ-promoters is affected by BCL11A through interaction with LCR including HS2. Therefore, we propose that these transcription factors and LCR are associated with the selective regulation of bovine γ-promoters, and the mechanisms for bovine globin switching.

Epidemiological survey of hantavirus infection in Japan and genetic variation of the viruses in *Myodes rufocanus*

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Hantaviruses are rodent-borne zoonotic agents which cause hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS). It is generally believed that hantaviruses have co-evolved with their rodent hosts and various serotypes are carried by specific natural hosts all over the world. In East Asia and Far East Russia, a large number of HFRS cases are

reported annually. Japan identifed HFRS cases related with Seoul virus transmitted by peridomestic brown rats (*Rattus norvegicus*) and laboratory rats from the 1960s to 1984. No HFRS patients have been reported since 1985; however, seropositive *R. norvegicus* has been detected in several locations. In addition, Hokkaido virus (HOKV) is distributed widely among *Myodes rufocanus* in Hokkaido, the main northern island of Japan. So far, no HFRS cases related with HOKV have been reported. In this paper, epidemiological surveys of rodents were carried out all over Japan, and genetic analyses of HOKV and its natural host, *My. rufocanus*, were performed.

To understand the recent epizootiology of hantavirus infection in Japanese rodents, firstly, 1658 rodents (including *R. norvegicus, R. rattus, Apodemus argenteus, A. speciosus,* and *My. rufocanus*) were captured in various regions of Japan from 1994 to 2011. All rodent sera were tested by ELISA using an antigen mixture of recombinant nucleocapsid proteins of Sin Nombre virus, Amur virus, and HOKV expressed in *Escherichia coli*. All of the 840 *Rattus* spp. and 113 wild rodents captured in southern regions of Japan excluding Hokkaido were seronegative, while among 705 wild rodents and insectivores from Hokkaido, 7.67% (27/352) of *My. rufocanus* and 1.19% (2/168) of *A. speciosus* had anti-HOKV antibodies. These results strongly suggest that the prevalence of hantavirus infection in wild rodents is extremely low in the southern regions of Japan. This might be the reason why no HFRS patients have been reported in recent years; however, HOKV infection is still prevalent in *My. rufocanus* in Hokkaido.

Genetic variation of HOKV was analyzed to gain insight into the evolution of this virus. Total RNA was extracted from lung tissues of seropositive rodents, the viral S gene was amplified by RT-PCR, and the nucleotide sequence was determined. Sequences of the mitochondrial DNA cytochrome *b* gene of the host rodents were also determined. Phylogenetic analyses of viral S and M genes indicated that HOKV sequences can be separated in 3 different virus groups depending on their geographical origin, such as Hokkaido, Sakhalin and Khabarovsk. On the other hand, among viral sequences from Hokkaido, no clear relationship was shown between the geographical distance of the virus origin and the genetic distance.

Although HOKVs from 3 areas share the same rodent hosts, the virus in Khabarovsk was located far from HOKVs in Hokkaido and Sakhalin on the phylogenetic tree based on the full-length viral S, M and L genes; therefore, HOKV carried by *My. rufocanus* may include phylogenetically different viruses.

Role of N-linked glycan of the envelope protein of tick-borne encephalitis virus

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Tick-borne encephalitis virus (TBEV) is a zoonotic disease agent that causes severe viral encephalitis in humans. TBEV is a member of the family *Flaviviridae*, genus *Flavivirus*, and is transmitted by tick bites. The envelope (E) protein of TBEV has one N-linked glycosylation consensus sequence, but there are few reports about the biological function of N-linked glycan

of the protein of TBEV. In this study, we investigated the function of the glycosylation of E protein using recombinant TBEV with and without N-linked glycan of E protein (TBEV Oshima IC-*pt* and Δ*Eg*, respectively) using an infectious cDNA clone.

Infectivity of the virion was not affected after removal of the N-linked glycan by N-glycosidase F. Δ*Eg* produced fewer virus progeny than *pt* in mammalian BHK cells. A similar level of E protein was secreted from cells infected with Δ*Eg* compared to *pt*, but the level of E protein with a conformational structure was markedly reduced. This indicates that the glycosylation of E protein contributes to the conformational stability of E protein and/or efficient transport in the viral maturation and secretion process.

In contrast, a similar level of progeny virus was secreted from the tick cell line, ISE6 cells, regardless of the glycosylation of E protein. Furthermore, it was revealed that the secretion pathway of TBEV in ISE6 cells was independent of Golgi apparatus and actin filament, which is essential for the secretion pathway of flavivirus in mammalian cells. These data indicate that TBEV is secreted through an unidentified pathway in tick cells.

Mice infected subcutaneously with Δ*Eg* showed no signs of disease. The viral multiplication of Δ*Eg* in peripheral organs was reduced compared to *pt*, leading to lower neuroinvasiveness. In contrast, intracerebral infection with Δ*Eg* resulted in a high mortality rate. These results suggest that glycosylation of E protein of TBEV is important for neuroinvasiveness in mice, due to efficient multiplication in the peripheral organs, and that it has little impact on neurovirulence. Additionally, mice infected subcutaneously with *pt* or Δ*Eg* could induce the same level of neutralizing antibodies.

In this study, we identified that the N-linked glycan of the E protein of TBEV is involved in the viral secretion process and attenuation in mice. These findings will be useful for further study to elucidate the mechanism of flavivirus multiplication, and to develop a live attenuated TBEV vaccine and efficient treatment agent targeting the N-linked glycan of viral protein.

Characterization of Ttck-borne encephalitis virus Oshima 08-AS strain isolated in Hokkaido in 2008

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Tick-borne encephalitis virus (TBEV), a zoonotic disease agent, causes severe encephalitis in humans. Recent serological surveys clarified that TBEV has been endemic in the southern part of Hokkaido over the last 10 years. In this study, we attempted to assess the risk of TBE in the region by analyzing the biological characteristics of the TBEV Oshima 08-AS strain isolated in Hokuto city in 2008.

The complete nucleotide sequence (11,100 nucleotides) of Oshima 08-AS was determined. The Oshima 08-AS strain had 36 nucleotide differences, resulting in 12 amino acid changes from the Oshima 5–10 strain isolated in 1995. All amino acid changes were located in the region coding for non-structural (NS) proteins. In virus-infected cells, there was no significant difference between virus multiplication of Oshima 08-AS and Oshima 5–10. In a mouse model, Oshima 08-AS showed higher morbidity and mortality

than Oshima 5–10. A relatively higher level of virus was detected in the brains of mice inoculated with Oshima 08-AS. In histopathological analysis, an early inflammatory response with the production of viral antigen in neural cells was observed in the brains of mice infected with Oshima 08-AS. Analysis using infectious cDNA indicated that several amino acids in NS1 (position 68) and NS5 (position 862 and 879), and three nucleotides in 3´-UTR were involved in the different pathology between Oshima 08-AS and Oshima 5–10. In summary, TBEV has been maintained at a low mutation rate in Hokuto city for more than 10 years, but it was shown that a few naturally occurring mutations caused altered pathogenicity of endemic TBEV. These results indicate that further monitoring of TBEV in this region is important.

Contribution and role of protein deacetylases against mitochondria-derived oxidative stress induced by menadione

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Mammalian cells are equipped with multiple intracellular signal transduction mechanisms to maintain their homeostasis in response to various stresses. The signaling mechanism through protein acetylation/deacetylation, which is regulated by histone acetyltransferases (HAT) and histone deacetylases (HDAC), respectively, is one such mechanism. The imbalance between oxidation and reduction in cells due to the increase of reactive oxygen species (ROS) induces oxidative stress, which is known to be involved in many diseases. Although recent studies suggest that HDAC are associated with the cellular response to oxidative stress, it remains unclear whether HDAC mediate cellular responses against mitochondria-derived oxidative stress. To elucidate this, the role of HDAC was investigated in cells exposed to mitochondria-derived oxidative stress by utilizing pharmacological inhibitors of different classes of HDAC. In this study, menadione was used as an inducing agent of mitochondria-derived oxidative stress.

When menadione-induced cell death was examined in human lung adenocarcinoma A549 cells, more cell death was observed in cells pretreated with a Class III HDAC inhibitor, splitomicin, before menadione than in cells treated with menadione alone; however, two Class I/II HDAC inhibitors, TSA and SAHA, had no influence on menadione-induced cell death. These results suggested that Class III HDAC, but not Class I/II HDAC, were involved in cell death caused by menadione. The contribution of apoptosis to menadione-induced cell death was then evaluated, and it was found that apoptosis was not the main mode of cell death. Analysis of the intracellular ATP level revealed a significant decrease in cells treated with splitomicin and menadione but not in cells treated only with menadione, indicating that necrosis was the primal mode of splitomicin-enhanced cell death. Since overactivation of poly (ADP-ribose) polymerases (PARP) is reported to be responsible in part for necrosis caused by the deprivation of intracellular ATP, PARP activity was examined by immunohistochemistry with anti-poly (ADP-ribose) antibody in A549 cells. Immunohistochemical analysis showed higher PARP activity in cells pretreated with splitomicin before menadione than in cells treated with menadione alone. It

was suggested that splitomicin enhanced PARP activity in A549 cells stimulated with menadione.

In conclusion, this study suggests that cells regulate mitochondria-derived oxidative stress via NAD^+, and that Class III HDAC and PARP play important roles in this regulatory mechanism. Considering that mitochondria-derived oxidative stress is linked with various diseases, including diabetes and myocardial infarction, the regulatory mechanism of oxidative stress proposed in this study might be involved in the pathophysiologies of many diseases.

Metabolic activation of promutagens in rat gastrointestinal tract

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The gastrointestinal tract is the organ first exposed to orally ingested xenobiotics. The first-pass effect or metabolic activation of drugs also occurs in the gastrointestinal tract; however, there are few reports on the metabolic pathway of chemicals in the gastrointestinal tract, and local metabolic activation or detoxification in the gastrointestinal tract is still unclear. In the present study, the expression and induction of xenobiotic-metabolizing enzymes and metabolic activation ability were investigated in the gastrointestinal tract.

In the gastrointestinal tract of rats (tongue, esophagus, stomach, jejunum, ileum, cecum, colon, rectum), various xenobiotic-metabolizing enzymes were found, including cytochrome P450 (CYP), microsomal epoxide hydroxylase, N-acetyl transferase, diphosphoglucuronosyl transferase and sulfotransferase. The expression levels of these enzymes were different among tissues. After administration of a CYP1A inducer (sudan III), the expression level of CYP1A mRNA or protein was induced. Interestingly, the tongue is the most sensitive organ to sudan III in CYP1A induction. Then, mutagen-activating ability was compared among S9 fractions of the gastrointestinal tract using 2-aminoanthracene (2-AA). Mutagen (2-AA) -activating capability was detected in all sections of the gastrointestinal tract of non-treated rats, and was slightly stronger in the cecum or ileum than in the other organs. After administration of sudan III, the level of mutagen (2-AA) activation in the duodenum and jejunum was especially increased.

In the present study, it is suggested that the difference in the expression levels of xenobiotic-metabolizing enzymes in the gastrointestinal tract may be responsible in part for organ-specific chemical sensitivity. The gastrointestinal tract is exposed daily to numerous xenobiotics, including mutagens; therefore, differences in the expression or induction ability of xenobiotic-metabolizing enzyme and the levels of mutagen activation may be partly responsible for cancer in the gastrointestinal tract.

Xenobiotic metabolism in amphibians: characteristics and inter-species differences

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Many researchers have reported the global decline of the amphibian population; chemical contamination is one of the causes. Xenobiotic metabolism is an important factor to determine sensitivity to chemicals because chemical toxicity is affected by metabolism; however, the details of xenobiotic metabolism in amphibians are still unknown. In this study, the characteristics of the conjugation reaction in amphibians were clarified and inter-species differences were found between amphibians and other vertebrates or among amphibians. In fish or mammals, it is reported that pyrene, a polycyclic aromatic hydrocarbon (PAHs), is mainly conjugated by glucuronide after hydroxylation, but in amphibians, the main conjugated metabolite of pyrene is sulfate conjugate. Moreover, glucose conjugation has been found only in Urodela species. Analysis of UDP-glucuronotransferase (UGT) indicated that the difference in glucose conjugation activity among amphibians was based on amino acid alterations in the cofactor binding region of UGT1A. Additionally, amphibians were exposed to 3-methylcholanthrene (3MC, a PAH) and the induction of xenobiotic metabolic enzymes, cytochrome P450 (CYP) 1A and UGT1A, was observed in the liver, stomach, intestine and skin. It was found that the expressions of CYP1A or UGT1A6 were markedly increased in the stomach or intestine, but not in the liver. These results suggested that xenobiotic metabolism in amphibians is amphibian-specific and different from in fish or mammals, and that inter-species differences in xenobiotic metabolism exist among amphibians.

Strain differences in cytochrome P450 in rats

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Knowledge of strain differences in drug metabolism is important for the selection of experimental rat strains for pharmacokinetic and toxicological studies; however, strain differences in cytochrome P450 (CYP), a drug-metabolizing enzyme, have not been fully reported. The focus of this study was strain differences in the activities, protein and mRNA expression of CYP in four rat strains: Wistar (WI), Sprague-Dawley (SD), Dark Agouti (DA) and Brown Norway (BN).

In the metabolism of warfarin as an enzymatic marker of CYP isoforms, DA showed higher activities of CYP2B1, CYP2C11 and CYP3A2 than WI. Western blotting analysis also showed that protein expressions of CYP2B1 and CYP3A2 in DA appeared higher than in WI. In addition,

RT-PCR analysis showed marked strain differences in the same fashion.

In terms of CYP1A1, ethoxyresorufin -O-deethylase activity, the enzymatic marker for CYP1A1, was highest in SD hepatic microsomes. RT-PCR showed that CYP1A1 mRNA expression in SD was higher than in other strains. On the other hand, the mRNA expression of aryl hydrocarbon receptor (AHR), the main transcriptional factor for CYP1A1, was lower in SD than in other strains.

To elucidate the inversion phenomena, the sequences of AHR, AHR nuclear translocator (ARNT) and the promoter region of CYP1A1 were investigated. Although the sequences of AHR and CYP1A1 promoter regions of WI were completely coincident with those of SD, SD had various mutation patterns in the C-terminal domain of ARNT, such as 1 amino-acid insertion and substitution. Moreover, the results of a gel shift assay showed that the nuclear extract from SD had higher binding affinity for CYP1A1 promoter region than WI, which might up-regulate the transcription of CYP1A1 mRNA.

This study showed significant strain differences in CYP activities and expressions, and in transcriptional factors for CYP.

Metal and metalloid contaminations in wild rat populations in Japan

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Heavy metal and metalloid contamination is a continuing environmental threat. Although in aquatic environments, waterbirds, fishes, and mollusks are frequently used as biomonitor species, reports on terrestrial animals are scarce. In this study, wild rats, as widely available terrestrial mammals, were proposed as a suitable sentinel animal for metal pollution. 98 brown rats (*Rattus norvegicus*) and 103 black rats (*Rattus norvegicus*) were collected from 16 regions of Japan. The sex of the animals was determined and age was estimated. The concentrations of seven metals (Cd, Co, Cr, Cu, Ni, Pb, Zn) and one metalloid (As) in liver and kidney were measured by atomic absorption spectrometry, and the effects of these contaminants were predicted by measuring the mRNA expression of selected biomarker molecules.

It was found that tissue residue patterns of urban wild rat populations are more influenced by habitat (chicken farms, roadside, etc.) than the region's soil contamination pattern. In addition, it was discovered that, in the Japanese samples, a high concentration of lead could be found in animals collected from Niigata, a high concentration of arsenic in Hyogo, and a comparatively high concentration of nickel in the case of rats from Chiba.

Comparison with the international literature revealed that, while arsenic, cadmium, and chromium showed comparatively low levels in the Japanese samples, lead, a heavy metal with high toxicity, was found in high concentrations in all examined organs of rats from Japan compared with other reported data.

To reveal the biochemical effect of metal pollution in rats, hepatic tissue residue metal contaminations were compared within a heavily polluted urban area (Tokyo), and samples from a low-contaminated uninhabited island (Oshima Oshima) and laboratory animals (Wistar rats).

We found that, in the cases of lead, zinc, cobalt, and chromium, significantly higher concentrations were measured in samples from Tokyo than in the other groups. In addition, metallothionein-1 (MT-1) and heme oxygenase-1 (HO-1) mRNA expression levels were significantly higher in Tokyo than in Oshima Oshima.

These results suggest that wild rats are ideal candidates for the tissue residue approach of metal and metalloid contamination monitoring in terrestrial environments. While there was a correlation between MT-1 and HO-1 expression levels and tissue metal contamination, since in wild environments these two molecules are induced by a wide variety of xenobiotics, they are more appropriate as general pollution indicators than metal- or metalloid-specific biomarkers.

Molecular survey of *Anaplasma* and *Ehrlichia* infections of rodents in Hokkaido

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Several species of the genera *Anaplasma* and *Ehrlichia* are causative pathogens of emerging infectious diseases in both humans and animals. In recent years, *Anaplasma phagocytophilum*, *Ehrlichia muris* and other pathogens of the family *Anaplasmataceae* have been newly detected in Japan. In Hokkaido, various epidemiological studies have been performed; however, the majority of these studies involved cows, deer and ticks, and there are few studies about rodents, although rodents are important vertebrate hosts of many *Anaplasma sp.* and *Ehrlichia sp.*. Therefore, we attempted to detect the prevalence of *A. phagocytophilum*, *E. muris* and *Anaplasma bovis* in 297 rodents captured in Hokkaido.

Molecular analysis of 285 bone marrow, 127 blood clot and 127 spleen samples were performed. For bone marrow analysis, 1 (0.35%) sample positive for infection by *A. phagocytophilum* and 6 (2.1%) samples positive for infection by *E. muris* were detected. For blood samples, no samples positive for *A. phagocytophilum* and 9 positive samples for *E. muris* were detected. For spleen samples, no samples positive for *A. phagocytophilum* and 4 samples positive for *E. muris* were detected. "*Candidatus* Neoehrlichia mikurensis" and "*Candidatus* Ehrlichia ovata" were detected in addition to PCR target pathogens.

To our knowledge, this is the first report of *A. phagocytophilum, E. muris* and "*Candidatus* N. mikurensis" infection in rodents in Hokkaido. *A. phagocytophilum* was detected in only 1 of 285 rodents, and *A. bovis* was not detected in this study; therefore, rodents may not be the main vertebrate hosts of *A. phagocytophilum* and *A. bovis* in Hokkaido. *E. muris* was detected in 6 of 285 rodents, suggesting that rodents may play a role in the enzootic maintenance of *E. muris*.

Regulatory mechanism of lipid metabolism in captive female Japanese black bears (*Ursus thibetanus japonicus*) during pre-hibernation and hibernation

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Since bears eat nothing during the entire hibernation period, they intake large quantities of food and accumulate body fat in autumn, and use their body fat for energy to survive during hibernation. It has been reported that body mass and body fat mass are highest in the pre-hibernating period in captive black bears (*Ursus thibetanus*); however, little is known about the regulatory mechanism of body mass and body fat in bears during pre-hibernation and hibernation. In this study, we measured plasma component levels and analyzed the gene expression of lipid metabolism-related enzymes in white adipose tissue (WAT) in female Japanese black bears.

Plasma glucose levels did not change seasonally. In contrast, plasma triglycerides (TG), total cholesterol, non-esterified fatty acids and glycerol levels in March were significantly higher than in June, and plasma very low density lipoprotein and low density lipoprotein levels tended to increase in March. In addition, plasma TG levels in November were significantly lower than in June. In WAT, the levels of FAS, G6PD, ME and DGAT2 mRNA expression were significantly increased in November and significantly decreased in March. On the other hand, mRNA expression in WAT was significantly increased for anti-lipogenic enzymes and fatty acid transport and catabolism-related enzymes in March compared with June.

These results indicate that lipogenesis and TG intake in WAT are up-regulated during pre-hibernation, and that lipolysis and release of lipids from WAT are facilitated during hibernation in WAT. Furthermore, this study showed that the expression of lipogenic genes was increased during pre-hibernation in captive black bears given an almost constant amount of food throughout the pre-hibernation period, suggesting that the mechanism of body fat accumulation in black bears is regulated not only by food intake, but also other physiological and metabolic factors.

Movements and genetic structure of brown bears (*Ursus arctos*) in Nemuro, eastern Hokkaido, Japan

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Concern about the increase of human-brown bear (*Ursus arctos*) conflicts is growing in

Shibetsu town, located in the northern Nemuro region of eastern Hokkaido. Appropriate measures for the conservation and management of bears to reduce human-bear conflicts are required; however, little is known about bear ecology, including movement, habitat use and genetic structure, which are essential for appropriate management. The objective of the present study was to reveal the movements and genetic structure of bears in the Nemuro region based on global positioning system (GPS) telemetry data and mitochondrial DNA (mtDNA) haplotypes.

From 2009 to 2011, 16 bears were captured, and 8 males and one female were tracked. Males traveled to and from the Shiretoko Peninsula and the southern Nemuro region. On the flat land, bears moved through windbreak forests and riparian forests, and these forests played a role as movement corridors. We investigated mtDNA haplotypes for 23 males and 7 females using samples obtained from captures, nuisance kills, hunting and hair-traps. All females were assigned to Cluster B, and it was suggested that Cluster B was distributed from the Shiretoko Peninsula to the Shibetsu region. On the other hand, males were assigned to either Cluster A or B. This result indicates that males born in the distribution area of Cluster A, the southern Nemuro region and central Hokkaido have moved to the Shibetsu region. Therefore, the Shibetsu region is the boundary area of the distribution areas of Clusters A and B, and this region has important implications for maintaining the genetic diversity of brown bears in Hokkaido as a boundary zone between Clusters A and B.

Thus, conservation of the bear population and their habitat in the Nemuro region is important for the conservation and management of Hokkaido brown bears. Further studies, however, are needed in order to reveal the more detailed characteristics of the movements and genetic structure of Hokkaido brown bears.

Analysis of the mechanism for the establishment of prion infection in Neuro2a mouse neuroblastoma cells

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A major component of the causative agent of prion diseases, called prion, is believed to be an abnormal isoform of prion protein, PrP^{Sc}. Thus, the production of PrP^{Sc} is often regarded as the propagation of prions. Recent research progress in this field has gradually uncovered that prion propagation in cells persistently infected with prions is closely associated with the membrane trafficking machinery of the cell; however, the process involved in the establishment of prion infection after the entry of prions into cells remains unclear. To address the initial step of prion infection of cells, in this study, PrP-res, a protease-resistant PrP^{Sc}, was inoculated into prion-permissive or -non-permissive cells to analyze the intracellular dynamics of PrP-res. PrP-res was purified from the detergent-resistant membrane fraction of brains from prion-infected mice and was labeled with Alexa Fluor 488 or 555 fluorescent dyes. The labeled PrP-res was inoculated into prion-non-permissive N2a-1 and prion-permissive N2a-3 cells, and PrP-res internalized into the cells was analyzed 6 hours after inoculation; however, the amount of internalized PrP-res appeared comparable regardless of cell type. Time-lapse imaging visualized the dynamic movement of

PrP-res in both cells, but no difference was observed in the movement of PrP-res in prion-permissive and non-permissive cells 6 h after inoculation. Next, the intracellular localization of internalized PrP-res was analyzed up to 72 h after inoculation, at which time newly generated PrP^{Sc} became detectable in peri-nuclear regions. Most PrP-res was co-localized with low density lipoprotein (LDL), a marker of the endo-lysosomal pathway, but not with transferrin, a marker of the endocytic recycling pathway, in both N2a-1 and N2a-3 cells. Given that newly generated PrP^{Sc} was detected in the peri-nuclear region as well as the extensive co-localization of PrP-res with LDL in both prion-permissive and non-permissive cells, PrP-res associated with the endo-lysosomal pathway was not expected to be involved in productive infection. This suggests that most internalized PrP-res undergoes abortive infection; therefore, differentiation of PrP-res that will enter the process of productive infection is essential for precise analysis of the initial step of prion infection in cells.

Analysis of PrP^{Sc} accumulation and glial cell activation in brains of prion-infected mice at the early stage of infection

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Prion diseases are fatal neurodegenerative disorders that are characterized by the vacuolation of neurons and neuropils, reactive astrogliosis, microglial activation and accumulation of an abnormal isoform of prion protein (PrP^{Sc}) in the central nervous system. These glial cells are known to be activated before clinical onset; however, how glial cells respond to prion propagation at the early stage of prion infection is largely unknown. Our laboratory has reported that anti-PrP monoclonal antibody 132, recognizing aa119–127 of mouse PrP, is useful for PrP^{Sc}-specific detection in prion-infected cells in combination with guanidium salt pre-treatment of fixed cells. In this study, this method was modified for PrP^{Sc}-specific detection in frozen tissue sections to analyze glial cell activation in brain regions where the accumulation of PrP^{Sc} becomes detectable in the early stage of infection. First, brain regions were investigated where the accumulation of PrP^{Sc}, i.e., prion propagation, can be detected in the early stage of infection. PrP^{Sc} became detectable in the medulla and thalamus of Chandler strain-infected mice as early as 30 dpi and 45 dpi, respectively. Double-staining of PrP^{Sc} and glial or pre-synaptic marker molecules revealed that at the early stage of infection, most PrP^{Sc} was present in regions close to synaptophysin-positive areas, a marker molecule of pre-synaptic regions. In the thalamus of Chandler-infected mice, astrocyte activation assessed by GFAP became detectable as early as 45 dpi, which was earlier than the appearance of microglial activation assessed by Iba-1. The precedence of astrocyte activation over microglial activation was also observed in the medulla. The same tendency was observed in the exact areas of the thalamus in which PrP^{Sc} is present in the early stage of infection. These results suggest that astrocytes recognize prion propagation directly or subtle changes in neurons by prion propagation and play an important role in neuropathobiology in the early stage of prion infection. Further studies on the activation state of glial cells in the early stage of infection will contribute to elucidate the pathobiological mechanisms of prion diseases.