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INFORMATION

Hokkaido University confered the degree of Bachelor of Veterinary Medicine to the following 42 graduates of the School of Veterinary Medicine on March 24, 2011.
The authors summaries of their theses are as follows:

Potentiality of fetomaternal microchimerism in mice

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During pregnancy, small numbers of cells are transferred between mother and fetus or between fetuses. This phenomenon is called fetus to mother (fetomaternal) microchimerism, mother to fetus microchimerism, or fetus to fetus microchimerism. In humans, it has been reported that female patients often develop or exhibit exacerbated autoimmune diseases after delivery resulting from the accumulation of fetus-derived cells in maternal tissues. The purposes of this study are to confirm the existence of fetomaternal microchimerism in mice and to clarify the dynamics of fetus-derived cells during postpartum maternal injury.

To detect the fetus-derived cells, polymerase chain reaction (PCR), reverse transcriptase-PCR (RT-PCR), and immunohistochemistry for GFP gene and protein were performed using C57BL/6 (B6) female mice mated with B6-Tg (CAG-EGFP) male mice on day 18.5 of pregnancy. Furthermore, to clarify the dynamics of fetus-derived cells during postpartum injury, B6 female mice mated with B6-Tg (CAG-EGFP) male mice were treated with streptozotocin (STZ) at 1 week after delivery.

During pregnancy, fetus-derived GFP gene was detected in several maternal organs showing individual variations. In mice after delivery, there was the expression of GFP gene in several organs, but positive organs were different among mice. In the group with STZ administration after delivery, GFP gene was detected at high frequency, especially in injured organs. In immunohistochemistry, GFP-positive cells were detected in lung of pregnant mice. They were also detected in liver, pancreas, and lung as epithelium-like cells in the STZ-treated group after devivery.

These results suggest that fetomaternal microchimerism occurs during pregnancy and delivery periods in mice, and that maternal injury after delivery increases the efficiency of microchimeric detection. It might be interesting to discuss the considerable relationship between fetomaternal microchimerism and postpartum diseases, including autoimmune diseases and tumorigenesis.

Analysis of urinary cellular patterns in murine glomerulonephritis

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The kidney is a nonregenerative organ composed of numerous functional nephrons and collecting ducts. Glomerular and tubulointerstitial damages decrease the number of functional nephrons and cause anatomical and physiological alterations resulting in renal dysfunction. It has recently been reported that nephron constituent cells are dropped into urine in several pathological conditions associated with renal functional deterioration. The author investigated quantitative and qualitative urinary cellular patterns in a murine glomerulonephritis model and elucidated the correlation between cellular patterns and renal pathology.

The urinary cytology and the renal histopathology were analyzed in BXSB/MpJ (BXSB, glomerulonephritis model) and C57BL/6 (B6, control) mice. Urinary cytology revealed that the number of urinary cells in BXSB mice changed according to the histometrical score of glomerulonephritis and urinary albumin; however, no correlation was detected in levels of blood urea nitrogen (BUN) and creatinine (Cre). Expression of specific markers for podocytes, distal tubules (DTs), and collecting ducts (CDs) were detected in BXSB urine. Immunopositive cells for WT1 (podocyte marker) and IL-1F6 (damaged DT and CD marker) in the kidney significantly decreased and increased in BXSB versus B6, respectively. In PCR array analysis for inflammatory cytokines and chemokines, *Il-10, Cxcl2*, *C3*, and *Il1rn* showed relatively higher expression in BXSB kidneys than in B6 kidneys. In particular, the highest expression of *C3* mRNA was detected in urine from BXSB mice. Furthermore, C3 protein and its mRNA were localized in the epithelia of damaged nephrons.

These findings suggest that epithelial cells of the glomerulus, DT, and CD are dropped into urine and that these patterns are associated with renal pathology progression. The author concludes that evaluation of urinary cellular patterns plays a key role in the early, noninvasive diagnosis of renal disease.

Adiponectin prevents cell motility induced by adipocyte-derived chemokines

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White adipose tissue (WAT) is essential for energy metabolism and functions in lipid storage. Recent findings indicate that WAT plays pivotal roles in the regulation of energy metabolism by

releasing and secreting fatty acids and a variety of cytokines, respectively. For example, leptin, the *obese* gene product exclusively expressed in adipose tissue, regulates appetite through its receptor present in the hypothalamus, while tumor necrosis factor-α (TNF-α) induces insulin resistance in WAT locally.

When an animal continues to ingest excess calories that exceed its energy expenditure, obesity develops, in which excess body fat is accumulated. It is now recognized that WAT in obese animals is in a state of chronic, low-grade inflammation with increased levels of various inflammatory cytokines such as TNF-α and macrophage infiltration.

Adiponectin (ADP) is a protein of 247 amino acids containing four domains, a putative signal sequence, a non-homologous sequence, a collagen-like domain, and a globular domain, similar to collagen type X and complement factor C1q, and secreted exclusively by adipocytes. Its circulating levels are lower in obese subjects than in lean subjects. Subjects with hypo-adiponectinemia exhibit the phenotype of metabolic syndrome, including insulin resistance and coronary artery disease. Administration of ADP has been shown to be beneficial in animal models of diabetes, obesity, and atherosclerosis, suggesting that ADP is an anti-inflammatory cytokine.

However, the precise roles and mechanisms of ADP have not been fully elucidated. Importantly, ADP has been shown to bind to some chemokines including monocyte chemoattractant protein-1 (MCP-1), but its role in chemotaxis function has not been explored yet. In addition, it is highly plausible that there is alteration in the balance between ADP and adipocyte-derived chemokines in WAT of obese animals. Therefore, in the present study, I examined the anti-chemotaxis function of ADP together with the expression of ADP and adipocyte-derived chemokines in obese mice.

Two groups of female C57BL/6J mice (4 weeks of age) were given either high fat diet (HFD) or control diet (ND) up to 23 weeks of age. The mice given HFD exhibited significantly increased body weight, WAT weight, and leptin mRNA expression in both peri-gonadal and retroperitoneal WAT compared with those of the mice given ND. In contrast, there were no differences in the mRNA expression in WAT of three chemokines, MCP-1, platelet factor 4 (PF4), and regulated upon activation, normal T cells expressed and presumably secreted (RANTES), between the two groups. Interestingly, the expression of ADP mRNA decreased in retroperitoneal, but not peri-gonadal, WAT in the obese mice; thereby, the ratio of PF4 to ADP increased in retroperitoneal WAT.

Chemotaxis assay was performed *in vitro* using HL-60 human promyelocytic leukemia cells. Hl-60 cells migrated through a 3-μm-pore membrane toward the three chemotactic factors used, but not to full-length and globular region of ADP. Hl-60 cells, however, tended to exhibit reduced migration toward the three chemotactic factors in the presence of either full-length or globular region of ADP. In particular, ADP prevented PF4-induced chemotaxis. Furthermore, there was selective and direct interaction between full-length ADP and PF4 using surface plasmon resonance method, while interaction between ADP and MCP-1 or RANTES remained to be elucidated.

In summary, the balance between ADP and adipocyte-derived chemokines in WAT of obese mice given HFD changed slightly, and ADP prevented PF4-induced chemotaxis *in vitro* through their direct interaction. Although the present results are not definitive, they suggest that ADP might prevent the action of adipocyte-derived chemokines *in vivo*, and therefore inhibit inflammatory processes such as macrophage infiltration.

High-fat diet impairs sleep quality and *prepro-orexin* gene expression in mice

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During the past two decades, the prevalence of overweight and obesity has become a major health problem of both developed and developing countries. Numerous studies indicate that obesity is often associated with sleep disorders, including sleep apnea syndrome, excessive daytime sleepiness, and insomnia. Since there is a possible link between sleep and obesity, it is important to understand the nature of physiological and molecular mechanisms that coordinate sleep-wake regulation and metabolic function. Recently, some mouse studies have shown changes in sleep-wake amount and architecture in genetic and diet-induced models of obesity. However, molecular pathways linking sleep-wake regulation and metabolic function are largely unknown. Therefore, in the present study, I performed a comprehensive phenotypic analysis of sleep at both the baseline and under sleep-deprived conditions in diet-induced obesity mice. In addition, I examined the involvement of the orexin system that plays a crucial role in regulating sleep-wake states and metabolic function in parallel with the sleep studies.

Twenty-four male C57BL/6J mice at the age of 4 weeks were randomly divided into three groups (n = 8/group), which were given high-fat diet and normal diet for 14 weeks (obese and control groups, respectively), and normal diet for 7 weeks and high-fat diet for the subsequent 7 weeks (moderately obese group). The body weights of mice in the moderately obese and obese groups ranged between 112–150% of the average body weight of the control group. Using these mice, I obtained five major findings that can be summarized as follows: 1) The amount of wakefulness was decreased but that of non-rapid eye movement (NREM) sleep was increased in diet-induced obesity mice. 2) Three sleep-quality measures were impaired in diet-induced obesity mice: sleep fragmentation and brief waking were dramatically increased but the diurnal rhythmicity of electroencephalogram delta power during NREM sleep was significantly blunted. 3) Body weight was negatively correlated with the amount of wakefulness but positively correlated with that of NREM sleep or the number of brief periods of waking. 4) The compensatory response to acute (i.e., 6 h) sleep deprivation in diet-induced obesity mice was comparable to that of control mice. 5) The expression of *prepro-orexin* gene was significantly decreased in obese mice, while those of *orexin receptor* gene types 1 and 2 were unaffected.

In summary, these results indicate that obese animals have increased sleep pressure and difficulties in maintaining wakefulness concomitant with impairment of the orexin system. Since these observations are significantly correlated with body weight, further studies on the role of orexin in animal models of obesity will clarify the mechanism and pathways linking sleep-wake regulation and metabolic function.

Diet-induced obesity affects maternal behavior in mice

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It is reported that neonatal infants from obese rat dams suffer a high fatality rate due to insufficient milk intake. One of the reasons for this phenomenon is obesity-induced lactation failure as evidenced by mouse experiments showing that diet-induced obesity in mice results in impaired ductal and lobuloalveolar development before and during pregnancy and reduced lactation. On the other hand, there are reports describing that obese women have shorter breastfeeding periods than lean women and that obese mothers spend less time interacting and feeding their infants than normal weight counterparts. The latter reports strongly suggest that diet-induced obesity affects maternal behavior; thereby, infants from obese rat dams suffer from insufficient milk intake. However, the relationships between obesity and maternal behavior are still unknown. In the present study, I examined quantitatively how obesity affected maternal behavior and tried to explore the mechanism behind the obesity-induced effects.

Female C57BL/6J mice (8 weeks of age), given a high-fat diet (HFD) for 16 weeks, significantly increased their body weight compared with that of mice given a control diet (ND). When they were mated, the conception rates, which were assessed by dividing the number of pregnant mice by the number of mice having a vaginal plug, in mice fed HFD and ND were 81% and 100%, respectively. Normal parturition rates, assessed by dividing the number of mice that delivered all fetuses in the uterus by the number of pregnant mice, in mice fed HFD and ND were 28% and 84%, respectively. These data indicated that the obese mice had difficulty in delivery.

It is of interest to note that pregnant mice build a nest at the mid- and late term of pregnancy. Indeed, the majority of mice fed ND (79%) built a deep nest suitable for nursing and the rest built a shallow one. In contrast, many of mice fed HFD (78%) failed to build any nest and no mice fed HFD built a deep one. Immediately after delivery, dams breastfeed infants. The majority of mice fed ND (79%) breastfed infants as judged by the presence of curd in the infant's stomach, while the majority of mice fed HFD (94%) did not. The averages of the infant's body weight from the dams fed ND and HFD were 1.21 g and 1.32 g, respectively, probably reflecting the difference in milk intake. These results indicated that the obese mice exhibited impaired nesting and breastfeeding performances. To further highlight maternal behavioral abnormalities, retrieving activity was examined in terms of whether dams retrieved infants after their forced removal. The retrieving rates, assessed by dividing the number of mice who retrieved infants by the number of mice tested, in mice fed HFD and ND were 13% and 60%, respectively.

There were no differences between mice fed HFD and ND in the plasma concentrations of glucose, non-esterified fatty acid, insulin, estrogen, progesterone, prolactin, and oxytocin, but there was for leptin, at one day after the delivery. I also examined the mRNA expression of prolactin and its receptor in the brain because the prolactin system is essential for the manifestation of maternal behavior such as nesting. Although prolactin signal was only detected in the pituitary glands, signal for prolactin receptor was detected in the choroid plexus and arcuate nucleus of non-obese mice. Interestingly, the

signal for prolactin receptor in the choroid plexus was enhanced after lactation, while in the arcuate nucleus it was unchanged.

In summary, the present study has demonstrated that diet-induced obesity in mice complicates delivery and impairs maternal behavior including nesting, breastfeeding, and retrieving activity, which appears before the delivery. The underlying mechanism by which obesity impairs maternal behavior remains unclear. I hope that ongoing analyses of the mRNA expression of prolactin and its receptor in the brain of mice fed HFD and ND and future analysis on the leptin system that regulates feeding behavior through its receptor present in the hypothalamus will reveal the mechanism involved.

Effect of hydrogen sulfide (H_2S) on rat primary afferent neurons

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Hydrogen sulfide (H_2S), which is generally known as a toxic gas, can be enzymatically synthesized in mammalian tissues. A growing body of evidence suggests that H_2S is a gaseous modulator in cardiovascular, gastrointestinal, and endocrine systems. Recently, it has been reported that H_2S stimulates capsaicin-sensitive primary afferent nerves innervating the rodent urinary bladder and trachea. However, the possible role of H_2S in primary afferent nerves is not yet clear. In this study, the effect of NaHS, an H_2S donor, on rat dorsal root ganglion (DRG) neurons was examined using Ca^{2+} imaging and whole-cell voltage-clamp techniques. NaHS concentration-dependently increased intracellular calcium concentration ($[Ca^{2+}]_i$) in all DRG neurons. In subpopulations of DRG neurons that were of small and medium size, NaHS evoked remarkable $[Ca^{2+}]_i$ increase. Most of the DRG neurons that showed remarkable responses to NaHS also responded to both capsaicin (a TRPV1 agonist) and mustard oil (a TRPA1 agonist). The remainder of DRG neurons showed only a small $[Ca^{2+}]_i$ increase induced by NaHS. The remarkable $[Ca^{2+}]_i$ responses to NaHS were significantly inhibited by the removal of external Ca^{2+}, ruthenium red (a non-selective TRP channel inhibitor), and HC-030031 (a TRPA1 inhibitor), but not by capsazepine, iodoresiniferatoxin, and SB366791 (TRPV1 inhibitors). Voltage-dependent Ca^{2+} channel blockers, ω-conotoxin GVIA (N-type), nifedipine (L-type), and mibefradil (T-type), failed to inhibit the NaHS-evoked $[Ca^{2+}]_i$ responses to NaHS. NaHS evoked an inward current in mustard oil-sensitive DRG neurons at a holding potential of −80 mV, which was also blocked by HC-030031. Our findings demonstrate that NaHS activates TRPA1 in rat DRG neurons. It is assumed that the Ca^{2+} influx through TRPA1 contributes to the NaHS-evoked remarkable $[Ca^{2+}]_i$ increase. H_2S may cause pain sensation by stimulating primary afferent nerves through TRPA1.

Analysis of reflex potential in neonatal mouse spinal cord

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There have been many reports on isolated neonatal rat spinal cord that reveal the effects of sedatives and analgesics on spinal cord and their mechanism. Although there are some reports on studies of mouse spinal cord that evaluate monosynaptic reflex potential (MSR), which reflects motility, the electrophysiological characteristics and the drug sensitivity of slow ventral root potential (sVRP), which is considered to reflect nociception, are unclear. The present study was conducted to establish an experimental system for recording spinal cord reflex potentials in mice and to examine their electrophysiological and pharmacological properties.

In isolated neonatal mouse (0–8 days old) spinal cord, electrical stimulation of lumbar dorsal root (L3–L5) evoked MSR and sVRP, equivalent to that of rat, which were recorded from the corresponding ipsilateral ventral root. The magnitude of both potentials increased in a stimulus strength-dependent manner, and sVRP from 7- or 8-day-old mice was small. Morphine depressed sVRP but not MSR in a concentration-dependent manner, as reported in rat. α_2 adrenoceptor agonists such as xylazine and dexmedetomidine depressed not only sVRP but also MSR as they do in rat, but the depressant effect of MSR was weaker than that in rat. Exogenous monoamines such as noradrenaline, dopamine, and 5-HT did not affect the concentration at which monoamines depressed reflex potential, but at high concentrations, they evoked depolarization of ventral root and depressed MSR and sVRP in a concentration-dependent manner. Endogenous monoamine releasers such as p-chloroamphetamine and methamphetamine in turn depressed reflex potentials without affecting ventral root potential.

The present study showed that MSR and sVRP can be recorded in mouse as well as in rat. Although the depressant effects of morphine and α_2 adrenoceptor agonists to sVRP in mouse were the same as those in rat, there is a species difference between rat and mouse. Further examination is required to determine the mechanism involved.

Role of urothelial layer in contraction of mouse bladder detrusor muscle

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The lower urinary tract, which is composed of the bladder and urethra, stores and eliminates urine, and is innervated by pelvic nerve (parasympasic), hypogastric nerve (sympathetic),

and pudendal nerve (somatic). It is suggested that bladder urothelium protects the tissues from electrolytes and toxin components in urine. In addition, bladder urothelium releases various factors by mechanical and chemical stimulations and regulates the activities of detrusor smooth muscles. Bradykinin has been suggested to be involved in pathologies such as cystitis, and its receptors are expressed in the urothelium. It is reported that bradykinin changes contractile response under pathological conditions.

In this study, the bradykinin-induced contractile responses of mouse bladder detrusor muscle in urothelium-denuded (U−) or urothelium-intact (U+) preparations were examined and compared with the responses to electrical stimulation, acetylcholine, and high KCl (HK).

In mouse bladder preparations, bradykinin, HK, and acetylcholine produced biphasic contractile responses, which consist of tonic (TC) and phasic contractions (PC). All stimulants produced larger TC response in U− than in U+. On the other hand, bradykinin, but not other stimulants, produced larger TC response in U+ than in U−. In the presence of a muscarinic receptor antagonist, atropine, or an adrenergic $\beta_1$receptor antagonist, prazosin, the contractile responses to bradykinin in U− and U+ were not changed. On the other hand, in the presence of an ATP P_{2X} receptor antagonist, α, β-methylene ATP, contractile response to bradykinin in U+ but not in U− was enhanced.

These results indicate that the urothelial layer affects the contractile response of bladder detrusor muscles. It is suggested that bradykinin releases ATP from the urothelium and suppresses the contractile response in detrusor muscle. The contractile response in detrusor muscle seems to be regulated by urothelial-derived factor and urothelial barrier function in urinary bladder.

Biological and chemical analyses of the host cell receptors for influenza viruses

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In order to reveal the relationship between the binding of influenza viruses to the receptors on the host cells and infectivity of viruses, the localizations of SAα2,3Gal and SAα2,6Gal receptors on the tissue cells of various species of birds and mammals were analyzed. It was found that the localizations of SAα2,3Gal and SAα2,6Gal receptors on the cells of trachea, jejunum, and colon were different depending on the species of birds and mammals. Then, bindings of A/chicken/Ibaraki/1/2005 (H5N1) (Ck/Ibaraki), which infects chickens and not ducks, and A/duck/Hokkaido/5/1977 (H3N2) (Dk/Hokkaido), which infects ducks and not chickens, to the trachea and colon were analyzed. The results indicate that Ck/Ibaraki and Dk/Hokkaido bound to both SAα2,3Gal and SAα2,6Gal receptors of each tissue. Experimental infection study revealed that the receptors on the infected cells were different between chickens and ducks. Ck/Ibaraki infected epithelial cells with SAα2,6Gal receptors lining the trachea and Dk/Hokkaido infected those with SAα2,3Gal receptors lining the colon. It was, thus, shown that binding of influenza viruses to SAα2,3Gal and SAα2,6Gal receptors is not strictly specific. Therefore, it was concluded that factors other than receptor specificity may affect the infection of host tissue cells by influenza virus.

Acquisition of pathogenicity of live attenuated classical swine fever vaccine strain GPE$^-$ by serial passages in pigs

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The live attenuated classical swine fever vaccine strain GPE$^-$ was established by serial passages of a virulent strain, ALD, in cells of swine, bovine, and guinea pig origin. To elucidate the mechanism of the attenuation of the ALD strain, GPE$^-$ strain, the virus obtained after 11 serial passages of GPE$^-$ in pigs, and ALD strain were compared for their pathogenicity in pigs and their proteins.

GPE$^-$ strain acquired pathogenicity after 11 serial passages in pigs. Three amino acid substitutions, T830A on E2 protein, and V2475A and A2563V on NS4B protein, were found in the virus passaged in pigs. To identify the amino acids responsible for the pathogenicity in pigs, full-length cDNA clone, pGPE$^-$, was constructed. Infectious virus generated in SK-L cells by transfection of complementary RNA to pGPE$^-$, vGPE$^-$, was confirmed to be identical to GPE$^-$ strain. Mutant viruses, vGPE$^-$/T830A and vGPE$^-$/A2563V, were generated by site-directed mutagenesis and reverse genetics. vGPE$^-$/A2563V grew poorly, equivalent to vGPE$^-$, in pigs. The other mutant, vGPE$^-$/T830A, grew more efficiently than vGPE$^-$ in pigs that did not show any clinical signs. The present findings indicate that multiple amino acids, not only on E2 but also on NS4B, are responsible for the exhibition of pathogenicity in pigs.

Molecular characterization of immunoinhibitory factors PD-1/PD-L1 in chickens infected with Marek's disease virus

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An immunoinhibitory receptor, programmed death-1 (PD-1), and its ligand, programmed death-ligand 1 (PD-L1), are involved in the immune evasion mechanisms of several pathogens causing persistent infections and neoplastic diseases. The blockade of the PD-1/PD-L1 pathway by antibodies specific to either PD-1 or PD-L1 resulted in the re-activation of immune reactions, and is expected to be applied to new therapies for infectious and neoplastic diseases. However, no functional analysis of these molecules has been reported for chickens. Thus, in this study, their expressions and roles were analyzed in chickens infected with Marek's disease virus (MDV), which induces immunosuppression in chickens.

PD-1 and two PD-L1-like molecules have already been identified in chickens, and these PD-L1 molecules were termed PD-L1 short and long. The deduced amino acid sequences of

chicken PD-1 and PD-L1 short and long showed low homologies with those of human and mouse PD-1 and PD-L1. However, functional domains, immunoreceptor tyrosine-based inhibitory motifs and immunoreceptor tyrosine-based switch motif in the intracellular domain of PD-1, were well conserved among chickens and other species.

The expression levels of PD-1 and PD-L1 were analyzed by real-time RT-PCR on splenocytes of oncogenic MDV-challenged chickens at the early cytolytic, the latent, and the tumor phases of MDV infection. The *PD-1* and *CTLA-4* expression levels were up-regulated at early cytolytic infection, while the *PD-L1* short and long expression levels were up-regulated at latent infection. These results suggest that, as immunologically relevant factors, PD-1 and CTLA-4 could be involved in MD pathogenesis at early cytolytic infection, and that PD-L1 short and long could contribute to the establishment and maintenance of MDV latency. Furthermore, the expression levels of *PD-1*, *PD-L1* short and long, and *CTLA-4* were increased at tumor sites of MDV-challenged chickens. These genes were also detected in spleens and tumor sites of MDV-infected chickens in the field. The expression levels of *PD-L1* short and long were markedly increased in the tumor sites. These results suggest that tumor cells transformed by MDV highly express these factors and thereby evade the immune responses of the host.

In order to clarify the immunoinhibitory functions of chicken PD-1/PD-L1, co-cultivation of Lee1 cells, which are from a T cell line constitutively producing IFN-γ, and DF-1 cells transiently expressing either PD-L1 short or long, was performed, and the *IFN-γ* expression level was analyzed in Lee1 cells by real-time RT-PCR. The *IFN-γ* expression was significantly decreased in Lee1 cells co-cultivated with DF-1 cells expressing PD-L1 long, suggesting that chicken PD-1/PD-L1 have immunoinhibitory functions.

In this study, chicken PD-1/PD-L1 were shown to have immunoinhibitory functions and contribute to the establishment and maintenance of MDV latency and MD pathogenesis. Further studies are desirable to identify the viral and host factors involved in the expression of PD-1 and PD-L1. These findings should be informative to develop new methods for prevention and therapy against a variety of persistent infections and neoplastic diseases in chickens.

Expression and functional analyses of an immunoinhibitory factor LAG-3 in bovine leukemia virus-infected cattle

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An immunoinhibitory receptor, lymphocyte activation gene-3 (LAG-3), which is mainly expressed in activated T cells, is involved in the immune evasion of several pathogens causing chronic infections and tumors. Previous studies showed that dual, rather than individual, blockade of LAG-3 pathway and another immunoinhibitory pathway, PD-1/PD-L1, augmented anti-virus or anti-tumor cytokine production, and the proliferation of antigen-specific $CD8^+$ T cells. For these reasons, dual blockade of the LAG-3 and PD-1/PD-L1 pathways is expected to be applied to new therapies for chronic infectious diseases and tumors. However, unlike human or mouse

LAG-3, no functional analysis of LAG-3 has been reported in domestic animals. Thus, in this study, cDNA encoding bovine LAG-3 was cloned and sequenced, and then its expression and roles in immune reactivation were analyzed in bovine leukemia virus (BLV)-infected cattle.

The full-length cDNA sequence encoding bovine LAG-3 was cloned and its deduced amino acid sequence showed high homology with those of human and mouse LAG-3. *LAG-3* mRNA was mainly expressed in T cells and up-regulated in peripheral blood mononuclear cells (PBMCs) by stimulation with concanavalin A or phytohemagglutinin.

Subsequently, the LAG-3 expression was analyzed by flow cytometry on PBMCs from BLV-infected cattle at the asymptomatic (AL), persistent lymphocytosis (PL), and enzootic bovine leukemia (EBL) stages. No difference in LAG-3 expression levels on $CD4^+$ and $CD8^+$ cells was observed among the different disease stages of BLV infection. In addition, the LAG-3 expression level did not correlate with white blood cell counts, viral load, and virus titer, which reflect the severity of BLV infection. On the other hand, major histocompatibility complex (MHC) class II molecule, which is the ligand of LAG-3, was strongly up-regulated with disease progression. Furthermore, dual, rather than individual, blockade of the LAG-3 and PD-1/PD-L1 pathways using anti-LAG-3 and anti-PD-L1 antibodies induced strong up-regulation of IL-2 and IFNγ production, and reduced the viral load in PBMCs in cattle at the PL stage.

In this study, it was suggested that LAG-3 is involved in the inhibition of T cell function through its binding and signaling from MHC class II molecule during BLV infection. Further investigations are necessary to develop new vaccination and therapy methods against BLV infection using anti-LAG-3 antibody and/or recombinant LAG-3 protein.

Functional analysis of Salp15 homologue in *Ixodes persulcatus*

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Ixodid ticks are harmful vectors for several pathogens causing infectious diseases that affect both human health and the livestock industry. Recent studies have focused on the development of an anti-tick vaccine that inhibits blood feeding of ticks. There have been several reports on the identification and characterization of tick molecules as effective targets for an anti-tick vaccine.

Salp15, a 15-kDa tick salivary gland protein, is known for its several suppressive activities against host immunity and its critical role in the transmission of Lyme disease agent in *Ixodes scapularis* and *I. ricinus*, the major vectors of the Lyme disease agent in North America and Western Europe. Salp15 inhibits the activation of $CD4^+$ T cells through the repression of both TCR-triggered calcium fluxes and IL-2 production. Furthermore, Salp15 adheres to *Borrelia burgdorferi* and specifically interacts with its outer surface protein C. The binding of Salp15 to *B. burgdorferi* protects it from antibody-mediated killing and facilitates the infection of mice. *I. persulcatus* serves as a vector of Lyme disease agents in Japan. Recently, we identified 2 Salp15 homologues in *I. persulcatus*,

Salp15 Iper-1 and *Iper-2*. In this report, we describe the function of Salp15 Iper in Lyme borrelia transmission.

To investigate the function of Salp15 Iper, recombinant Salp15 Iper (rSalp15 Iper) was prepared in both bacterial and insect cells. rSalp15 Iper was recognized by tick-immunized hamster sera, indicating that it is a secretory protein exposed to the host animals. Solid-phase overlay assay and indirect fluorescence assay showed that Salp15 Iper binds to OspC from *B. burgdorferi*, *B. garinii*, and *B. afzelii*. Importantly, this binding protected the spirochete from antibody-mediated killing *in vitro*. In addition, Salp15 Iper tended to facilitate infection of mice. Although a vaccination trial with rSalp15 Iper was carried out to examine the role of Salp15 Iper in tick blood feeding, Salp15 Iper antiserum did not influence tick feeding. Thus, further characterization of tick molecules, including Salp15 Iper, which protects the spirochete from antibody-mediated killing, could lead to the development of new strategies to prevent both transmission of tick-borne diseases and blood feeding by ticks.

Distribution of ticks and prevalence of tick-borne protozoan parasites in Myanmar

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Piroplasms are tick-transmitted, intracellular and hemoprotozoan parasites that cause piroplasmosis in various mammals, especially cattle. *Theileria* parasites of *T. sergenti*/*buffeli*/*orientalis* are transmitted by *Haemaphysalis* spp. ticks, whereas *Babesia bigemina* and *B. bovis* are mainly transmitted by *Rhipicephalus* (*Boophilus*) spp. ticks. Theileriosis and babesiosis in cattle are highly epidemic in Southeast Asian countries, including Thailand, Viet Nam, and Malaysia. In a previous study, we detected *B. bovis* and *Theileria* DNA from the blood of cattle in Myanmar, suggesting that piroplasmosis is also epidemic in this country. In the present study, the distribution of ticks and the prevalence of tick-borne protozoan parasites in Myanmar were investigated.

Ticks were collected at three cities, Napyitaw, Pyin Oo Lwin, and Mandalay, in the central region of Myanmar in January, 2009, at two cities, Yangon and Pathein, in the southern part of Myanmar in December, 2009, and at Myitkyina in the northern part of Myanmar in July, 2010. Morphological observation indicated that ticks collected at Nepyitaw, Mandalay, and Pyin Oo Lwin belonged to the subgenus *Rhipicephalus* (*Boophilus*) and ticks at Yangon, Pathein, and Myitkyina belonged to the subgenus *Rhipicephalus* (*Boophilus*) or the genus *Haemaphysalis*. Although 93.2% (96/103) of *Boophilus* ticks were positive for PCR amplification of *cytochrome oxidize C subunit I* (*COI*) gene using primers common to *Haemaphysalis* and *Rhipicephalus*, none (0/10) of the *Haemaphysalis* ticks was positive for this. The sequence of one of the PCR products of *Boophilus* ticks (480 bp) was 90 and 92% identical to those of *B. microplus* and *B. annulatus*, respectively.

Total tick DNAs were examined by PCR for *18S rRNA* gene of *B. bovis*, *Spe*I-*Ava*I DNA fragment of *B. bigemina*, and major piroplasm

surface protein gene of *Theileria* parasites. As a result, the prevalence of *B. bovis* was 20.1% (20/90) in *Boophilus* ticks and 19.6% (9/46) in *Haemaphysalis* ticks. The prevalence of *B. bigemina* was 30.2% (29/96) in *Boophilus* ticks and 52.5% (24/46) in *Haemaphysalis* ticks. The prevalence of *Theileria* parasites was 17.7% (17/96) in *Boophilus* ticks and 0% (0/46) in *Haemaphysalis* ticks.

These results suggest that *Rhipicephalus* (*Boophilus*) ticks collected in Myanmar are closely related to *R.* (*B.*) *microplus* and transmit *B. bovis* and *B. bigemina* in Myanmar. Further studies are needed to reveal the prevalence and transmission of piroplasm parasites in cattle in Myanmar.

Epidemiological studies of *Babesia* and *Theileria* infections in cattle in Myanmar

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In the Republic of the Union of Myanmar, there have been very few epidemiological studies of hemoprotozoan diseases in livestock. In the present study, an epidemiological survey was performed on *Babesia bovis*, *B. bigemina*, and benign *Theileria* infections in cattle in central, southern, and northern regions of Myanmar using FTA® cards for direct blood sampling followed by PCR amplification of each species-specific target gene.

A total of 521 cattle DNA samples were examined by nested PCR for *18S rRNA* gene of *B. bovis* and *Spe*I-*Ava*I DNA fragment of *B. bigemina*, and by PCR for major piroplasm surface protein gene of *Theileria* parasites. Prevalences of *B. bovis*, *B. bigemina*, and benign *Theileria* parasites were 20.7%, 36.7%, and 49.5%, respectively, indicating that piroplasmoses are widespread in Myanmar. Statistical analyses indicated that prevalence rates of both *Babesia* species were significantly higher in the central region than in other regions, while the prevalence rate of *Theileria* was higher in southern and northern regions. In terms of cattle breed, positive rates of all three hematoprotozoan parasites were significantly lower in the Zebu breed than the Holstein-Friesian. In addition, the positive rate of *B. bigemina* was higher in cattle under one year of age than over one year of age, whereas the positive rate of *Theileria* was higher in cattle over one year of age.

These results suggest that the breed, age, and location are important factors for the prevalence of piroplasmoses in Myanmar. However, the influence of grazing status and seasons on hemoprotozoan infections in cattle awaits further epidemiological studies in other regions in Myanmar.

Cloning and expression of *Trypanosoma congolense* major surface protease B2

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Major surface proteases (MSPs) are zinc-binding metalloproteases, which have been identified in most Trypanosomatidae protozoan parasites. The function of MSPs varies among genera and species, even though the parasites are taxonomically closely related. Recently, it was shown that *Trypanosoma congolense* produced MSPs, which were classified into six families (TcoMSP-A, B1, B2, C, D, E). However, the functions of TcoMSPs have remained unclear. Therefore, the present study aimed at cloning and molecular characterization of TcoMSP-B2.

First of all, a partial cDNA sequence of *TcoMSP-B2* was obtained from the *T. congolense* EST database. Then, the full-length *TcoMSP-B2* sequence (1,623 bp) was cloned after RT-PCR. The predicted amino acid sequence indicated that TcoMSP-B2 was a polypeptide of 57.7 kDa containing a metalloprotease catalytic motif (HEXXH). The predicted amino acid sequence of *TcoMSP-B2* had higher identity with that of *T. brucei* MSP-B (TbMSP-B) (58%) than TbMSP-A and TbMSP-C (41% and 34%, respectively). In addition, the characteristics of TcoMSP-B2, such as signal peptide site and hydrophobic region, were similar to those of TbMSP-B. Therefore, it was thought that TcoMSP-B2 was an orthologue of TbMSP-B. Meanwhile, it was reported that the nucleotide sequence of *TcoMSP-B1* had a great similarity with that of *TcoMSP-B2*, but *TcoMSP-B1* had an additional 54 bp at the 5' end sequence compared with *TcoMSP-B2*. Thus, RT-PCR was performed to examine whether *TcoMSP-B1* was transcribed into mRNA. As a result, mRNA of *TcoMSP-B1* was not detected in all stages in the lifecycle. This implied that the *TcoMSP-B1* gene does not exist in *T. congolense*.

Although RT-PCR analysis detected mRNA in all stages in the lifecycle, Western blotting analysis using antisera against recombinant TcoMSP-B2 showed that TcoMSP-B2 (about 65 kDa) was specifically expressed in the epimastigote form (EMF). Immunofluorescence microscopy demonstrated that TcoMSP-B2 was expressed on the cell surface of EMF. These results indicated that the expression of TcoMSP-B2 was post-transcriptionally regulated. The discrepancy between the predicted (57.7 kDa) and observed (65 kDa) masses of TcoMSP-B2 might be explained partially by glycosylation of the TcoMSP-B2, as shown in MSPs of *Leishmania* spp. Indeed, TcoMSP-B2 has four potential N-glycosylation sites, two potential O-glycosylation sites, and a GPI anchor binding site.

In conclusion, it was indicated that TcoMSP-B2 is a GPI-anchored cell surface glycoprotein and zinc-binding metalloprotease specifically expressed in EMF. *T. congolense* expresses a different cell surface molecule from one stage in the lifecycle to another. Therefore, there is a possibility that TcoMSP-B2 sheds the cell surface molecule, as TbMSP-B does. Since EMF is a proliferative stage parasitizing in the proboscis of tsetse fly, there is a possibility that TcoMSP-B2 interacts with factors derived from tsetse fly.

Analysis of genetic locus responsible for X-ray hypersensitive 1 (*xhs1*) in the LEC rat

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Challenge for establishment of naïve induced pluripotent stem cell lines of miniature pig as a laboratory animal; Improvement of the reprogramming of pig embryonic fibroblast by small-molecule compounds

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Molecular cloning of canine vascular endothelial growth factor receptor-3 and analysis of its expression in splenic and cutaneous hemangiosarcoma

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Vascular endothelial growth factor receptor-3 (VEGFR-3) is a member of the receptor tyrosine kinase superfamily, and its function is indispensable for lymphangiogenesis and angiogenesis processes, which are closely related to inflammation and the growth and metastasis of tumors. While the predicted gene sequence of canine VEGFR-3 (cVEGFR-3) is available, reports concerning cVEGFR-3 are limited. In particular, the expression profile of cVEGFR-3 in normal tissue and tumor is unknown. In the present study, therefore, molecular cloning of cVEGFR-3 was performed by reverse transcription-polymerase chain reaction (RT-PCR), and changes in the expression of cVEGFR-3 in splenic and cutaneous hemangiosarcoma were investigated by quantitative real-time RT-PCR (qRT-PCR) and immunohistochemistry (IHC).

First, two different cVEGFR-3 genes were cloned and named as cVEGFR-3-1 and cVEGFR-3-2. The lengths of the cVEGFR-3-1 and cVEGFR-3-2 genes were 4098 and 4074 bp, and

the predicted polypeptides were 1365 and 1357 amino acids long, respectively. These two genes included seven immunoglobulin-like domains, a transmembrane segment, a juxtamembrane segment, and a protein kinase domain, the same as the human VEGFR-3 gene. Moreover, the nucleotide sequence and the predicted amino acid sequence of these genes were found to share high homology with those from human, mouse, rattus, and bovine VEGFR-3. These results indicated that these cloned genes were genes of VEGFR-3 of dogs.

Furthermore, the deletion of 8 amino acids in the C-terminal tail, which results in the reduction of a tyrosine residue, was found in the cVEGFR-3-2 gene. Accordingly, the activity of cVEGFR-3-2 might be different from that of cVEGFR-3-1. In addition, cVEGFR-3-1 and -2 shared higher homology with human VEGFR-3 isoforms 1 and 2, respectively. Thus, cVEGFR-3-1 and cVEGFR-3-2 might be isoforms of VEGFR-3 of dogs.

In spleen, skin, mammary gland, lung, liver, kidney, adrenal gland, urinary bladder, ovary, uterus, duodenum, small intestine, and large intestine from healthy dogs, the transcription of the cVEGFR-3 gene was observed. In particular, its transcription in spleen, mammary gland, lung, adrenal gland, and ovary, which should contain many vessels and lymphatics, and should show active angiogenesis, lymphangiogenesis, and hematopoiesis, seemed to be more abundant than that in other organs.

Additionally, mRNA and protein expression profiles for cVEGFR-3 in splenic hemangiosarcoma were examined by qRT-PCR and IHC. As results, the levels of the cVEGFR-3 mRNA and protein were almost the same as those in normal spleen in some cases, and were higher than those in normal spleen in other cases. It is speculated that the elevated expression of cVEGFR-3 might be related to the degree of differentiation and the proliferating potential of tumor. In cutaneous hemangiosarcoma, mRNA and protein expressions of cVEGFR-3 were higher than those in normal skin. However, since only one case could be analyzed in the present study, more cases should be analyzed in further study.

In conclusion, the cVEGFR-3 genes were cloned and two isoforms of canine VRGFR-3 were found. It was also shown that cVEGFR-3 should be expressed in various organs in healthy dogs. Moreover, it is possible that the expression of cVEGFR-3 is elevated in some canine hemangiosarcoma cases. Further study is necessary to elucidate the association between the level of cVEGFR-3 and the malignancy, pathology, and prognosis of tumors.

Molecular cloning and transcription analysis of the heat shock protein 90 gene from *Babesia gibsoni in vitro*

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Heat shock protein 90 (Hsp90) is a 90 kDa heat shock protein. *Babesia gibsoni* is a causative pathogen of canine babesiosis. There are no reports about the Hsp90 of *B. gibsoni* (BgHsp90). In the present study, therefore, molecular cloning of BgHsp90 by reverse transcription-polymerase chain reaction (RT-PCR) was performed. Moreover, mRNA expression profiles for BgHsp90 exposed

to either high or low temperature were examined by quantitative real-time RT-PCR (qRT-PCR).

At first, BgHsp90 gene was cloned and sequenced. The length of the gene was 2,264 bp, and the predicted polypeptide was 716 amino acids long with a calculated molecular weight of 82.8 kDa. There were two introns, the sizes of which were 120 bp and 257 bp, in the BgHsp90 gene. The nucleotide sequence and the predicted amino acid sequence of BgHsp90 were compared with those of other organisms. As a result, BgHsp90 was found to have high homology with Hsp90s of *B. bovis* and *Babesia* sp. BQ Lintan, which are also *Babesia* parasites.

Moreover, in addition to the sequences of BgHsp90, the nucleotide and predicted amino acid sequences of Hsp90 from other organisms were included in the phylogenetic analysis. A phylogenetic analysis based on the entire coding region of the Hsp90 gene and complete amino acid sequences of the Hsp90 polypeptide showed that BgHsp90 lies within a phylogenetic cluster with Hsp90s from *Babesia*, *Theileria*, and other protozoans, and Hsp90-α from mammals. This result indicated that BgHsp90 might be an ortholog of Hsp90-α.

In addition, to examine the change in the transcription of the BgHsp90 gene in the cultured *B. gibsoni* after a shift in temperature, qRT-PCR was performed. When the cultured *B. gibsoni* was incubated at 43°C for 1 hr, the transcription of the BgHsp90 gene was significantly increased. In contrast, the transcription of the BgHsp90 gene was not increased when *B. gibsoni* was incubated at 4°C and 32°C for 1 hr. This result indicated that the exposure of *B. gibsoni* to elevated temperature might result in increasing transcription of the BgHsp90 gene.

In conclusion, the present study revealed the nucleotide and predicted amino acid sequences of Hsp90 from *B. gibsoni*. Additionally, it was shown that the transcription of the BgHsp90 gene was elevated under heat shock. However, further studies are necessary to clarify the functions and roles of BgHsp90.

Cloning and expression analysis of canine reprogramming factors and induction of canine dermal fibroblasts for the generation of canine induced pluripotent stem cells

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Induced pluripotent stem (iPS) cells can be generated from somatic cells by transduction with four defined factors in several species, but there is little information about canine reprogramming factors and generation of iPS cells. In this study, the expression patterns of SOX2, KLF4, MYC, and OCT3/4 genes were analyzed in normal tissues and these genes were cloned and then induced to canine dermal fibroblasts by retrovirus-mediated transduction. I detected the expression of KLF4 and MYC in 10 tissues but SOX2 was undetectable. This result is similar to the expression pattern of orthologous genes in mouse and human. The cloned sequence of SOX2 was shorter than the predicted sequence in NCBI by 6 bp, that of KLF4 was longer by 276 bp, and those of MYC and OCT3/4 were the same as the predicted sequences. The sequence

identities of canine SOX2, KLF4, MYC, and OCT3/4 with orthologous genes in mouse and human were very high. After the introduction of the four canine factors to the canine dermal fibroblast (CDF), CDFs grew more efficiently and formed non-silencing colonies at day 7. At day 6, CDFs, exposed to the four canine factors and human NANOG and human LIN-28, formed a flat and silencing colony and the colony displayed tightly packed morphology similar to canine ES cells. After picking up the colony on SNL feeder cells, it could not maintain its appearance. Other conditions could not induce CDFs to form colonies, and RT-PCR revealed that the introduced CDFs did not express any factors related to pluripotency. These results confirmed that the four canine factors could not induce CDFs to generate canine iPS cells, and that the generation of canine iPS cells, in contrast to those of mouse and human, needs additional factors.

A study on extracellular matrix metabolic factor of equine tendon and cartilage

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Tissues composing anatomical framework, such as bone, tendon, and cartilage, contain a significant amount of extracellular matrix (ECM), which is an important factor in maintenance of characteristic function of these structures. In osteoarthritis (OA), an imbalance between matrix metalloproteinases (MMPs) and their inhibitors has been implicated in degenerative processes of tissue structure. Reversion-inducing cysteine-rich protein with Kazal motifs (RECK), a membrane-anchored MMP regulator, has recently been shown to be up-regulated in human OA cartilage, which indicates that RECK may also be an important factor in equine tendon and cartilage, where active turnover of ECM by MMPs occurs. The objective of this study was to obtain basic information on ECM metabolism and its regulatory mechanism with the intent of analyzing pathophysiological changes in tendon and cartilage in the early phase of equine OA. For this purpose, the expression patterns of equine MMPs and tissue inhibitor of metalloproteinases (TIMP) in equine tenocyte and chondrocyte were examined. Additionally, we confirmed the presence of RECK in horses and characterized its function in equine OA.

Tendon and cartilage were sampled from two skeletally normal thoroughbred horses, from which equine tenocyte and chondrocyte cell cultures were obtained. First, expression of RECK gene in equine tenocyte and chondrocyte was confirmed by reverse transcription PCR (RT-PCR). Second, cells were stimulated using tumor necrosis factor (TNF)-α or interleukin (IL)-1β for 0, 12 h, 1, 2, 4, and 7 days, and changes in gene expression of RECK and other proteins associated with ECM metabolism were investigated by a real-time PCR-based relative quantification technique.

As a result, we found that RECK gene was expressed in equine tenocyte and chondrocyte, the expression level of which was modified by the stimulation in both cell types. In tenocytes, the expression of MMP-2 and 13 was increased by the stimuli, while the increase in MMP gene expression was less profound in chondrocytes

than in tenocytes; these expression patterns indicate that MMP gene expression was seen in an earlier phase of inflammation in chondrocytes. The expression pattern of TIMP genes suggested that the timing and the regulatory mechanism of their expression are different in each cell type. This study demonstrated the presence of RECK in equine tendon and cartilage. The expression level of RECK gene changed with stimulation by the inflammatory cytokines. These data suggested that RECK plays a role in ECM metabolism of equine inflammatory articular disease.

The expression level and timing of MMP and TIMP genes were different between tenocytes and chondrocytes. This suggested that the mechanism controlling the proteins associated with ECM metabolism is different in each tissue. In addition to MMPs and TIMPs, RECK may also play an important role in joint disease, and further investigation of its function is warranted in future research of ECM metabolism and its regulatory mechanism in OA.

Evaluation of cytotoxicity and mechanism of action of rapamycin against canine transitional cell carcinoma

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Canine transitional cell carcinoma (TCC) of the urinary bladder is a highly invasive tumor, and the therapeutic efficacy of surgery and/or chemotherapy for this tumor is limited. More effective therapies are therefore desired to improve the quality of life and expected life span of affected animals.

Rapamycin, an immunosuppressive drug, inhibits a protein that plays an important role in the phosphatidylinositol 3-kinase (PI3K)/Akt cell proliferation pathway. This particular protein is called mammalian target of rapamycin (mTOR). Recently, mTOR inhibitors have been intensively studied as potential anti-tumor agents in human medicine, but there have been few studies of the anti-tumor effect of mTOR inhibitors in veterinary medicine. The aim of this study was to evaluate the potential effects of rapamycin as a therapeutic option for dogs with TCC of urinary bladder.

Firstly, the anti-tumor effect of rapamycin against canine TCC cells was examined by administering rapamycin to tumor-bearing mice transplanted with canine TCC cells. *In vitro* antiproliferative assays were performed using MTT colorimetric method and nucleic acid quantification technique. Rapamycin showed a significant anti-proliferative effect in cultured tumor cells. Secondly, piroxicam, which is a commonly employed therapeutic agent against canine TCC, was used in combination with rapamycin to enhance the anti-tumor effects of rapamycin. The cytotoxicity of the combination was evaluated by *in vitro* assay. No enhancement of the effect of rapamycin by piroxicam was however detected.

It is postulated that inhibition of mTOR by rapamycin results in compensatory activation of Akt that supports the survival of rapamycin-treated tumor cells. Akt inhibitor, LY294002, was therefore used with rapamycin to boost the *in vitro* cytotoxicity effect of rapamycin. Using rapamycin with LY294002 should cause a synergistic anti-tumor effect against canine TCC cells. No significant difference in Akt phosphorylation with supplementation of rapamycin was observed by Western blotting. This may have

been due to poor visualization of phosphorylated Akt in this experimental condition.

In conclusion, rapamycin had cytotoxic effects against multiple canine TCC cell lines, and the effect could be enhanced synergistically by an Akt inhibitor. These results suggested that rapamycin used with Akt inhibitors has potential as an effective therapeutic tool for dogs with TCC of the urinary bladder.

The origin of antibodies induced in cerebrospinal fluid by intrathecal immunization

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Intrathecal immunization is a method by which antigens are directly inoculated into the intrathecal (intracerebral, intracerebroventricular, and subarachnoid) area and can induce antibody response not only in the peripheral blood but also in the cerebrospinal fluid (CSF). In this study, I examined the antibody titers in the blood and CSF after intrathecal immunization and analyzed CNS histopathologically to clarify the origin of antibodies in the CSF. Furthermore, I examined whether intrathecal immunization could rescue rabid rabbits.

Firstly, I measured antibody titers in the blood and CSF after intrathecal immunization with inactivated rabies vaccine. Antibody titers did not increase after the first immunization but they did after the second immunization and then reached the same level as that in the peripheral blood. Lymphocytes (T cells and B cells), plasma cells, and macrophages infiltrated the area around blood vessels in both brain parenchyma and meningis. Furthermore, upregulation of IgG mRNA in the brain of intrathecally immunized rabbits suggested antibody production in the CNS. Influx of antibody from the peripheral blood is also considered. Therefore, the origin of antibodies induced in the CSF by intrathecal immunization is considered to be both antibody production in the brain and influx from the peripheral blood.

Then, I vaccinated rabbits intrathecally using the peptide derived from phosphoprotein of rabies virus. However, I could not identify increased antibody titers in the CSF even after second intrathecal immunization. The result suggested that intrathecal vaccination using peptide derived from phosphoprotein of rabies virus does not induce antibody response in the CSF.

Finally, I tried to rescue rabid rabbits with intrathecal immunization. Rabid rabbits were vaccinated intrathecally with inactivated rabies vaccine and the rabid rabbits at the terminated stage were anesthetized and kept under the control of an artificial respirator. However, all the rabbits died and the efficacy of intrathecal immunization against rabies was not demonstrated.

Further studies are needed to induce massive migration of antibody-producing cells into the brain and to induce high antibody titer rapidly in the CSF to treat rabid animals.

Prophylactic efficacy of mouse rabies by induction of antigen-producing cells into the central nervous system

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Rabies is a viral zoonotic disease that has 100% mortality after the presentation of neurological symptoms. Rabies virus invades the hosts' central nervous system (CNS) via the peripheral nerves through the bite of rabid animals. Subcutaneous or intramuscular vaccinations are not effective treatment for rabid humans or animals because serum antibodies do not neutralize the viruses within the CNS. Therefore, establishment of effective post-exposure vaccination and/or treatment is strongly required.

Intrathecal immunization is a method to inject antigens directly into subarachnoid spaces including ventricles or brain parenchyma. In the present study, we demonstrated that intracerebral (IC) immunization of mice could induce higher antigen-specific antibody titer in blood more rapidly than subcutaneous (SC) immunization of mice. However, this was not sufficient to exert a prophylactic effect as post-exposure immunization, which indicated the importance of rapid induction of high antibody titer not only in blood but also in the CNS for prevention of rabies.

Since the cross-linking of peripheral and brain immune systems was expected from our previous research and other reports, we next immunized mice via the IC route after SC immunization. As a result, antibody-producing cells were induced in the CNS by IC immunization after SC immunization. In addition, this immunization could prevent rabies virus spread within the brain after the inoculation of a lethal dose of live viruses. Surprisingly, some of the IC-immunized mice survived after showing neurological signs including leg paralysis. The result of the present study suggested that intracerebral immunization is a possible measure for the treatment of rabies.

Genome diversity of fowl glioma-inducing virus and construction of its full-length infectious molecular clone

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Fowl glioma-inducing virus (FGV) belongs to avian leukosis virus (ALV) subgroup A, and causes so-called fowl glioma, perineurioma, and cerebellar hypoplasia in chickens. In the first part of this study, to clarify the process by which FGV acquires unique pathogenicity in the nervous system, the prevalence of FGV was examined by FGV-specific nested PCR and histopathological analysis in Japanese native chickens, which were bred in captivity in Kumamoto from the middle of the Showa period for ornamental purposes, and in chickens at Nagoya University bred for

research purposes. FGV proviral DNA was detected in 36 (31.3%) of 115 chickens in Kumamoto, and 4 FGV mutants were isolated. Another ALV strain, Km_5666, in which FGV-specific region was not detected by the nested PCR, was also isolated from a brain affected by fowl glioma. The *env* region of this strain showed high identity (95%) with the corresponding region of FGV, but the 5'LTR, *gag*, and *pol* regions showed high similarity (more than 96%) to endogenous ALV *ev-1*. In addition, two ALVs (Km_5843, Km_5845) were also isolated from the other brains with glioma-related lesions, and the 5'LTR, *gag*, and *pol* regions of these strains showed high similarity (97%, 96%, 98%, respectively) to ALV *ev-1*. These results suggest that FGV mutants and the other glioma-inducing ALVs probably derived from ALV *ev-1* were prevalent in Japanese fowls in Kumamoto, which appears to show the genome diversity of neuropathogenic ALVs. On the other hand, no ALV was isolated from any birds in Nagoya. In the second part of this study, in order to establish a system with which to analyze the molecular mechanism of FGV virulence, an infectious FGV molecular clone was generated by fusion PCR and limited enzymatic digestion. After the molecular clone was transfected into cultured cells, mature viral particles were detected in the supernatant by electron microscopy, and p27 antigen was detected in the virus fluid by ELISA. In addition, genomic analysis showed that the nucleotide sequence of the virus isolated from the infected cells was identical to that of the molecular clone. These results suggest that the constructed infectious molecular clone is useful to analyze FGV virulence.

Individual *in vitro* maturation and culture of bovine oocytes using microwells

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Developing methods of individual or small group culture of bovine oocytes enables the production of transferable embryos from a small number of oocytes and the investigation of the mechanisms of maturation, fertilization, and development of oocytes. The objectives of this study were to determine the effects of individual *in vitro* maturation (IVM) and *in vitro* culture (IVC) using microwells on the developmental competence of oocytes to blastocysts. In this study, I used microwell plate (MW-P) for IVM and well of the well dish (WOW-D) for IVC. In experiment 1, the cleavage and blastocyst rates of oocytes matured individually in a droplet decreased ($P < 0.01$), but those of oocytes matured in MW-P increased ($P = 0.10$) compared with those of oocytes cultured in a group. In experiment 2, no significant differences in cleavage and blastocyst rates were observed between individual IVC in WOW and group IVC. In experiment 3, oocytes were individually matured, fertilized, and cultured using MW-P, droplet, and WOW-D, respectively. Although there was no significant difference in blastocyst rates between experimental groups, the timing of blastocyst formation was later in oocytes cultured individually than in oocytes cultured in a group ($P < 0.01$). These results suggested that individual IVM in MW-P and IVC in WOW-D improve the developmental competence of oocytes to blastocysts.

Separation of good quality sperm by Percoll density gradient centrifugation of bovine frozen semen

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Frozen-thawed bovine semen includes sperm with different morphologically characteristics and motilities depending on each bull and each ejaculate. For *in vitro* fertilization (IVF), it is necessary to separate good quality sperm that has normal morphology and high motility. Although Percoll density gradient centrifugation (Percoll method) is used generally for sperm separation, there have been no studies that investigated the relationship between concentration of Percoll for density gradient and the efficient separation of good quality sperm. In addition, sex-sorted X- and Y-sperm are present at a lower number ($2 \sim 3 \times 10^6$ sperm), only 1/5 or less that of normal frozen sperm. For IVF using sex-sorted sperm, it is important not only to separate good quality sperm but to make concentrated sperm suspension.

Firstly, the efficacy of the Percoll method on sperm separation was investigated. The Percoll method reduced the proportions of dead and abnormal sperm and increased the proportion of good quality sperm in the sediments after centrifugation. Secondly, the effect of Percoll concentration (30/45%, 30/60%, and 45/90%) on sperm separation was examined, and then IVF was performed using sperm separated by different Percoll concentrations. Sperm separated by 45/90% Percoll method showed higher normality, motility, and fertilizability than those separated by 30/60% and 30/45% Percoll.

The sperm recovery and the proportions of sperm with normal morphology and high motility obtained using a fine neck tube (FNT) with 45/90% Percoll were not different from those using a conventional conical tube. In addition, it was easy to make concentrated sperm suspension in FNT after separation from sex-sorted sperm.

The present results indicate that 45/90% Percoll method is the most effective for the separation of good quality sperm. In addition, 45/90% Percoll method using FNT could separate good quality sperm from sex-sorted sperm and make concentrated sperm suspension.

In vitro growth (IVG) culture of bovine oocytes derived from early antral follicles: Effects of fibroblast growth factor (FGF) 7 in IVG medium and maturational culture system on developmental competence of IVG oocytes

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In mammals, there are a large number of immature oocytes in the ovaries. *In vitro* growth (IVG) culture can accomplish the production of superior domestic animals and facilitate the study of oocyte growth mechanism. However, maturational and developmental competences of bovine IVG oocytes derived from early antral follicles are lower than those of oocytes grown *in vivo*. In the present study, to improve the developmental competence of bovine IVG oocytes, fibroblast growth factor (FGF) 7 was added to the growth culture medium, and the effects of maturational culture system and pre-maturational culture of IVG oocytes on developmental competence after *in vitro* fertilization were investigated.

At first, the effects of FGF7 on the growth of oocytes and the proliferation of granulosa cells were investigated after 4 to 16 days of culture. As a result, oocyte diameter and cell numbers of granulosa cells were increased after 8 days of culture in the presence of FGF7. However, the proportion of oocytes that survived was significantly decreased after 16 days of culture regardless of FGF7 addition. Secondly, I examined the effects of maturational culture system on the maturation and subsequent development of the oocytes obtained after 14 days of IVG culture in the presence or absence of FGF7. IVG oocytes were cultured for maturation in droplets or micro-well. Some oocytes were cultured in the medium supplemented with IBMX before maturational culture in micro-well. Regardless of FGF7 addition to IVG culture, the maturation rate of oocytes cultured in micro-well with pre-maturational culture was higher than that of oocytes without pre-maturational culture and equivalent to that of oocytes grown *in vivo*. After *in vitro* fertilization, cleavage and blastocyst rates of oocytes matured with pre-maturational culture were found to be higher than those without pre-maturational culture regardless of FGF7 addition to IVG culture.

The present study demonstrated that FGF7 addition to the growth culture medium promotes oocyte growth and granulosa cell proliferation. In addition, oocyes grown *in vitro* showed a high maturation rate equal to that of oocytes grown *in vivo*, and their developmental competence was improved when matured in micro-well with pre-maturational culture.

Endoplasmic reticulum (ER)-associated degradation of R664X mutant of anion exchanger 1 (AE1): Interactions of R664X AE1 with ER chaperones and dislocation channel proteins

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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 of the polypeptide in the ER and its retrotranslocation into the cytosol, leading to its degradation by the proteasome system. Previous studies have demonstrated that the ERAD of an R664X mutant of the bovine anion exchanger 1 (AE1), a causative mutant of hereditary spherocytosis in cattle, is largely different from that for ΔF508-CFTR, being *N*-glycosylation-independent and ubiquitylation-independent. The purpose of the present study was to identify the ER components that participate in recognition and dislocation of R664X AE1. To do this, candidate proteins, including calnexin, BiP, derlin-1, derlin-2, derlin-3, Bap31, Sec61β, and gankyrin, were co-transfected into HEK293 cells with R664X AE1, WT AE1, and/or ΔF508-CFTR, and intracellular localization and protein-protein interactions of these proteins were analyzed. The major findings were 1) R664X AE1 bound to calnexin and BiP, and 2) R664X AE1 was associated with derlin-1, Bap31, and Sec61β. R664X AE1 formed a complex with calnexin through peptide-based binding. R664X AE1 showed more profound binding with derlin-1 and Bap31 than WT AE1 and ΔF508-CFTR. Remarkable reduction in the AE1 content was observed in the cells co-transfected with derlin-1. These ER proteins basically exhibited reticular co-localization with R664X AE1 in the ER throughout the cells. These findings indicate that R664X AE1 is recognized by calnexin and BiP, resulting in retention in the ER, and retrotranslocated from the ER through dislocation channels formed with derlin-1, Bap31, and Sec61.

Association between the $Gly^{492} \rightarrow Ser$ mutation in the GLAST gene and the red cell low glutamate transport phenotype characteristic of Japanese Shiba dogs

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Dog red cells have a subtype of Na^+, K^+-dependent glutamate transporter, GLAST. However, some Japanese dogs have low glutamate transport (LGluT) red cells, lacking GLAST in the membranes, as an autosomal recessive phenotype. Although these dogs frequently have the $Gly^{492} \rightarrow Ser$ mutation (G492S), it does not completely cosegregate with the LGluT phenotype. Hence, it has been proposed that a heterologous combination of G492S mutation and other defects is associated with LGluT. In the present study, we assessed the linkage between G492S and LGluT to validate the genetic model of LGluT inheritance in Japanese dogs. First, we examined the prevalence of G492S in a dog population and found that G492S was almost exclusively observed in Shiba dogs. Then, we examined the glutamate transport phenotypes and G492S genotypes in other Shiba dog colonies and confirmed the higher prevalence of G492S and LGluT in these dogs. Linkage analyses using microsatellite markers in chromosome 4 revealed that G492S is not conclusively associated with LGluT. Therefore, the genetic model that LGluT is caused by a combination of G492S and other causative mutations is applicable for these dogs. Interestingly, the expression level of GLAST was remarkably decreased in LGluT cells during reticulocyte maturation compared with that of normal cells, demonstrating that GLAST is removed more extensively in LGluT cells at the reticulocyte stage. We also assessed primary structures of red cell membrane skeletal proteins, but could not find mutations specific for LGluT. In conclusion, LGluT phenotype and G492S mutation should be characteristics of Shiba dogs, and this breed is suitable for study of LGluT inheritance. Moreover, understanding of the mechanisms of GLAST protein reduction during reticulocyte maturation would be useful for clarification of the molecular basis of the LGluT phenotype.

Functional difference between the γ-globin promoter and the β-globin promoter in bovine globin switching

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Globin switching in the birth period is a physiological response involving globin chain substitution of the fetal γ-chain with the adult β-chain. Globin switching in humans is controlled

by a competitive mechanism that involves sequence-specific association of transcriptional factors to the γ- and β-promoters and the locus control region (LCR). However, bovine γ- and β-globin possess promoter sequences that are very similar to each other and the switching mechanism remains unknown. The purpose of the present study was to identify transcription factors to analyze the mechanisms for differential promoter activation of γ- and β-globin genes in cattle. Electrophoretic mobility-shift assay, immunoblotting, and the promoter reporter assay demonstrated that erythroid Krüppel-like factor (EKLF) bound to and activated both γ- and β-globin promoters. Affinity chromatography and mass spectrometry analyses showed that PARP-1 and GATA-1 bound to the γ- and β-globin promoter -163 ～ -108 regions and suggested that these proteins are crucial for proper globin switching. DNA methylation assays and bisulfite sequencing showed that genomic DNA methylation had some roles in β-globin promoter activation and suggested some regulating mechanism in bovine γ- to β-globin switching. In conclusion, these findings indicated that EKLF has an essential role in the activation of both γ- and β-globin genes and suggested that the interactions with EKLF, PARP-1, GATA-1, and DNA methylation are involved in generation of the functional difference of γ- and β-globin gene promoters in globin switching in cattle.

Construction of a replicon and an infectious cDNA clone of the Sofjin strain of tick-borne encephalitis virus

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Tick-borne encephalitis virus (TBEV), a zoonotic disease agent, causes severe viral encephalitis named tick-borne encephalitis (TBE) in humans. In October 1993, a human case of encephalitis was diagnosed as TBE in Hokkaido and TBEV Oshima strain was isolated from sentinel dogs, wild rodents, and ticks in the same area. The Oshima strain was categorized into the Far-Eastern subtype by genetic analysis. The Far-Eastern subtype is highly pathogenic and causes a severe and debilitating encephalitic disease in humans. In our previous study, the Sofjin-HO strain, the prototype strain of the Far-Eastern subtype, was shown to be more pathogenic than the Oshima strain. The amino acid identities between the two strains are more than 98%. Therefore, if we compare the Sofjin strain with the Oshima strain at the molecular level, it should provide significant information to reveal the viral molecular determinant involved in the virulence, which should increase the understanding of the pathogenicity of TBEV. Replicons and infectious cDNA clones are useful to investigate the genetic determinants in flavivirus pathogenesis. In this study, we constructed a replicon and an infectious cDNA clone of the Sofjin strain.

The replicons and the infectious cDNA clones of the Sofjin strain were constructed by the insertion of the viral cDNA into a low-copy plasmid. In the cells transfected with the replicon RNA, the production of the viral non-structural proteins and the replication of the replicon RNAs were observed. The recombinant viruses derived from the infectious cDNA replicated in BHK cells and caused a severe and debilitating encephalitic

disease in a mouse model.

In the parental Sofjin strain, four amino acid differences according to the viral quasispecies were observed, and the effects of these differences on viral properties were analyzed. The difference in E protein, amino acid (aa) position 496, and NS4A protein, aa position 58, were found to affect viral replication by IFA and real-time PCR in replicon-transfected cells. The recombinant virus with glutamic acid at aa position 122 of E protein showed high neurovirulence in mice, indicating the involvement of this residue in the pathogenicity of the parental Sofjin strain.

In conclusion, our results demonstrated that the replicon and the infectious cDNA clone of the Sofjin strain were useful for the analyses of the viral characteristics. These replicon and infectious cDNA will be used to identify the viral molecular determinant involved in the difference between the Sofjin and the Oshima strain, increasing the understanding of the pathogenicity of TBEV.

The glycosylation of the E protein of West Nile virus affects its pathogenicity in chick model

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West Nile virus (WNV) is an RNA virus, and a member of the family *Flaviviridae*, genus *Flavivirus*. WNV is transmitted by mosquito bite, and is maintained in the enzootic transmission cycle between mosquitoes and wild birds. WNV infection causes neurologic illness in humans and horses, which are accidental hosts. In recent years, highly pathogenic WNV has been reported worldwide, and it has become a major concern for public health.

Birds are important for the epidemiology of WNV in nature. However, their susceptibility to WNV varies depending on species, and the pathogenicity of WNV to birds is unclear. It has been suggested that the glycosylation of the E protein of WNV is related to the high pathogenicity of recent isolates of WNV in birds. In this study, the pathogenicity of the WNV glycosylated variant was analyzed in young domestic chicks to evaluate the effect of the glycosylation of the E protein on the pathogenicity to birds.

The viremia titers of the glycosylated variant in 2-day-old chicks exceeded 10^5 PFU/ml, and the viral titers of the glycosylated variant in the hearts, spleens, and kidneys were significantly higher than those of the non-glycosylated variant. In addition, it was shown that the neutralizing antibody response and the cytokine (IFN-α, LITAF, TNFSF15, IL-1β, IL-6, and IFN-γ) responses were not affected by the glycosylation of the E protein. In 3-week-old chicks infected with the WNV glycosylated variant, no clinical symptoms were observed, and no virus was detected in the serum and organs. These data indicate that the high pathogenicity in young chicks was due to the high rate of multiplication in the serum and organs, but not to the neutralizing antibody response and the cytokine responses. Japanese encephalitis virus (JEV), closely related to WNV, showed no virulence in 2-day-old chicks and lower titer of this virus was detected in the serum and organs, indicating the difference in the multiplication rate in organs between WNV and JEV.

In this study, it was shown that young

domestic chicks that show high susceptibility to WNV can be a useful animal model for WNV infection in wild birds. In addition, the glycosylation of the E protein of WNV was proved to be important for the viral proliferation in peripheral organs such as heart, leading to the high pathogenicity in birds. These findings will be useful for further study to develop efficient treatment and vaccines for birds against WNV.

Characterization of hantaviruses in Mexican wild rodents

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Hantavirus causes two forms of severe human diseases, hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS). HPS is a respiratory syndrome caused by Sin Nombre virus (SNV), Andes virus (ANDV), and other hantaviruses distributed over South and North America and carried by specific natural rodent hosts. Although there has been no report of HPS in Mexico, the country is adjacent to the southwestern states of U.S.A. where HPS is endemic. In addition, there are hantaviruses among rodents in Mexico. Because the economic relationship between Japan and Mexico is expanding year by year, it is necessary to determine the characteristics of the Mexican hantaviruses. In this paper, we extend the knowledge of the hantavirus genetic diversity and the antigenic characteristics among wild rodents in Mexico.

We identified 3 unique genotypes of the hantaviruses, which we designated herein as Montano virus (MTNV) from *Peromyscus aztecus*, Carrizal virus (CARV) from *Reithrodontomys fulvescens*, and Huitzilac virus (HUIV) from *R. megalotis*.

Next, we prepared recombinant nucleocapsid proteins of MTNV, CARV, and HUIV (MTN-rNP, CAR-rNP, and HUI-rNP). The three Mexican hantavirus rNPs had similar reactivity to the immune and virus-infected sera and the monoclonal antibodies (MAbs). All the Mexican hantavirus rNPs showed cross-reactivity against the anti-PUUV serum and the anti-SNV-rNP serum. It is suggested that the Mexican hantaviruses have similar antigenicity to each other.

There are no MAb against the Mexican hantaviruses. To prepare the MAb against the Mexican hantaviruses, four MAbs (2F11, F8B5, G8B4, and 7E6) were generated by the immunization of the MTN-rNP. Three of them were shown to recognize a linear epitope in the MTNV nucleocapsid protein while one was shown to recognize a conformational epitope. We analyzed the reactivity of the hantavirus rNPs to the MAbs. All the MAbs reacted with the Mexican hantavirus nucleocapsid proteins and three MAbs (2F11, G8B4, and 7E6) reacted with SNV-rNP. Our result indicated that the antigenicities of the three Mexican hantaviruses are almost identical to those of each Mexican virus and similar to that of SNV.

Three MAbs (2F11, G8B4, and 7E6) recognized the N-terminal region of MTN-rNP. Although 2F11 and 7E6 were shown to recognize an overlapping region, G8B4 and F8B5 were shown to recognize different regions. These MAbs were applied to ELISA to detect hantavirus nucleocapsid protein. This ELISA could detect the Mexican hantaviruses and SNV. Therefore, this ELISA can be applied as a specific diagnostic method for New World hantavirus infection.

Intermittent hypoxia induced radioresistance in rat glioma cells

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Hypoxia has been shown to be a feature associated with tumor resistance to ionizing radiation. Historically, tumor hypoxia refers to chronic hypoxia due to the limitation of oxygen diffusion. However, recent studies reported that fluctuations in oxygen pressure varying within several hours lead to so-called intermittent hypoxia. This temporary hypoxia is mainly due to unstable blood flow caused by functional or structural abnormalities of tumor vessels, and is suggested to contribute to tumor progression by promoting tumor cell migration and angiogenesis. However, in rat glioblastoma, the existence and the effect of intermittent hypoxia on cellular radioresistance remain unknown. In this study, I therefore examined whether intermittent hypoxia occurs in intracranial C6 glioma *in vivo*, and also investigated how intermittent hypoxia affects cellular radioresistance by comparing the effect of intermittent hypoxia in tumor cells with that of persistent hypoxia in a culture system.

First, PET imaging was performed using [^{18}F]-FMISO as a hypoxia tracer in an attempt to detect time-dependent transition of hypoxic region. However, changes of the distribution of [^{18}F]-FMISO were hardly observed because of the low signal-noise ratio of scan images. I next evaluated intermittent hypoxia in intracranial glioma using immunohistochemistry. Two different hypoxia markers, pimonidazole and EF5, simultaneously or with a time interval of 6 hours, were injected into C6 glioma-bearing rats. Paraffin sections of glioma were immunostained with these markers. When these markers were co-injected, they showed the same distribution in the tumor tissue. On the other hand, when pimonidazole was injected 6 hours prior to EF5, there were some areas labeled only with pimonidazole or EF5, indicating the existence of intermittent hypoxic areas in C6 intracranial glioma. Furthermore, radiation-induced reproductive cell death was examined by colony formation assay *in vitro*. C6 cells were incubated under normoxia, persistent hypoxia for 4 hours, or intermittent hypoxia with 4 cycles of 1-hour hypoxia interrupted by 30-minute reoxygenation, followed by X-irradiation in normoxic conditions. The treatment of the intermittent hypoxia significantly enhanced radioresistance compared with that of the persistent hypoxia or normoxia. To reveal the mechanism behind this, I analyzed the expression of HIF-1α by western blot, the amount of DNA double-strand breaks, the amount of cellular glutathione (GSH), and the enzymatic activity of superoxide dismutase (SOD). However, I did not observe any differences in the data between intermittent hypoxia and persistent hypoxia.

In conclusion, the present data showed the existence of intermittent hypoxia in transplanted intracranial C6 glioma and the enhancement of radioresistance of tumor cells exposed to intermittent hypoxia; however, I could not explain why this radioresistance of C6 cells was induced after exposure to intermittent hypoxia *in vitro*.

p66Shc CH2 domain regulates radiosensitivity in SCCVII cells via interaction with hsp72

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Various environmental stresses cause cellular damage that potentially threatens cellular integrity. To protect against these stresses, cells are equipped with defense mechanisms, which are essential for the maintenance of cellular homeostasis. Previous studies have shown that X-irradiated cells generate intracellular reactive oxygen species (ROS), which are associated with apoptotic signal transduction. Recent evidence indicates that p66Shc, an adaptor protein belonging to the ShcA family, is involved in the regulation of intracellular ROS level. Because it is suggested that intracellular ROS level affects cellular radiosensitivity, I investigated whether p66Shc plays a crucial role in determining cellular radiosensitivity.

In this study, it was demonstrated that X-irradiation-induced cell death of SCCVII/WT p66, a stable cell line expressing wild-type p66Shc, was higher than that of SCCVII/vec, a control cell line, indicating that p66Shc influenced cellular radiosensitivity. Since p66Shc has a unique amino-terminal region, collagen-homology 2 (CH2), distinct from other ShcA isoforms, I hypothesized that the CH2 domain interacted with other proteins and these interactions had an influence on cellular sensitivity toward X-irradiation. To test this hypothesis, I prepared recombinant protein containing CH2 domain, named His×6-p66 CH2, and searched for the interacting proteins in cell extract. LC-MS/MS analysis showed that it associated with heat shock protein 72 (hsp72). To examine the relationship between this interaction and radiosensitivity, I tested the effect of a small-molecule inhibitor of hsp72, PES, on cellular radiation-induced cell death in SCCVII/WT p66. As a result, PES enhanced the radiosensitivity in SCCVII/WT p66, but not in SCCVII/vec.

These results indicated that p66Shc plays a role in promoting radiation-induced cell death and that cytosolic hsp72 reduces the function of p66Shc through their interaction. Previous studies showed that p66Shc regulates oxidative stress and hsp72 is required for protection against stresses, suggesting that the association between p66Shc and hsp72 could regulate cellular responses against various stresses not limited to X-irradiation. However, it is still unclear how this protein interaction affects the cellular stress sensitivity, and further research is necessary to clarify this.

Relationship between delayed reactive oxygen species production and cell cycle control after X-irradiation in human lung carcinoma A549 cells

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Reactive oxygen species (ROS) induced by X-irradiation have been reported to be associated with various biological effects such as apoptosis, genomic instability, and bystander effects. Although several studies have shown that X-irradiation upregulates mitochondria-derived ROS production, its mechanism remains unclear. On the other hand, it is well known that X-irradiation causes cell cycle arrest at the G2/M phase under the control of G2 checkpoint. Recent studies indicated that the cell cycle in host cells plays a role in the regulation of mitochondrial size, number, and function. I therefore hypothesized that the cell cycle was involved in the regulation of intracellular ROS level, mitochondrial content, and mitochondrial function in X-irradiated cells. To test this hypothesis, I performed flow cytometric analyses using a double-staining technique with Nuclear-ID™ Red DNA Stain for cell cycle and a specific fluorescent probe (DCFDA for intracellular ROS level, MitoTracker™ Green FM for mitochondrial content, and $DiOC_6(3)$ for mitochondrial membrane potential) in human lung adenocarcinoma A549 cells.

When intracellular ROS level, mitochondrial content, and mitochondrial membrane potential were analyzed in each cell cycle fraction (G1, S, and G2/M) of A549 cells without or with 10 Gy of X-irradiation, fluorescence intensity of intracellular ROS, mitochondrial content, and mitochondrial membrane potential in the G2/M phase were higher than those of G1 or S phase regardless of exposure to X-irradiation. In addition, when A549 cells irradiated with 10 Gy were incubated for 12 h and ROS level of each cell cycle fraction was compared with that of an identical cell cycle fraction of non-irradiated cells, I found that X-irradiation caused an increase of ROS level in each cell cycle fraction. To investigate how long the cell cycle was arrested at the G2/M phase by X-irradiation, I analyzed the transient time of each cell cycle phase with or without X-irradiation using cells synchronized at the G1/S to S phase by thymidine treatment. The result showed that the period of G2/M phase in irradiated cells was about three times longer than that in non-irradiated cells.

The present result suggested that radiation induced accumulation of mitochondria-rich cells by a G2 checkpoint mechanism, and that radiation-induced enhancement of intracellular ROS level was due to the long-term accumulative effect of ROS leaked from mitochondria. In conclusion, X-irradiation enhanced mitochondrial content and function through the accumulation of a cell population in the G2/M phase, leading to the increase of intracellular ROS level.

Effects of toxic metal contamination on wild rats in mining areas in Zambia

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Mining is a major industry in Zambia, which has two major mining sites: a lead-zinc (Pb-Zn) mine in Kabwe city and a copper-cobalt (Cu-Co) mine in the Copperbelt Province. To examine the effects of mining activities and metal pollution on sentinel wildlife, wild black rats (*Rattus rattus* and *R. tanezumi*) were captured in Kabwe and Chingola (in the Copperbelt Province), as well as in Lusaka (as a non-contaminated site). Wild black rats in Kabwe had accumulated significantly higher concentrations of Pb and Cd in various organs than rats from Lusaka. In Chingola, significantly higher concentrations of Cu, Co, Pb, and Cd had accumulated in wild black rats than in rats from Lusaka. Mining activities could have contributed to metal pollution in these areas. Body weight and renal Pb concentration were negatively correlated, suggesting growth suppression by Pb. To examine the effects of metals on molecular reactions, metallothionein (MT)-1, MT-2, and heme oxygenase (HO)-1 mRNA expression levels were measured. Metallothionein-1 and -2 mRNA expression levels of wild black rats from Kabwe were significantly higher than those of rats from Lusaka, and were positively correlated with Cu, Pb, and Zn levels, while negatively correlated with As, Co, and Cr levels. Furthermore, DNA methylations in the Kabwe population were significantly lower than those in the Lusaka population. DNA methylation was negatively correlated with liver Pb concentration. These results suggest that wild black rats in Zambian mining sites were exposed to metals that accumulated in their organs, causing biological reactions (growth suppression, induction of MT transcription, and reduction of DNA methylation). In addition, wild black rats appear to be adequate sentinels for metal pollution.

Mechanism and species difference of dioxin toxicity in avian species

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Avian species are one of the groups of animals at high risk from dioxins. It was reported that numerous avian species, especially fish-eating birds and raptors, accumulated high levels of dioxins. Although large differences in dioxin sensitivity have been reported among avian species, the reason for this has not been fully explained. The aim of this study is to characterize the bird aryl hydrocarbon receptor (AHR), known to be related to dioxin toxicity,

and clarify the species differences in birds. AHRs of 18 avian species were analyzed, and it was found that the two amino acids reported to contribute to determining dioxin sensitivity, did not correlate to the taxonomy or phylogeny of AHR protein sequence. In addition, two different AHR isoforms, AHR1 and AHR2, were compared in terms of mRNA expression ratio in each species, and it was revealed that dioxin-sensitive birds dominantly express AHR1. From these results, two avian species were identified as species at high risk from dioxin. Transcriptional ability was evaluated in these species, and it was revealed that AHR1 of the ostrich in particular is associated with high sensitivity.

Additionally, the mechanism causing avian heart defects was clarified. AHR is known to induce CYP1A expression with ligand binding. We focused on the COX-2 mediating pathway that is clearly separated from the CYP1A inducing pathway. Fertile chicken eggs were injected with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and COX-2 selective inhibitor, NS398, and chick heart failure was investigated on day 10. The increases in chick heart to body weight ratio, atrial natriuretic factor (ANF) mRNA expression, and enlarged ventricle caused by TCDD were abolished with co-treatment of NS398. This result suggested that TCDD-induced heart defects are mediated by a COX-2 requiring pathway.

The effect of metal pollution in cattle by mining activity in Zambia

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The Republic of Zambia is rich in mineral resources, such as zinc (Zn) and lead (Pb), and thus mining has been a key industry in Zambia. The toxic effects of metals on humans and animals have been a concern around mining areas in this country. Although the exploitation of Zn and Pb in Kabwe, one of the main mining areas in Zambia, stopped in 1994, diffusion of the toxic metals to contaminate the soil in the area is still a big problem. However, the effect of these toxic metals on animals living around Kabwe has never been investigated.

Lead (Pb) is known to have immune and neuro-system toxicity. Therefore, the effect of Pb on animals and humans in Kabwe is considered to be important. Furthermore, a recent report from JECFA indicated that Pb has no threshold because of its toxicity. Therefore, in the present study, we paid attention to cattle, which is one of the most widespread and important domestic animals in Zambia, and focused on toxic metal pollution. In addition, we also paid attention to immunity-related factors of cattle to understand the immune toxicity of these metals, especially Pb.

We took cattle blood samples in Kabwe ($n = 12$) and Lusaka ($n = 10$) and measured metal concentrations using an atomic absorption spectrometer and an inductively coupled plasma mass spectrometer. Furthermore, we measured mRNA expression of metal-responsive protein and some cytokines in white blood cells using real-time RT-PCR. For *in vitro* study, we cultured peripheral blood mononuclear cells (PBMCs) from cattle ($n = 1$) that were exposed to lead acetate (3, 6, 12, 25, 50 μM) for 24 hours and, subsequently, mRNA expressions of metal-

responsive protein and some cytokines were analyzed by real-time RT-PCR.

Pb and Cd concentrations in cattle blood from Kabwe were significantly higher than those from Lusaka. The mRNA expressions of MT-2, TNF-α, IFN-γ, IL-1β, IL-6, and iNOS were significantly different in Kabwe cattle from Lusaka cattle. There were correlations between Pb and mRNA expressions of MT-2, TNF-α, IL-6, IL-1β, and iNOS. Furthermore, in *in vitro* study using cattle PBMC, Pb exposure led to changes in the expressions of MT-2, TNF-α, IL-1β, and iNOS, which were similar to the field results.

In summary, the results (MT-2, TNF-α, IL-1β, iNOS) of the field study in Kabwe cattle were in accordance with the results of the laboratory experiment. These results suggested that the Pb exposure was the cause of the changes in immunity-related factors in cattle bred in Kabwe.

The isolation of mouse mesenchymal stem cells and analysis of their therapeutic effect on mice infected with prion

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Prion diseases are intractable diseases and the treatment of these diseases is urgently required. Since progressive neurodegeneration is a characteristic feature of these diseases, the protection of neurons from degeneration and regeneration of damaged nervous tissues is required for functional recovery. Mesenchymal stem cells (MSCs), known as one of the pluripotent stem cells, are a potential tool for regenerative medicine/cell therapy. Transplantation of immortalized human bone marrow-derived MSCs prolonged the survival of mice infected with prion even when transplanted after the clinical onset, suggesting the possible application of MSCs for the treatment of prion diseases (Song, C.-H., Honmou, O., Nakamura, K., Hamada, H., Furuoka, H., Hasebe, R., and Horiuchi, M. *J. Virol.,* 83: 5918–5927, 2009). However, that study was carried out by xenotransplantation of human MSCs into mice. In order to analyze the effect of MSCs on prion diseases under allogeneic transplantation, mouse MSCs were isolated and characterized from bone marrow, compact bone, and adipose tissue, and transplanted into prion-infected mice to evaluate the therapeutic potential. MSCs isolated from bone marrow, compact bone, and adipose tissues expressed Sca-1, a marker for the self-renewal of stem cells, and also expressed molecules that are reported to be non-specific markers for MSCs, such as CD44 and CD106. However, flow cytometric analysis also showed that isolated MSCs consisted of heterogeneous cell populations. Multipotent ability of MSCs isolated from bone marrow and compact bone was demonstrated by *in vitro* adipogenesis and osteogenesis. MSCs derived from bone marrow and compact bone specifically migrated to brain homogenates from prion-infected mice, determined by *in vitro* migration assay, but those derived from adipose tissue did not migrate. MSCs derived from each tissue were transplanted into hippocampus or intravenously to prion strain-infected mice at the time of clinical onset (at 120 days post-inoculation) to analyze the therapeutic potential. Intra-hippocampal transplantation of bone marrow-derived MSCs significantly prolonged the survival of mice infected with the Chandler prion

strain compared with that of mock-treated control (161.8 ± 9.9 vs. 152.3 ± 6.2 days, $p < 0.05$). However, intravenous transplantation did not appear to prolong the survival. Although MSCs used in this study consisted of heterogeneous cell populations, MSCs could prolong the survival of the Chandler prion-infected mice after the intra-hippocampal transplantation. This indicates that the usage of a particular MSC population, which can be obtained by improving the methods for MSC isolation, will improve the therapeutic potential. The identification of such a specific MSC population will greatly contribute to the establishment of treatment for prion diseases using regenerative medicine/cell therapy.