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INFORMATION

Hokkaido University conferred the degree of Bachelor of Veterinary Medicine to the following 42 graduates of the School of Veterinary Medicine on March 25, 2013. The authors summaries of their theses are as follows:

Investigation of an efficient culture condition for the differentiation of murine skeletal myoblasts

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During fetal development, with the activation of muscle regulatory factors (MRFs), skeletal myoblasts differentiate into multinuclear myotubes subsequent to mature myofibers having contracting ability. Recent studies on skeletal myoblast differentiation were performed to develop edible meat *in vitro* or for the treatment of skeletal muscle-related diseases; however, the current myoblast culture system has some problems in the efficiency of skeletal muscle differentiation. The present study investigated efficient culture conditions for skeletal myoblast differentiation.

A murine myoblast cell line, C2C12, was used. Differentiation was induced in Dulbecco's modified Eagle's medium (DMEM) for 6 days with the following modifications: animal species of serum (fetal calf or horse), serum concentration (0.01–4%), and insulin concentration (0–100 nM). Differentiation of C2C12 was evaluated by their morphology, the protein expression levels of myosin heavy chain (MyHC) and MRFs (*Myh1*, *Myod1*, *Myog*). Furthermore, the effect of three-dimensional culture on C2C12 differentiation was analyzed using a collagen gel system.

On the sixth day after differentiation culture, C2C12 differentiated into myotubes, and their length and nuclear numbers increased depending on both serum and insulin concentrations. In particular, the use of horse serum tended to increase the length of myotubes compared to fetal calf serum. Although the width of myotubes increased in an insulin concentration-dependent manner, there was no effect with serum. MyHC protein levels in differentiated C2C12 increased in a serum and insulin concentration-dependent manner, and the highest value was observed in those cultured with 4% horse serum. For MRF gene expressions, those in *Myh1* and *Myog* were higher when horse serum was used rather than fetal calf serum after 3 days of differentiation, and the opposite results were obtained for *Myh1* and *Myod1* expressions after 6 days of differentiation. Under culture conditions with 100 nM insulin and fetal calf or horse serum, the use of 2% or 4% horse serum tended to show the highest value in all examined indices for myogenic differentiation, including morphometry, MyHC protein levels, and MRF expression. It was noteworthy that contraction was also observed under these conditions. There was no improvement in C2C12 differentiation by the three-dimensional culture system.

In conclusion, during the differentiation culture of myoblasts, the length and fusion degree of myotubules were affected by the serum and insulin concentrations, and their thickness was only affected by the latter. From these findings, it is proposed that differentiation

medium containing 2–4% horse serum with 100 nM insulin differentiates myoblasts into longer myofibers containing rich myosin.

Analysis of duodenojejunal flexure formation in mice

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During mammalian gastrointestinal development, the gut tube elongates and forms flexures at the point of the physiological umbilical hernia. The gastrointestinal morphology has a species-specific running pattern, indicating genetic control. In this study, the developing mouse gut was observed to elucidate the mechanism of gut flexure formation.

In postnatal mice, although the running and flexure patterns of the intestines differed among individuals, part of them was common, such as the duodenojejunal flexure (DJF). In the examined prenatal mice (embryonic day 10.75–13.75), every gut tube had the same pattern among individuals at the same embryonic days. In particular, the DJF was independently and clearly observed and suitable for subsequent analyses. According to the existence and position of the flexure, the developing DJF was morphologically classified into 3 stages. At the expansion stage, no visible flexure was observed. However, the proliferation rate and the nuclear size of the mesenchymal cells differed between the left-right and dorso-ventral intestinal axes; in particular, the former showed higher values on histoplanimetry. At the flexure formation stage, the DJF started to bend toward the dorsal direction along the body axis. Simultaneously, since the DJF showed counterclockwise rotation along the antero-caudal intestinal axis, the right lateral side of the DJF along the intestinal axis corresponded with the top of the flexure. Furthermore, the outer bending side of the flexure showed a thinner gut wall, more squamous mesothelium, and a higher proliferation rate in the mesenchymal and epithelial cells than on the inner side. At the flexure elongation stage, the DJF elongated around the stomach. No contribution of the dorsal mesentery to DJF bending was observed during the examined period. For analysis of the genes associated with gut development, the mRNA expressions of *Aldh1a2* and *hedgehogs* were higher on the ventral side along the body axis at the expansion stage and were detected in the developmental gut tube, including the DJF.

In conclusion, these results suggest that the gut tube twists and bends along the left-right intestinal axis to form the DJF along the dorso-ventral body axis. It is proposed from this study that the asymmetry of cell morphology and proliferation along the left-right intestinal axis mediated by retinoid and hedgehog signaling induces DJF formation, not tension from the dorsal mesentery.

Vision of tetrachromats: Study on plumage colors of jungle crow (*Corvus macrorhynchos*) and common crow (*Corvus corone*) using a digital UV camera

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1. Humans are unable to distinguish sexual and species differences from the plumage colors of some birds that are known to be dichromatic for avian perception. Teterachromatic birds possess ultraviolet (UV)-sensitive cones and UV vision can provide great advantages to identify, discriminate, investigate, qualify and quantify various objects over human vision under natural conditions. We investigated potential sexual and species differences in plumage colors in the jungle crow (*Corvus macrorhynchos*) and common crow (*Corvus corone*), both of which are indistinguishable with human vision, utilizing a newly assembled digital UV camera.
2. Visible images and UV images of crows were photographed concomitantly under visual or UV light and the images were converted to an 8-bit gray-scale. Several regions of interest (ROI) were set, cut and pasted onto a new canvas and the UV/Visible ratio value of the brightness of each pixel was calculated. From the ratio values, pseudo-color images were created with a 16-color look-up table. Species or sexual plumage color differences were assessed with the UV/Visible ratio and pseudo-colors, which can be indicatives of hue differences.
3. Regional differences were found in some ROI for both species and sex. The average ratio in the cranial region of wings tended to be higher than in the caudal region. The cranial region of the wing has previously been demonstrated to show iridescent structural colors, indicating that the region reflects more UV than visible due to the microstructure of the plumage.
4. Neither species showed sexual differences in color variation under the present experimental conditions. The back and chest regions of female common crows showed higher ratio values than those of female jungle crows, and the cranial region of the left wing of male jungle crows showed a higher value than that of male common crows. These differences can be utilized as clues to distinguishing each species.
5. The current study indicated that reproducing tetrachromats' vision with UV photographs combined with visible images could be a powerful tool to evaluate color differences in some species or even in general objects that humans cannot perceive. Further investigation with a refined and improved technique would enable better understanding of the physiological significance of UV vision in tetrachromats.

Individual variation of near-ultraviolet vision in humans

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1. Based upon a preliminary finding that some humans can detect UV but others cannot, we conducted an examination to examine whether humans can detect and recognize UV. We utilized an in-house assembled optical scope with visible and infrared-absorptive filters to make it possible to path pure UV only. Subjects were requested to observe UV images through the scope and self-judged whether they could identify an artificial mark or not. Their age, sex, birthplace, astigmatism, and eyesight were noted afterwards in a personal to analyze potential factors that may be involved in UV vision. In addition, they were requested to select their perceived color from a color table to characterize the color recognition produced by UV.

2. In agreement with the preliminary finding, it became evident that some subjects could detect UV but others could not. The percentage of subjects who could detect UV increased as the intensity of UV given increased or as the period of dark adaptation before the start of the examination increased.

3. Subjects with astigmatism or good eyesight, or coming from high UV-exposure locations in Japan showed better UV vision but there were no sex differences.

4. In subjects who could detect UV, the degree of color saturation tended to be suppressed as UV intensity decreased. The same trend was found as the period of dark adaptation before the start of the examination increased and the recognized hue became bluish.

5. All these results indicate that individual variation exists in UV vision, which may be affected by several factors. Both cone and rod cells may be involved in UV detection in humans depending on various environmental conditions and the final color image created in the visual cortex can be determined by the relative contribution of each photoreceptor cell. Under low UV intensity, signals from rod cells may become predominant.

6. The present findings provide new clues to understand the physiology of human vision even for UV perception, which has been generally thought to be invisible to humans.

Analysis using an acceleration data logger of behavioral changes before calving in dairy cows

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1. Accurate prediction of calving time in dairy cows is highly beneficial to prepare for the potential incidence of dystocia, but reliable detection of parturition signs and its methodology remain to be established. In this study, we attempted to record and quantitatively analyze behavioral changes before calving using tri-axial acceleration data loggers attached to the back between the sacrum to caudal vertebra of dairy cows restricted to a calving stall for 4 days before and on the day of calving.

2. On the calving day, a significant decrease in mean standing duration and an increase of mean lying duration were observed. Diurnal variation was found in walking activity, but not in the walk ratio. Both walking activity and the walk ratio tended to increase gradually from 24 hours before calving and reached significantly elevated levels 3 hours before calving ($154 \pm 81\%$ and $84 \pm 56\%$ increase, respectively). The increase of walking activity was suggested to be due to increases of standing duration and the walk ratio. The duration of tail raising increased significantly from 18 hours before calving. In addition to behavioral changes, we assessed physiological and morphological changes. Rectal temperature dropped 54–30 hours before calving. Close to calving, relaxation of the sacrosciatic ligaments, congestion of the vulva and distension of the teats and udders were observed; however, the onset of these changes varied by several days among individuals.

3. The present results led us to conclude that the mean standing duration, the mean lying duration, walking behavior and tail raising can be useful indicators to predict the calving time with high accuracy. Physiological and morphological signs as longer-term indices can be used as additional clues to predict calving.

Effect of proinsulin C-peptide on proliferation of human breast cancer MCF7 cells

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Proinsulin C-peptide shows beneficial effects on microvascular complications of type 1 diabetes. These effects are thought to be mediated by a specific receptor for C-peptide, but the possible receptor structure has not been defined yet. Recently, it was reported that

C-peptide has the capacity to activate α-enolase, a glycolytic enzyme, through specific interactions. As α-enolase acts as a cell surface receptor for plasminogen, it may conceivably also serve as a receptor for C-peptide *in vivo*.

The α-enolase gene, *ENO1*, also encodes c-Myc promoter-binding protein-1 (MBP1), which downregulates the activity of *c-MYC* proto-oncogene. α-Enolase is the longer form (49 kDa) localized in both cytoplasm and nuclei, while MBP1 is shorter (37 kDa) and is found mostly in the nuclei. It is interesting to note that α-enolase has the ability to suppress *c-MYC* expression.

Furthermore, it has been shown that C-peptide is internalized and transferred in the nuclei within one hour after stimulation of cells. Taken together with the above, the hypothesis is proposed that a C-peptide-α-enolase complex forms on the cell surface, and is internalized and transferred in the nuclei, resulting in the suppression of *c-MYC* expression by α-enolase, which leads to cell proliferation inhibition. The aim of this study was to find clues to confirm the hypothesis.

Human breast cancer MCF7 cells, known to secrete α-enolase, were used in the study. Treatment of the cells with C-peptide dose-dependently suppressed cell growth in culture without affecting apoptotic signals and cell viability. C-peptide also induced transient activation of extracellular signal-regulated kinase (ERK), transient increase of the *c-MYC* gene and c-Myc protein expression within one hour after C-peptide stimulation, and decreased c-Myc protein expression 4 to 6 hours after stimulation. Prior treatment with an ERK kinase (MEK) inhibitor synergistically increased C-peptide-induced *c-MYC* gene expression, suggesting that the increase of *c-MYC* gene expression by C-peptide was independent of ERK activation.

In summary, C-peptide induced a cytostatic effect on human breast cancer MCF7 cells and increased the expression of *c-MYC* proto-oncogene and its translation product c-Myc shortly after stimulation. These results might be a clue to verify the hypothesis, but further study is necessary for confirmation.

Reduced glutathione inhibits three isozymes of enolase

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Increase in intracellular Ca^{2+} concentration and 5-HT release induced by hydrogen sulfide via transient receptor potential A1 in RIN14B cells

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Recently, hydrogen sulfide (H_2S), like nitric oxide and carbon oxide, is suggested to be a gasotransmitter in the mammalian body. H_2S is produced from L-cysteine by cystathionine-γ-lyase and cystathionine-β-synthase, and acts on several receptors and channels, such as NMDA receptors, K^+ channels, and voltage-dependent T-type Ca^{2+} channels. H_2S is also reported to activate transient receptor potential (TRP) cation channels, TRPV1 and TRPA1. Some secretory cells, such as pancreatic β-cells and enterochromaffin cells, express TRPA1, but the effect of H_2S on these cells is unknown. In this study, the effects of H_2S on intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and 5-HT release in RIN14B cells (rat pancreatic δ-cell line) were investigated by using a Ca^{2+} imaging technique and high performance liquid chromatography. NaHS, an H_2S donor, increased $[Ca^{2+}]_i$ in a concentration-dependent manner. The response to NaHS was significantly inhibited by the removal of extracellular Ca^{2+}, ruthenium red (a non-selective TRP channel antagonist) and HC030031 (a selective TRPA1 antagonist), but not by 5'-iodoresiniferatoxin, SB366791 (TRPV1 antagonists) and voltage-dependent Ca^{2+} channel blockers. Dithiothreitol and tris-(2-carboxyethyl) phosphine, reducing agents of disulfide bond, also inhibited the Ca^{2+} response to NaHS. NaHS increased 5-HT release from RIN14B cells in a concentration-dependent manner. 5-HT release by NaHS was inhibited by the removal of extracellular Ca^{2+}, HC030031 and dithiothreitol. These results indicate that H_2S increases $[Ca^{2+}]_i$ in RIN14B cells through the activation of TRPA1, and causes the release of 5-HT. H_2S is suggested to open TRPA1 channels via covalent modification of cysteine residues. Since RIN14B cells are used as models of pancreatic δ-cells or enterochromaffin cells, H_2S may be a secretagogue in these cells.

Inhibitory effect of adrenaline α_2 receptor agonists on reflex potentials in mouse spinal cord

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Adrenaline α_2 receptor agonists, dexmedetomidine (DEX) and xylazine (XYL), are clinically used for sedation and analgesia in animals and humans. Although α_{2A} receptors in the spinal cord are thought to mediate analgesic effects of these agonists, the lack of specific

antagonists for receptor subtypes makes it difficult to identify the receptors responsible for the effects.

In this study, we examined the effect of α_2 agonists on isolated spinal cord preparations of mice. DEX and XYL inhibited both slow ventral root potential (sVRP), which is thought to reflect a nociceptive pathway, and monosynaptic reflex potential (MSR). sVRP was more sensitive to these agonists than MSR. Guanfacine (α_{2A} agonist) inhibited sVRP, while (R)-(+)-m-nitrobiphenyline (α_{2C} agonist) inhibited MSR. The inhibitory effect of DEX on sVRP but not MSR was antagonized by atipamezole (α_2 antagonist) and efaroxan (imidazoline I_1 receptor antagonist). Idazoxan (I_2 antagonist) did not affect MSR and sVRP inhibition of DEX. There was no difference in the inhibitory effects of MSR by DEX and XYL between wild and α_{2A} KO mice. The inhibitory effect of DEX on sVRP was significantly decreased but not abolished, while that of XYL was slightly attenuated.

These results indicate that α_{2A} receptors play a major role in sVRP inhibition by DEX but not XYL. MSR inhibition of DEX and XYL, and sVRP inhibition of XYL are suggested to be mediated by receptors other than α_{2A} receptor. Imidazoline I_1 receptors may also mediate sVRP inhibition by DEX.

Increase in extracellular adenosine level by inhibitors of adenosine metabolic enzymes and removal of extracellular Ca^{2+} in rat spinal astrocytes

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Adenosine (ADO) derived from astrocytes plays an important role as a neuromodulater in the CNS. ADO is usually produced by the degradation of released ATP in the extracellular space and increased ADO is incorporated into the cells by nucleoside transporters (NTs), while the intracellular ADO level is regulated by adenosine kinase (AK) and adenosine deaminase (AD). Although it has been reported that the inhibition of AK and AD activities or the removal of external Ca^{2+} increased extracellular ADO ($[ADO]_o$), the mechanism underlying the $[ADO]_o$ increase is unclear. In this study, we examined the contributions of NTs and ATP release to $[ADO]_o$ increase in cultured rat spinal astrocytes. The mixture of AK and AD inhibitors (ABT-702 and erythro-9-(2-hydroxy-3-nonyl)adenine, EHNA, respectively) increased $[ADO]_o$ but not $[ATP]_o$, which was suppressed by ENT2 inhibition. On the other hand, Ca^{2+} removal increased not only $[ADO]_o$ but also $[ATP]_o$, both of which were reduced by carbenoxolone (gap junction inhibitor). The $[ADO]_o$ increase by Ca^{2+} removal was diminished by POM-1 (ecto-ATPase inhibitor) and rather enhanced by ENT1 and ENT2 inhibition. These results suggest that ENT2 is responsible for ADO release when intracellular ADO is increased, while the removal of external Ca^{2+} releases ATP, which is extracellularly degraded to ADO. Gap junction channels may be involved in ATP release by Ca^{2+} removal.

Classical swine fever virus infection and immune response in pigs

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The pathogenicity of classical swine fever virus (CSFV) is determined by virus replication and the immune response following virus replication in pigs. In the present study, virus replication and the immune response in pigs inoculated with mutant or recombinant viruses generated from the infectious cDNA clone of a live attenuated GPE^- vaccine virus were analyzed.

In pigs inoculated with $vGPE^-$/T830A; V2475A; A2563V, virus recovery occurred from systemic tissues, and excessive expression of mRNAs of interferon-α (IFN-α), interleukin (IL)-6 and IL-10 was detected in tonsils. In pigs inoculated with $vGPE^-$/N136D; T830A; V2475A; A2563V restored the capacity to inhibit type I IFN induction by amino acid substitution at position 136 in N^{pro}, and more viruses were recovered from systemic tissues than from pigs inoculated with $vGPE^-$/T830A; V2475A; A2563V. There was less IFN-α mRNA in tonsils than in those of pigs inoculated with $vGPE^-$/T830A; V2475A; A2563V. No significant difference was found in virus titers between pigs inoculated with $vGPE^-$/T830A; V2475A; A2563V and $vGPE^-$/T830A; Eystrup NS4B carrying NS4B gene derived from highly virulent CSFV strain Eystrup and 3 out of 5 pigs inoculated with $vGPE^-$/T830A; Eystrup NS4B died. In contrast, mRNAs of not only IFN-α, IL-6 and IL-10 but IFN-γ, IL-1α and TNF-α were strongly expressed in tonsils of pigs inoculated with $vGPE^-$/T830A; Eystrup NS4B.

These results indicate that the overproduction of cytokines such as IFN-α and IL-6 following virus replication is responsible for the pathogenicity such as leukopenia and thrombocytopenia in pigs infected with CSFV. In addition, these results reveal that regulation of the host immune response by N^{pro} and NS4B is responsible for pathogenicity of CSFV in pigs.

Analysis of pathogenicity and genetic characteristics of END-negative classical swine fever viruses isolated from domestic pigs in Thailand

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Classical swine fever virus (CSFV) has been divided into 2 groups that show the “exaltation of Newcastle disease virus” (END^+) or not (END^-). END^+ strains interfere with interferon alpha and beta (IFN-α/β) secretion in cells by viral N^{pro} protein and END^- strains do not. END^- strains

had not been isolated in the field until 1992. Four END$^-$ CSFVs (KPP/93, RBR/93, NKRS/98 and NKS/98 strain) were isolated from domestic pigs in Thailand from 1993 to 1998. In this study, the genes of 4 END$^-$ strains were sequenced and their pathogenicity in pigs was analyzed. These isolates have been classified into genotype 1.1 by phylogenetic analysis. The IFN-α/β bioassay *in vitro* revealed that a single amino acid substitution L40H or double amino acid substitutions S17P, and N61K in N^{pro} were responsible for the acquisition of N^{pro}-mediated suppression of IFN-α/β induction. In pigs inoculated with KPP/93 strain, thrombocytopenia, petechia in the kidney and swollen mesenteric lymph nodes were observed; however, none of the pigs inoculated with this virus showed any clinical signs and only a small amount of virus was recovered from blood and tissue samples. In addition, the cytokine response in the pigs was lower than that in pigs inoculated with a highly virulent strain. These findings indicate that the END$^-$ virus replicated inefficiently in swine cells and thus showed low pathogenicity in pigs.

Roles of hemagglutinin in the penetration of influenza virus into host cells

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Feral water birds, represented by ducks, are a natural reservoir of influenza A viruses. Duck influenza viruses do not transmit to chickens directly, but via domestic water birds and terrestrial birds. The hemagglutinin (HA) of influenza viruses is a possible target for antiviral drugs because of its key roles in the initiation of infection. It was found that Stachyflin, which is thought to be a fusion inhibitor, showed anti-virus activity against H1 and H2 but not H3 influenza viruses; however, its activity has not been tested against H4-H16 influenza viruses. In the present study, we examined the activity of Stachyflin to inhibit the replication of each virus of the 16 HA subtypes and identified factors responsible for the susceptibility of the viruses to this compound. In addition, as a factor responsible for determination of the host range of avian influenza viruses, the optimal pHs for fusion and acid stability of viruses were analyzed.

In chapter 1, it was found that Stachyflin has antiviral activity against not only H1 and H2 but also H5 and H6 viruses, as well as A(H1N1) pdm09 virus in an *in vitro* assay. It was also found that Stachyflin inhibited the growth of A/chicken/Ibaraki/1/2005 (H5N2) virus, in addition to A/WSN/1933 (H1N1) virus, in mice. Substitution of amino acid residues was found on the HA2 subunit of Stachyflin-resistant viruses. Docking simulation indicated that D37, K51, T107, and K121 are responsible for constructing the binding pocket for the compound. In addition, analyses of the 3-dimensional structure indicated that the binding pocket in the HA of Stachyflin-susceptible virus strains was different from that of insusceptible virus strains.

In chapter 2, A/duck/Mongolia/54/2001 (H5N2) (Dk/Mongolia) and A/chicken/Ibaraki/1/2005 (H5N2) (Ck/Ibaraki) replicated only in their primary hosts, and the optimal pH for the fusion of Ck/Ibaraki was significantly higher than that of Dk/Mongolia. In addition, the growth of Ck/Ibaraki was more extensive than Dk/Mongolia in CK cells, in which endosomal pH was higher

than that of MDCK cells, which was caused by differences in the optimal pH for fusion and the function of M1 and/or M2 of these viruses. Accordingly, the correlation between the host range and optimal pH for fusion was analyzed for 57 avian influenza viruses isolated from ducks or chickens. In consequence, no correlation was found among the host range, optimal pH for fusion, and acid stability of viruses. Indeed, it was reported that optimal pH for fusion of the H5N1 highly pathogenic avian influenza viruses had effects on the pathogenicity on ducks and chickens; however, no relationship was found in the present study.

The present study revealed that Stachyflin binds to the binding pocket in the HA and inhibits the fusion of the viral envelope with endosomal membranes, resulting in inhibition of the replication of H1, H2, H5, and H6 viruses. In addition, the present results should provide useful information for the development of the HA inhibitors. Furthermore, it was demonstrated that the optimal pH for fusion and the acid stability of viruses are not necessarily correlated with the host range or the pathogenicity of avian influenza viruses. Further studies should reveal the factors involved in the determination of the host range and pathogenicity of avian influenza viruses.

Roles of immunoinhibitory factors PD-1/ PD-Ls in the tumorigenesis of Marek's disease virus and the anti-tumor effect of its vaccine

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An immunoinhibitory receptor, programmed death 1 (PD-1), and its ligands, programmed death ligand 1 and 2 (PD-L1, PD-L2), are involved in the immune evasion mechanism for several pathogens causing persistent infections and neoplastic diseases. Blockade of the PD-1/ PD-Ls pathway by recombinant proteins or antibodies specific to those molecules results in the re-activation of immune reactions, and is expected to be applied to new therapies for chronic infectious disease and neoplastic diseases. These molecules have been already identified in chickens and are suggested to be involved in the pathological mechanism and tumorigenesis of Marek's disease virus (MDV). MDV establishes latency in $CD4^+$ T cells and subsequently causes malignant T cell lymphomas, one of the characteristics of Marek's disease (MD). MD has been successfully controlled by vaccination. MD vaccine achieved unparalleled success in preventing the disease and is known as the first effective immune prophylaxis against cancer. Thus, revealing details of its protection mechanism would help to develop novel anti-tumor vaccines for other neoplastic diseases; however, its mechanism has not been completely elucidated. Thus, in this study, the roles of PD-1/ PD-Ls in the tumorigenesis of MDV and the anti-tumor effect of MD vaccine were analyzed.

The immunoinhibitory function of PD-L1 via PD-1 has been identified, while functional analysis of PD-L2 has not been performed, although its regulatory effect was suggested based on analysis of its amino acid sequence. The expressions of PD-1 and its ligands are induced by an activated immune response. In this study,

soluble PD-1, PD-L1 and PD-L2 proteins (PD-1-Ig, PD-L1-Ig, PD-L2-Ig) were prepared and used for analysis of their expressions and functions. The expression levels of PD-1, PD-L1 and PD-L2 were increased on activated splenocytes, determined by flow cytometry using those soluble proteins. This result suggests that the PD-1/PD-L pathway is also required to regulate immune responses after activation in chickens, as previously observed in mice and humans. Furthermore, PD-1-Ig, as well as PD-L1-Ig and PD-L2-Ig, was shown to bind to the cell surface of MD cell line cells activated with IFN-γ, suggesting that MD tumors also use the PD-1/PD-L pathway for immune evasion.

In order to clarify the roles of PD-1-Ig in tumorigenesis by MDV, cells were isolated from tumor lesions of a chicken with clinical MD, and cultured in the presence of PD-1-Ig. Although blockade of the inhibitory signal from PD-Ls is known to induce tumor-specific T cells to effectively reject neoplastic cells, promoted cell proliferation and increased MDV load were observed in these cells cultured with PD-1-Ig in this study. The reason for these unexpected findings is still unclear, but may be due to the fact that both the target cells for MDV transformation and for activation by PD-1-Ig are T cells. Thus, PD-1-Ig showed the ability to block the inhibitory signals provided by PD-Ls on neighboring cells. In addition, a positive signal via PD-L1 by PD-1 was reported by a few groups and could be involved in this observation.

It has been reported that the expression levels of *PD-1*, *PD-L1* and *PD-L2* are increased in chickens infected with virulent MDV; however, in this study, upregulation was not observed in vaccinated or vaccinated chickens challenged with virulent MDV. This result suggests that the immunoinhibitory signal provided by the PD-1/PD-Ls pathway could contribute to the anti-tumor effect of MD vaccines.

MDV disrupts the host immune response intricately after infection, and the mechanism of its immune regulation is complicated. Further studies would be desirable to identify the viral or host factors related to the immune evasion of MDV and the anti-tumor effect of MD vaccine. This would be informative to develop new methods for the prevention of and therapy for a variety of chronic infections and neoplastic diseases.

Identification and functional analysis of an immunosuppressant molecule, sL2, in *Ixodes persulcatus* ticks

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Ixodid ticks are harmful vectors of several pathogens causing infectious diseases and afflicting human public health. As vectors, these ticks are known to transmit *Borrelia burgdorferi*, one of the *Spirochaetaceae*, causing Lyme disease. These economically important ticks can be controlled by the use of acaricides, but these can cause many problems, for example, environmental/food contamination and the emergence of acaricide-resistant ticks. Thus, recent studies have focused on the development of new strategies to control ticks, including an

anti-tick vaccine that blocks blood feeding and the oviposition of ticks. There have been several reports on the identification and characterization of tick molecules as effective targets for the anti-tick vaccine.

Sialostatin sL2 (sL2) is a tick salivary gland protein, derived from *Ixodes scapularis*. sL2 inhibits the activation of cysteine protease and has anti-inflammatory activity and suppressive action on cytotoxic T cells. It was reported that the number of ticks attached to hosts and the amount of blood-feeding were significantly reduced in hosts vaccinated with recombinant sL2 derived from *I. scapularis* as compared to control unvaccinated hosts, showing that sL2 can be a candidate molecule for an anti-tick vaccine. In Japan, *I. persulcatus* is a major vector of Lyme disease, but little information is available on the molecules, including sL2, of this tick. Thus, in this research, sL2 homologues were identified in *I. persulcatus*, and their biological functions were studied.

Two cDNA clones, whose nucleotide and deduced amino-acid sequences show high similarity to those of *sL2* of *I. scapularis*, were isolated from salivary glands of fed female *I. persulcatus* ticks and designated as *sL2*-1 and *sL2*-2. Reverse transcriptase PCR analysis showed that sL2-1 was expressed specifically in the salivary glands, while *sL2*-2 was in the whole body, and these genes were expressed throughout the life cycle stages of the ticks. In addition, *sL2*-1 and *sL2*-2 were expressed even before feeding and were continuously expressed while feeding the ticks.

To investigate the function of sL2, recombinant sL2 (rsL2) was prepared: Nus/rsL2-1 is expressed in *Escherichia coli* and FLAG/sL2-2 is expressed in COS-7 cells, respectively. Nus/sL2-1 was recognized by tick-infested hamster sera, determined by Western blotting, indicating that sL2 was the secretory protein exposed to the host animals. In addition, FLAG/sL2-2 appeared to inhibit IFN-γ production from bovine PBMCs stimulated with Concanavalin A.

Thus, *I. persulcatus* has factors similar to sL2 of *I. scapularis*, and this molecule seems to be involved in tick-feeding and the transmission of pathogens by suppressing host immune responses such as the production of cytokines. Using *I. persulcatus* rsL2, it will be necessary to further investigate its functions, such as the inhibition of other cytokine production and the proliferation of immune cells in the hosts. This research will be required to develop an effective vaccine to control *I. persulcatus* in the field.

Clinical usefulness of Transcranial Doppler ultrasound in dogs

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Transcranial Doppler ultrasound (TCD) is a noninvasive modality used to evaluate intracranial circulation by measuring cerebral blood flow; however, little is known about the clinical usefulness of TCD in veterinary medicine. The purpose of this study was to clarify the clinical usefulness of TCD for the evaluation of canine intracranial diseases.

Firstly, the repeatability of TCD variables in the basilar artery was investigated in six normal dogs. The variability of the resistance index (RI), pulsatility index (PI), and the ratio of systolic to

diastolic mean velocity (Sm/Dm) were satisfactory in conscious and anesthetized dogs. Ultrasound contrast media (microbubbles) enhanced Doppler signals from cerebral arteries. It was indicated that contrast medium caused higher values in recorded velocities, but no changes in RI, PI, and Sm/Dm.

Secondly, 50 clinical dogs with neurological symptoms were enrolled in this study to evaluate the association between TCD measurements and MRI findings. Based on MRI findings, 17 dogs were categorized into Group I (no abnormal findings), 20 dogs into Group II (intracranial structural disease without suggestive findings of intracranial hypertension), and 10 dogs into Group III (intracranial structural disease with suggestive findings of intracranial hypertension). RI, PI, and Sm/Dm were significantly higher in Group III than in Group I and Group II. In particular, high Sm/Dm was strongly related to findings suggestive of intracranial hypertension. On the other hand, there were no differences in TCD variables between Group I and Group II.

In conclusion, these results show that TCD has adequately high repeatability in dogs. Furthermore, TCD measurements of RI, PI, and Sm/Dm may be valuable to evaluate intracranial hypertension in dogs with intracranial diseases.

Induction and detection of canine T helper 17 cells isolated from peripheral blood mononuclear cells

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T helper (Th) 17 cells are $CD4^+$ T cells characterized by their production of the pro-inflammatory cytokine interleukin-17A (IL-17A). Th17 cells are thought to play important roles in various inflammatory and immune-mediated disorders in humans and mice. Differentiation of murine Th17 cells from naïve T cells is controlled by transforming growth factor (TGF)-β and IL-6; however, there are contradictory reports as to the role of TGF-β in the differentiation of human Th17 cells and very little is known about the mechanism of Th17 cell differentiation in dogs. The aim of this study was to induce and detect canine Th17 cells from peripheral blood mononuclear cells (PBMC).

Firstly, PBMC was stimulated for 72 hours with anti-CD3 antibody, anti-CD3 antibody + IL-2 or PMA + ionomycin. IL-17A mRNA expression in activated PBMC was analyzed by quantitative real-time PCR (qRT-PCR). Among these three culture conditions, IL-17A mRNA expression was most increased in PBMC stimulated with anti-CD3 antibody + IL-2.

Next, the effect of TGF-β and IL-6 on canine Th17 cells differentiation was evaluated. PBMC was stimulated with anti-CD3 antibody + IL-2 for 24 or 72 hours in the presence or absence of TGF-β and IL-6. PBMC was also stimulated with PMA + ionomycin for the same incubation period. IL-17A mRNA and protein expression in activated PBMC were analyzed by qRT-PCR and ELISA. TGF-β and IL-6 were unable to induce IL-17A mRNA and protein expression with 24-hour and 72-hour incubation periods. IL-17A mRNA expression was higher in PBMC stimulated with anti-CD3 antibody + IL-2 than with PMA + ionomycin in both incubation periods. In contrast, IL-17A protein expression was more abundant in supernatants from PBMC stimulated with PMA + ionomycin when

compared anti-CD3 antibody + IL-2 at 24 but not 72 hours.

Lastly, canine Th17 cells in PBMC stimulated with anti-CD3 antibody + IL-2 or PMA + ionomycin for 24 hours were detected by flow cytometry using anti-human IL-17A monoclonal antibody. The percentage of $CD4^+$ cells producing IL-17A was increased after stimulation under both culture conditions.

In the present study, canine Th17 cells were successfully induced from PBMC with anti-CD3 antibody + IL-2 stimulation regardless of the presence of TGF-β and IL-6. In addition, canine Th17 cells were successfully detected by flow cytometry. These methods are useful for analysis of the roles of Th17 cells in the pathogenesis of canine inflammatory and immune-mediated disorders.

Uptake of diminazene aceturate in diminazene aceturate-resistant *Babesia gibsoni* isolate *in vitro*

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Diminazene aceturate (DA) is the first-line agent for the treatment of canine babesiosis; however, the development of DA-resistant *Babesia gibsoni* isolates has been reported recently, making treatment of this disease difficult. In *Trypanosoma* spp. the loss of P2 nucleoside transporter, which is an adenosine transporter, in a DA-resistant strain was reported. Due to structural similarities between DA and adenosine, it is possible that loss of this P2 nucleoside transporter could result in the reduction of DA uptake. However, adenosine transporters such as the P2 nucleoside transporter have not been investigated in *B. gibsoni*, and their role in the development of DA resistance has not been elucidated. In the present study, the amount of DA and adenosine uptake were measured in wild-type and DA-resistant isolates of *B. gibsoni*.

Firstly, DA amounts in the wild type, which was cultured in 200 ng/ml DA for 24 hours, and the DA-resistant isolate, which was cultured in 200 ng/ml DA continuously, were measured by high performance liquid chromatography. The results showed that the DA amount in the DA-resistant isolate was significantly lower than in the wild type.

Secondly, to clarify the mechanism of the reduction of DA in the DA-resistant isolate, [^{3}H] adenosine was added to cultures of the wild-type and DA-resistant isolates, and [^{3}H]adenosine uptake was observed using a liquid scintillation counter. The results showed that adenosine uptake by the DA-resistant isolate was higher than by the wild type, suggesting that the activity of adenosine transporter in the DA-resistant isolate was not suppressed.

Finally, to observe direct inhibition of the adenosine transporter by DA, the wild type was exposed to various concentrations of DA, and its adenosine uptake was measured. Although the number of parasites decreased at a high concentration of DA, adenosine uptake was not affected, indicating that DA could not directly inhibit the adenosine transporter in *B. gibsoni*.

In the present study, it was shown that the DA amount in the DA-resistant *B. gibsoni* isolate was reduced; however, its adenosine uptake was not suppressed. These results suggest that the adenosine transporter in *B. gibsoni* is not

involved in the mechanism of DA resistance, and that *B. gibsoni* could have obtained DA resistance through mechanisms different from *Trypanosoma* spp. Further investigations of the cause of the reduction in DA in DA-resistant *B. gibsoni* isolate are necessary.

Therapeutic and diagnostic usefulness of oncoprotein DEK in canine bladder transitional cell carcinoma

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Canine transitional cell cancer (TCC) is one of the most common bladder tumors encountered in dogs. Identification of a molecular target for early diagnosis and new therapy for this tumor are expected. To identify a new therapeutic target gene for canine TCC, highly expressed genes in human TCC were selected and their usefulness as molecular targets was evaluated.

First, three potential therapeutic genes, DEK, Pim-1 and Survivin, were screened by RT-PCR using four canine TCC cell lines. DEK mRNA was the only gene detected in all cell lines.

Secondly, DEK mRNA expression in normal tissues was screened by RT-PCR, and no expression was detected in normal bladder tissue. Also, DEK protein expression in normal bladder tissues, cystitis and TCC tissues were analyzed by immunohistochemistry. The DEK-positive cell rate was significantly higher in TCC (71.7%) than in normal bladder (0.6%). On the other hand, cystitis tissues showed a relatively high DEK-positive cell rate (51.1%).

Thirdly, to evaluate the roles of DEK in cell viability, apoptosis and chemosensitivity in canine TCC, the influence of DEK siRNA transfection into three canine TCC cell lines was evaluated. The DEK mRNA and protein expression were downregulated in all cell lines by siRNA. Apoptosis was detected by Annexin V staining and highly induced by transfected cells. Cell proliferation and attenuated chemoresistance to carboplatin were evaluated by MTT assay and significantly high cell growth inhibition was detected.

These results suggested that DEK is a potential molecular target gene in canine bladder TCC. Further investigation using a transplantation animal model and ideal combination drug application is expected. Also, detection of DEK expression in the inflammatory environment may be useful to identify new diagnostic and therapeutic options for canine TCC.

Characterization of phenotypical changes in cultured equine chondrocytes by inflammatory stimulus

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It is very difficult for cartilage to maintain its phenotype during cell culture. Although hyaline cartilage cultured with IL-1β has been considered an in vitro model of osteoarthritis, it is said that its phenotype changes to that of fibrocartilage without particular stimulation, especially in monolayer culture.

The objective of this study was to investigate whether phenotypical changes occur during simple culture without IL-1β. We also investigated the effect of CTGF, a new type of drug for osteoarthritis, using markers of cartilage metabolism.

Cartilage was sampled from two skeletally normal Thoroughbred horses, from which equine cartilage cell cultures were obtained. We first confirmed the expression of hypoxia-inducible factors 1 alpha and 2 alpha (HIF-1α, HIF-2α), collagens type I alpha 1, II alpha 1 and X alpha 1 (COL1A1, COL2A1, COL10A1), aggrecans, runt-related transcription factor-2 (Runx2), vascular endothelial growth factor (VEGF) and connective tissue growth factor (CTGF).

Second, the expression of a fibrocartilage marker COL1A1 and hyperprophic markers HIF-2α, COL10A1, and Runx2, a hyaline cartilage marker COL2A1 and a new angiogenesis factor VEGF A were evaluated in in vitro culture for 96 hours. In addition, the effect of IL-1β and CTGF on expression levels of these markers was examined. Expressions of HIF-2α, Runx2 and COL10A1 increased with incubation time and the addition of IL-1β. Expressions of Runx2 and COL10A1 decreased by adding CTGF.

Although phenotype of hyaline cartilage was thought to change into that of fibrocartilage with monolayer culture, findings of this study suggest that phenotype of hyaline cartilage could change to that of hypertrophic cartilage with monolayer culture. Therefore, it is suggested that monolayer culture of chondrocytes with IL-1β might serve as a model of osteoarthritis.

Measurement of intracellular ferrous content in canine histiocytic sarcoma cells and evaluation of cytotoxicity of dihydroartemisinin against canine histiocytic sarcoma

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Canine histiocytic sarcoma (CHS) is an aggressive malignancy of antigen-presenting dendritic cells or phagocytic macrophages. CHS has metastatic potential and its prognosis is poor

owing to widespread disease at the time of presentation. Chemotherapy has shown some efficacy in slowing disease progression. More effective antitumor drugs are desired to improve the quality of life and expected life span of affected animals. Dihydroartemisinin (DHA) is a derivative of Artemisinin that was isolated from a plant, *Artemisia annua*. It has been widely used as an antimalarial compound and has cytotoxicity against various tumor cell lines. The active endoperoxide bridge in its structure reacts with iron to form free radicals. Compared with the corresponding normal cells, cancer cells have a higher iron influx rate, which makes them more susceptible to the cytotoxic effect of DHA. CHS is expected to contain a high amount of iron and to be damaged effectively by DHA. The aim of this study was to measure the amount of iron in CHS and to evaluate cytotoxic effects of DHA on CHS cell lines.

The amount of iron in CHS tissue was measured by ICP-MS. CHS tissue was also stained by Berlin blue to detect ferric ion. It was shown that CHS tissue contained much more ferric ion than other tumor tissue.

The potentiating influence of the cytotoxicity of DHA by ferric ion was evaluated by MTT assay. CHS cells were damaged effectively by DHA in DMEM with higher iron concentration. This cytotoxicity was inhibited by chelating iron with deferoxamine. These results suggested that DHA should be further evaluated as a novel therapeutic agent for CHS.

Pathogenicity and prevalence of fowl glioma-inducing avian retroviruses considered to be derived from *ev*-1

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Fowl glioma-inducing virus (FGV) belongs to avian leukosis virus subgroup A (ALV-A). Whereas ALVs generally induce hematopoietic neoplasms in chickens, FGV induces fowl glioma, perineurioma and cerebellar hypoplasia. Recently, ALVs considered to be derived from endogenous retrovirus *ev*-1 have been isolated from Japanese fowl affected with fowl glioma in Kumamoto. In Chapter I, in order to evaluate the pathogenicity of Km_5666 strain, which is one of these isolates, C/O specific-pathogen-free chickens were inoculated *in ovo* with 5×10^4 TCIU of Km_5666 or culture medium as a negative control. Km_5666 induced perivascular lymphocytic infiltration (five of 12 birds; 42%), periventricular proliferation of immature round cells (12%), mild astrogliosis (25%) and fowl glioma (12%). Glioma was recognized only in 140-day-old chickens and these brain lesions were milder than those induced by Sp strains in the previous study (Avian Pathology 40: 499–505, 2011). Two (12%) of the inoculated chickens also revealed morphological abnormalities of myocardial fibers. Intracerebral viral RNA levels of Km_5666 were lower than those of Sp-40 at any examined age. These results suggest that Km_5666 has the ability to induce fowl glioma but its pathogenicity is lower than that of Sp strains and this strain also could induce myocardial abnormality. The difference in pathogenicity to the nervous system was considered to be related to the replication rate within the infected brains. In Chapter II, the prevalence of fowl glioma-inducing ALVs derived from *ev*-1 in Japanese fowl in Kumamoto

and Yamaguchi was examined by viral isolation and sequence analysis of isolates. Fifteen (21%) of 73 chickens examined histopathologically had fowl glioma. Viral isolation was performed in 151 samples, including 121 chickens and 22 eggs in Kumamoto and 8 chickens in Yamaguchi, and 55 strains (36%) were totally isolated from them. Twenty-nine (52%) of these isolates lacked an FGV-specific region. Phylogenetic analysis based on the regions of *gag*, *pol*, *env*SU and 3'LTR showed that all 12 isolates in Kumamoto grouped together in a cluster different from the cluster of FGV. The *gag* and *pol* regions of the 12 isolates had 98–99% identity to those of *ev*-1. In contrast, the *pol* regions of 2 isolates in Yamaguchi had 99% identity with that of *ev*-1 but the *gag* regions showed high identity (95%) to that of Rous sarcoma virus. These results in Chapters I and II suggest that fowl glioma-inducing ALVs showing high similarity to *ev*-1 are mainly prevalent among Japanese fowl in Kumamoto.

Enhancing effect of antibody production against rabies virus by uridine 5'- triphosphate in mice

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Extracellular nucleotides, such as adenosine 5'-triphosphate (ATP) and uridine 5'- triphosphate (UTP) are known to act as "find-me" signals for phagocytic cells, bind membrane-associated P2 purinergic receptors, and induce various physiological reactions. In this study, the production of antibody in mice immunized by inactivated rabies vaccine containing nucleotides was investigated. Injection of inactivated rabies vaccine with UTP induced significantly higher serum antibody production in mice, which was not observed by ATP inoculation. The enhancement of antibody production by UTP was mediated by P2Y4 receptor stimulation. In an air-pouch experiment, the infiltration of monocytes and macrophages was increased and gene expression of IL-4 and IL-13 was upregulated in the regional lymph nodes by UTP treatment. These results suggested that UTP added to vaccine enhanced the activation of Th2 cells and the humoral immune response. It was also indicated that UTP could directly activate immune cells in an *in vitro* experiment. Survival rate of UTP-treated mice before rabies virus challenge was slightly higher than that of mice without UTP treatment. In conclusion, UTP, one of the "find-me" signals, can enhance antibody production against rabies virus in mice when used as a vaccine adjuvant.

Effect of antibodies on the clearance of rabies virus from virus-infected cells

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Rabies is a lethal zoonotic disease that is caused by rabies virus infection. No effective therapy is available and the mortality rate is extremely high after showing neurological signs. A high titer of virus-neutralizing antibody has been detected in the cerebrospinal fluid of a few survivors of rabies, but it is still unclear how the clearance of viruses from infected neurons occurs. In the present study, the proliferation of rabies virus was analyzed after the addition of anti-rabies virus serum or anti-rabies virus antibody to virus infected-cultured cells and the localization of the antibody was examined. By incubation with anti-serum or antibody, the foci of virus-infected cells were localized in a small area and the number of virus-positive cells was significantly decreased. Positive signals of immunoglobulin G were observed in virus-infected cells and some were co-localized with virus antigens near the cell membrane. The amount of viral structural protein released into cultured cell media was significantly reduced by antibody treatment. These results suggested that the anti-rabies virus antibody could inhibit the spread of the virus (neutralization) and also inhibit the budding of progeny viruses from the cell surface. Further, the possibility of virus digestion within infected cells by prolonged antibody treatment was suspected, but the detailed mechanism of intracellular neutralization could not be elucidated.

Evaluation of frozen-thawed bovine sperm by flow cytometry

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Flow cytometry is rapid and objective for assessing the membrane function of bovine sperm. This study attempted to develop a quartet staining method that simultaneously assessed sperm viability, acrosome integrity and mitochondrial membrane potential using SYBR-14, propidium iodide (PI), phycoerythrin-conjugated peanut agglutinin (PE-PNA) and MitoTracker Deep Red (MTDR).

By fixing sperm with formaldehyde, the fluorescence of SYBR-14, PE-PNA and PI was maintained for 2 hr. Since the characteristics of sperm evaluated by flow cytometry and fluorescein microscopy were similar, flow cytometry was proved to not detect debris as sperm. Also, adding MTDR to the triple staining method enabled an accurate assessment of sperm viability, acrosome integrity and mitochondrial membrane potential simultaneously, since there was no interference between the fluorochromes.

The quartet staining method was applied to evaluate the quality of post-thaw sperm obtained

from 10 bulls. The rate of sperm that was viable, and had an intact acrosome membrane and high mitochondrial membrane potential correlated with the rate of sperm with rapid progressive motility.

In conclusion, the quartet staining method can simultaneously assess three sperm membrane functions accurately.

Effects of follicle-stimulating hormone and bone morphogenetic protein-4 on in vitro growth of bovine oocytes derived from early antral follicles

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In mammalian ovaries, there are a large number of growing oocytes, most of which degenerate during the growth phase of follicles. Establishment of an in vitro growth (IVG) culture system for growing oocytes will enhance the production of superior domestic animals. However, maturational and developmental competences of bovine IVG oocytes derived from early antral follicles are lower than those of oocytes grown in vivo. Moreover, in vitro steroidogenesis of oocyte-granulosa complexes (OGCs) from early antral follicles is not clear. Follicle-stimulating hormone (FSH) and/or bone morphogenetic protein-4 (BMP-4) are known to be essential for the growth of follicles. Therefore, in the present study, the effects of FSH and/or BMP-4 on oocyte growth, maturational competences and steroidogenesis of OGCs were investigated.

FSH and/or BMP-4 were added to the growth culture medium on Day 4 of IVG culture, and OGCs were cultured for a further 8 days. As a result, FSH did not affect the survival and growth of IVG oocytes, but BMP-4 decreased them. Although the germinal vesicle (GV) stage of oocytes was GV0–1 before IVG culture, more than 50% of oocytes arrived at GV2–3 stages after IVG culture, regardless of FSH and/or BMP-4 addition. In the presence of FSH and BMP-4, the GV2–3 stage rate (90%) of oocytes was equal to in vivo-grown oocytes. After maturational culture, regardless of FSH and/or BMP-4 addition, 70–80% oocytes reached metaphase II (MII). In the presence of BMP-4 alone, the MII rate (81%) of oocytes was equal to that of in vivo-grown oocytes (92%). Estradiole-17β (E_2) production of OGCs increased until Day 8, but decreased thereafter, regardless of FSH and/or BMP-4 addition. In the presence of FSH and/or BMP-4, OGCs produced less E_2 than without them. Progesterone (P_4) production of OGCs increased throughout the culture period. Addition of FSH enhanced OGCs to produce P_4. E_2/P_4 ratio in culture medium tended to be low with the addition of FSH, but high with BMP-4.

The present study demonstrated that addition of FSH tended to reduce the E_2/P_4 ratio in culture medium, but promoted the progress of the GV stage of IVG oocytes. Addition of BMP-4 decreased oocyte viability and growth, but tended to raise the E_2/P_4 ratio in culture medium, and improved the MII rate, which was equal to that of in vivo-grown oocytes.

Determination of a candidate gene for HK red cell phenotype in Japanese dogs

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Canine red cells normally show a low intracellular K^+ (LK) phenotype; however, some Japanese dogs have high intracellular K^+ (HK) red cells as an autosomal recessive phenotype. Since these cells do not lose Na,K-ATPase completely during maturation and exhibit similar characteristics to reticulocytes, terminal differentiation of HK red cells is thought to be impaired by a single HK causative gene. In the present study, we determined that *TSPO2* was a candidate gene for the HK phenotype by a genome-wide association study using canine SNP chips. Subsequent sequencing analysis showed that dogs from two distinct pedigrees possessed two different alleles with mutations in *TSPO2* that resulted in amino acid substitutions: one allele had C40Y mutation and the other allele contained triple mutations V89F/ΔF98/T120I. Analysis of genomic DNA demonstrated that the dogs possessing HK red cells were homozygotes for the C40Y allele or compound heterozygotes for the C40Y and V89F/ΔF98/T120I alleles. Wild-type TSPO2, C40Y TSPO2, and V89F/ΔF98/T120I TSPO2 were similarly localized in the endoplasmic reticulum and the nuclear membrane in transiently transfected HEK293 cells. V89F/ΔF98/T120I TSPO2 was a more labile than the wild type and C40Y TSPO2 when transfected cells were exposed to cycloheximide. In stable transfectants of K562 cells, TSPO2 expression was transiently increased when the cells were induced into the erythroid lineage by hemin. The subsequent decline in the TSPO2 level was faster in cells expressing the V89F/ΔF98/T120I mutant than in cells expressing wild-type or C40Y TSPO2. The intracellular deposition of free cholesterol was evident in K562 cells expressing wild-type TSPO2 but not in cells expressing C40Y or V89F/ΔF98/T120I mutants. Furthermore, TSPO2 polypeptides were detected in erythrocytes and reticulocytes from LK dogs but not from HK dogs. These findings suggest that instability of the mutant TSPO2 and consequently impaired cholesterol metabolism during erythroid development and maturation are involved in the molecular cause of the HK red cell phenotype.

Bovine kidney AE1 anion exchanger: analyses of its tissue distribution and basolateral sorting signals

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The kidney form of anion exchanger 1 (kAE1) is known to play pivotal roles in

regulating the acid-base balance by transporting HCO_3^- generated in α-intercalated cells into the circulation in a cooperative manner with the secretion of H^+ by vacuolar ATPase (V-ATPase) in the collecting duct. Recent studies on the molecular pathogenesis of familial distal tubular acidosis in humans due to various AE1 gene mutations revealed that a tyrosine residue Y359 and the C-terminal YXXΦ motif with the sequence Y904DEV are essential for the proper targeting of kAE1 to the basolateral membrane. This finding led us to the assumption that bovine kAE1 has different features in its distribution in renal tubules and membrane polarization, because bovine AE1 lacks tyrosine residue corresponding to Y359 in human kAE1and has phenylalanine instead (F373), while the C-terminal sequence is conserved as the sequence Y923DEV. The purpose of the present study was to determine the localization of bovine kAE1 in the kidney and to examine the roles of the tyrosine residue and the C-terminal motif in polarized sorting of bovine kAE1. Immunofluorescence microscopy demonstrated that kAE1 was present in the cortex and outer medulla and was localized in the basolateral membrane of intercalated cells deficient in the principal cell marker aquaporin 2. In the cortex, intercalated cells possessing kAE1 showed no signals for V-ATPase, whereas the principal cells had apical signals for V-ATPase. By contrast, the cells positive for kAE1 contained V-ATPase in both the apical and basolateral membranes. In transfected cells, wild-type kAE1 (kAE1 WT) showed plasma membrane localization in HEK293 and non-polarized MDCK cells and basolateral localization in polarized MDCK cells. In contrast, bovine kAE1 F373Y displayed a loss of polarized sorting and localized to both apical and basolateral membranes. Moreover, an alanine substitution for Y923 resulted in no change in its basolateral sorting in polarized MDCK cells but caused intracellular retention of the protein. These results demonstrate that bovine kAE1 would have a pivotal role in maintaining the acid-base balance and suggest that the mechanisms for its basolateral sorting and the functional relationship with V-ATPase are different from those in human kAE1.

Studies on clinical pathobiology and molecular basis for Poodle macrocytosis

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Poodle macrocytosis is a congenital disorder with dysplastic features of red blood cells and ineffective erythropoiesis that occurs mostly in Toy and Miniature Poodles. Affected dogs do not have associated clinical signs and are not anemic, while the animals exhibit marked macrocytosis. Although marked dyserythropoiesis and the morphologic changes resemble those associated with megaloblastic anemia due to vitamin B12 or folate deficiency in humans, the nature of the hematopoietic defect has not been defined. The purpose of the present study was to characterize the pathological features in more detail and to find the causative gene for this hematopoietic disorder in a Toy Poodle. The dog had typical macrocytosis with a mean corpuscular volume of 99–106 fl and no anemic manifestations, and a large number of red cells possessed multiple

Howell-Jolly bodies of various sizes. Bone marrow erythroblasts showed megaloblastic morphology, including increased mitosis, karyolysis, binucleation, multinucleation, irregular nuclear shapes, apoptotic nuclear fragmentation, nuclear-cytoplasmic asynchrony, and nuclear bridging. Moreover, neutrophil hypersegmentation was noted. Although these findings were highly representative of pathological changes in folate or vitamin B12 deficiency in humans, serum levels of folate and vitamin B12 and the folate content in red cells were within or higher than the reference range. Red cell membrane proteins in the affected dog showed no significant changes and had no indices for the shortened life span of the red cells. Analysis of the bone marrow cDNAs and genomic DNA in the affected dog showed that several genes for enzymes involved in the folate metabolic pathway, including *SHMT1*, *SHMT2*, and *MTHFD1*, had changes in the nucleotide sequence that led to amino acid substitutions.

Quantification of the virus gene in the follicular fluid of persistently infected cattle with bovine viral diarrhea virus

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Bovine viral diarrhea virus (BVDV) is widespread throughout the world and causes significant economic losses in dairy farms. Persistently infected (PI) cattle, which are the source of BVDV infection in a herd, do not exhibit typical clinical symptoms and it is difficult to detect PI cattle in a herd based on clinical manifestations. Transmission of the virus via reproductive technology, such as semen production and embryo transfer, has been previously reported; however, the detailed mechanism of vertical transmission of BVDV has not been clarified. In the present study, the quantity of BVDV in the follicular fluid, serum and leucocytes of PI cattle was assessed. In addition, the relationship between BVDV distribution and infection of ova, embryos and reproductive disorders was discussed for a BVDV eradication program.

The amount of the virus in follicular fluid, serum and leucocytes was calculated based on the copy number of the BVDV gene using quantitative reverse transcription polymerase chain reaction (RT-PCR). The amount of BVDV per 1 μl follicular fluid was significantly greater than in serum and leucocytes. No significant correlation was recognized between BVDV in the blood and follicular fluid. In 11 of 12 PI cattle, the greatest amount of BVDV was present in follicular fluid. Also, there was no correlation among the quantities of BVDV in each sample and BVDV subgenotype, age, history of pregnancy and the clinical symptoms. On the other hand, a negative correlation was recognized between the amount of BVDV in follicular fluid and the concentration of estradiol in blood. In PI cattle, it might be necessary to investigate other hormonal concerns.

In conclusion, BVDV invaded follicular fluid more than the peripheral circulation in PI cattle. This might influence the direct infection of ova and embryos or reproductive disorders. In addition, in acute infection or injection of live vaccine, after the removal of BVDV from blood by the host immune response, the possibility of BVDV remaining in the follicular fluid should be

considered. This is also important for BVDV control.

Seroepizootiological survey of tick-borne encephalitis in Japan and Russia and the role of interferon antagonist activity in the pathogenicity of tick-borne flaviviruses

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The tick-borne flavivirus group (TBFV) includes several important human and animal pathogens, such as tick-borne encephalitis virus (TBEV) and Omsk hemorrhagic fever virus (OHFV). There is no specific treatment for TBFV or for the control of TBFV infection, so it is important to specify the endemic area and to design an effective vaccination plan. In this study, (i) we conducted a seroepizootiological survey of wild rodents in 3 areas of Japan and the Samara area of Russia to investigate the distribution of TBEV; (ii) we also investigated the role of interferon (IFN) antagonist activity in the pathogenicity of TBFV.

TBEV is a zoonotic disease agent that causes severe encephalitis (tick-borne encephalitis; TBE) in humans. More than 5000 cases of TBE are reported annually in Russia, and the first human case of TBE was confirmed in 1993 in southern Hokkaido; however, surveys of endemic foci of TBEV have been conducted in limited regions. In this study, epizootiological surveys of TBEV were conducted in previously unexamined areas of Japan and Russia. Three hundred seven rodent sera were collected in Shari, Furano and Yamagata in Japan and 151 in Samara in Russia. One *Myodes rufocanus* in Shari, Hokkaido, Japan and one *Apodemus flavicollis* in Samara, Russia were identified to have specific antibodies against TBEV. This is the first study to indicate the presence of endemic foci of TBEV in Shari and Samara. These information will be useful for further analysis and the prevention of TBE.

It has been reported that several flavivirus possess IFN antagonist activity, but the role of this activity in the pathogenicity of tick-borne flaviviruses is not well understood. In this study, we tried to construct viruses with reduced IFN antagonist activity and investigated their pathogenicity.

In NA cells treated with IFN-α, infection with TBEV or OHFV resulted in reduction of the activation of interferon-stimulated regulatory elements (ISRE) promoter, and inhibition of the phosphorylation and nuclear transport of STAT1. Similar results were obtained in cells expressing the non-structural 5 (NS5) protein of TBEV or OHFV, indicating that NS5 is a critical element in the suppression of IFN-mediated JAK-STAT signaling. An alanine substitution was introduced at amino acid position 380 of the NS5 protein of TBEV and OHFV (380A), and the characteristics of their IFN antagonist activity was compared with those of the wild-type (wt) viruses. IFN-α treatment reduced the production of the 380A virus, while that of the wt viruses was not affected. The inhibitory effects of the 380A viruses on the JAK-STAT signaling pathway were markedly reduced compared with those of the wt viruses.

In a mouse model, subcutaneous infection with the 380A virus of TBEV Oshima 5-10 or

OHFV resulted in lower mortality than with the wt virus. Similar reduced pathogenicity was observed in intracranial infection with the 380A virus of TBEV Oshima 5-10. In mice infected subcutaneously with the 380A virus of TBEV Oshima 5-10, a low level of viremia and delayed viral replication in the brain were observed as compared with those infected with the wt virus. This low replication in organs could contribute to the reduced pathogenicity in mice.

In summary, our results demonstrated that the reduction of IFN antagonist activity induced the attenuation of tick-borne flavivirus *in vivo*. These findings will be useful for further study to elucidate the mechanism of the pathogenesis of tick-borne flaviviruses and to develop a live attenuated vaccine and efficient treatment agent targeting the IFN antagonist activity of tick-borne flavivirus.

Analysis of type I interferon responses to hantavirus infection in MRK101 cells derived from the kidney of the grey red-backed vole (*Myodes rufocanus bedfordiae*)

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Hantaviruses are maintained in Rodentia, Soricomorpha, and Chiroptera species in nature, and cause human diseases such as hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS). Hantaviruses are asymptomatic and persistent infections in wild rodents. The mode of virus infection and innate immune responses in host rodents remain to be elucidated. Hokkaido virus (HOKV), one of the hantaviruses, is carried by the grey red-backed vole (*Myodes rufocanus bedfordiae*), and MRK101 cells have been established from the kidney of *M. rufocanus*.

In this study, type I interferon (IFN) responses following hantavirus infection in MRK101 cells were investigated. The nucleotide sequences of *Ifna*, *Ifnb*, *Mx2*, *Oas1*, *Isg15*, and *Actb* genes in MRK101 cells were cloned and determined. Poly (I: C) transfection and Sendai virus (SeV) infection of the cells induced the expression of type I IFN- (IFN-α, IFN-β) and IFN-stimulated genes (ISGs: Mx2 OAS1, ISG15). Next, cells infected with hantaviruses, including HOKV, Puumala virus (PUUV), and Seoul virus (SEOV), were analyzed for the mRNA expression levels of IFN-α, IFN-β and Mx2 at 6, 12, 24, 48, 72, and 96 hours post-infection. HOKV infection induced IFN-α, but not IFN-β and Mx2, with a low level of progeny virus replication. In contrast, infection with PUUV and SEOV induced IFN-β and Mx2 with an increased level of virus replication. Furthermore, in PUUV- or SEOV-infected cells, Mx2 protein and virus nucleocapsid protein (NP) were partly accumulated in the perinucleus.

The results suggest that (i) type I IFN responses in MRK101 cells are intact, and (ii) the mode of type I IFN responses varies depending on the infected hantaviruses. The MRK101 cell line can be a useful tool for studying various virus infections in the cells of host rodents, including hantavirus infection and innate immune responses in host rodents.

Mechanism of cell protective effect by mitochondrial overexpression of DNA repair enzyme apurinic/apyrimidinic endonuclease 1

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The increase of oxidative stress accompanied by the increase of reactive oxygen species (ROS) *in vivo* damages various biomolecules, leading to the reduction of cell viability. Mitochondria are known to be involved in the regulation of oxidative stress as a source as well as a target of ROS. Although it has been reported that mitochondrial overexpression of apurinic/apyrimidinic endonuclease 1 (APE1), a protein with dual functions of DNA repair and redox regulation, shows a protective effect against oxidative stress, its mechanism has not been determined yet; therefore, this study aimed to elucidate the cell protective mechanism of mitochondrial APE1 against oxidative stress.

Three different expression vectors for mitochondria-targeting APE1 – wild-type (mtAPE1), redox activity-deficient (C65S), and endonuclease activity-deficient (E96A) – were generated and introduced into b.End3 cells. When cell viability after H_2O_2 treatment was evaluated, it was found that the expression of mtAPE1 showed a protective effect against H_2O_2, and that both functions of redox activity and endonuclease activity in APE1 were required for this protective effect. Because recent studies have indicated that the redox status in mitochondria is important for determining cellular susceptibility against oxidative stress, it was analyzed in b.End3 cells overexpressing mitochondrial APE1 variants. It was revealed that the expression of mtAPE1 suppressed mitochondrial oxidation after H_2O_2 treatment. On the other hand, this effect was not observed in cells expressing of C65S or E96A. Furthermore, to evaluate the effect of mtAPE1 overexpression for mitochondrial DNA (mtDNA) damage after H_2O_2 treatment, it was analyzed using quantitative PCR. Although the expression of all mtAPE1 variants reduced mtDNA damage after H_2O_2 treatment, C65S and E96A were less effective than mtAPE1 to mitigate H_2O_2-induced mtDNA damage.

In summary, the overexpression of APE1 in mitochondria showed a cell protective effect against oxidative stress, and it was suggested that this effect was due to mtDNA protection by both endonuclease activity and redox activity in APE1. Because mitochondrial dysfunction is known to participate in the pathophysiology of diseases such as diabetes and myocardial infarction, the results of this study might be helpful to prevent and to develop treatment for these diseases.

Radioprotective effect of hydrogen sulfide in Chinese hamster V79 cells

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It has been demonstrated that gaseous molecules, such as nitric oxide and carbon monoxide, have various physiological effects. In addition, hydrogen sulfide (H_2S) was recently discovered as the third endogenous gaseous molecule. Although it has been reported that one of the physiological effects of H_2S is to protect cells from radiation-induced cellular damage, its mechanism remains unknown. This study aimed to reveal the effects and the mechanism of H_2S for radiation-induced cellular damage.

To examine whether pretreatment with a slow-releasing H_2S donor, GYY4137, influences radiation-induced cell death, a colony formation assay was performed in Chinese hamster lung fibroblast V79 cells. Pretreatment of GYY4137 for 6 h significantly increased the surviving fraction of V79 cells after irradiation. This result suggested that H_2S released from GYY4137 inhibited radiation-induced cell death. To evaluate the effect of H_2S on radiation-induced DNA double-strand breaks (DSB), we performed a 53BP1 focus formation assay. Pretreatment with GYY4137 for 6h significantly decreased the average number of 53BP1 foci in a cell, indicating the inhibition of radiation-induced DSB formation. The treatment of V79 cells with culture media preincubated with GYY4137 for 6h just before irradiation also decreased the average number of 53BP1 foci in a cell. This result suggested that H_2S released from GYY4137 inhibited radiation-induced DSB formation if it existed in cells and/or the surrounding environment when irradiated. The reaction rate constant of H_2S with ·OH or $\cdot O_2^-$ was evaluated by the electron spin resonance (ESR)-spin trapping method with DMPO and calculated with kinetic competition analysis. The reaction rate constants of H_2S with ·OH and $\cdot O_2^-$ were $1.6 \times 10^{10}\ M^{-1}\cdot s^{-1}$ and $1.5 \times 10^{2}\ M^{-1}\cdot s^{-1}$, respectively. The rate constant of H_2S with ·OH was approximately six times higher than that of ethanol, which is a well-known ·OH scavenger. These results suggested that H_2S was highly effective as a ·OH scavenger, but not as a $\cdot O_2^-$ scavenger.

In conclusion, this study suggested that H_2S had radioprotective effects via the reduction of radiation-induced DSB by scavenging ·OH. It was considered that exogenously and endogenously H_2S acted as a radioprotective factor.

Relationship between tumor radioresistance induced by intermittent hypoxia and cell cycle control

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It is well known that hypoxic cells exist in regions with poor vascularity in solid tumors, however, recent studies reported that fluctuations in oxygen pressure within several hours were generated by unstable blood flow due to vasodilation and vascular occlusion and then induced temporary hypoxia in nearby vessels, so-called intermittent hypoxia. Several studies revealed that intermittent hypoxia contributed to tumor malignant progression by promoting cell migration and angiogenesis. Furthermore, intermittent hypoxia has been shown to make cells more radioresistant than persistent hypoxia in rat glioma C6 cells *in vitro*. In this study, to investigate whether this intermittent-hypoxia-induced radioresistance occurs across multiple cell lines, the effect of intermittent hypoxia on the radiosensitivities was examined in human lung adenocarcinoma A549 cells and mouse squamous carcinoma SCCVII cells, in addition to C6 cells. The difference in biological mechanisms behind tumor radioresistance between intermittent hypoxia and persistent hypoxia was also explored, focusing on DNA repair capacity and cell cycle control.

Reproductive cell death by X-irradiation was assessed by the colony formation assay. C6, A549 and SCCVII cells were exposed to persistent hypoxia for 6 hours or to intermittent hypoxia with 6 cycles of 1-hour hypoxia interrupted by 30-minute oxygenation, followed by X-irradiation under normoxia. In all cell lines, intermittent hypoxia enhanced radioresistance compared with persistent hypoxia or normoxia. Next, to clarify the involvement of DNA repair capacity in intermittent-hypoxia-induced radioresistance, focus formation of γ-H2AX and 53BP1 was examined to measure temporal changes in DNA damage after X-irradiation. However, the result of this experiment did not show any differences among normoxia, intermittent hypoxia and persistent hypoxia. Flow cytometric analysis of the cell cycle demonstrated that intermittent hypoxia increased the fraction of early S phase cells and decreased that of G2 phase cells compared with normoxia and persistent hypoxia. These data suggested that tumor cells treated with intermittent hypoxia achieved radioresistance by decreasing the population of the relative radiosensitive early S phase and increasing that of the radioresistant G2 phase.

In conclusion, the present data showed that tumor radioresistance caused by intermittent hypoxia was observed widely in multiple tumor cell lines, and one of the reasons for this radioresistance was the adjustment of their cell cycle to the radioresistant phase, although further studies are necessary to clarify in more detail the mechanism behind this radioresistance.

Characteristics and interspecies differences in xenobiotic metabolism in fish species: Biophylaxis to polycyclic aromatic hydrocarbon

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The Organization for Economic Cooperation and Development (OECD) test guideline is used as an uniform testing method for chemical risk assessment. In the OECD test guideline, specific fish species are used as standard test organisms; however, even in those test organisms, there is still insufficient information about xenobiotic metabolism. In this research, species differences in the metabolism of polycyclic aromatic hydrocarbons (PAHs) among 14 freshwater fish species were examined.

Animals were exposed to pyrene, which is a typical PAH, for 24 hours. Identification of pyrene metabolites by HPLC and mass spectrometry indicated that pyrene-glucuronide and pyrene-sulfate were major metabolites in all the fish species. The ratio of pyrene-glucuronide to pyrene-sulfate and the total amount of pyrene conjugates excreted from each fish showed clear species differences. For example, although *Colisa labiosa* produced a small amount of pyrene metabolites, *Fundulopanchax gardneri* excreted many more metabolites. There was no correlation between the total amount of excreted metabolites and the liver weight of the fish.

To determine the kinetic parameters of pyrene metabolism in fish, enzymatic activities (cytochrome P450 1A, uridine diphosphate glucuronosyltransferase, sulfotransferase) were measured using liver microsomes and cytosols of *C. labiosa*, *F. gardneri*, *Oryzias latipes* and *Danio rerio*. It was found that kinetic parameters of three enzymes were different among 4 fish species. In particular, *C. labiosa* showed the lowest enzymatic efficiency of CYP1A, UGT and SULT-dependent activities. These results were in accordance with the small excreted amount of pyrene metabolites in *C. labiosa in vivo*. This indicated that both Phase-I and Phase-II metabolic activities potently contributed to making a species differences in the excretion levels of pyrene in fish. Moreover, during the 1-week excretion period for pyrene metabolites, a novel (but unidentified) metabolite was observed only in *C. labiosa*, suggesting that *C. labiosa* has a unique pattern of metabolic reaction compared to other fish species.

As observed above, from this research, it was revealed that there are large differences in PAH metabolism among fish species, even between OECD standard test organisms *D. rerio* and *O. latipes*. It is therefore necessary to clarify inter-species differences of xenobiotic metabolism for ecotoxicological assessments.

Characteristics and species differences of xenobiotic metabolism in birds: comparative metabolism of anticoagulant rodenticide, warfarin and a polycyclic aromatic hydrocarbon (PAH), pyrene

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Various chemicals, such as rodenticides, veterinary drugs, pesticides and environmental pollutants, have caused poisoning incidents in wild birds all over the world. Xenobiotic metabolizing ability is one of the most important factors which determine the sensitivity against chemical substances; however, there is little information about xenobiotic metabolism in birds compared to mammals. Thus, this study focuses on the characteristics and species differences of xenobiotic metabolism in birds compared to mammals.

In this study, warfarin, anticoagulant rodenticide, and pyrene, a representative PAH, were used as model compounds.

I. Warfarin metabolic activity was compared among rats, chickens, crows, ostriches and mallards using liver microsomes. Kinetic parameters were determined and compared with warfarin detoxification ability. Warfarin metabolic activity varied between rats and birds, and even between bird species. 4'-Hydroxylated warfarin was the dominant metabolite in bird species. It has already been reported that the CYP2C subfamily is highly expressed in chicken liver and that CYP2C produces 4'-warfarin in mammals. Thus, it is possible that CYP2C is mainly involved in metabolizing anticoagulants such as warfarin in bird species.

II. Male white leghorn chickens (9 weeks), Japanese quail (1 year) and male Wistar rats (8 weeks) were exposed orally to 4 mg/kg pyrene. After injection, plasma, feces, urine, and bile were collected. Pyrene metabolites in these samples were extracted and analyzed using high-performance liquid chromatography (HPLC) with a fluorescence detector. HPLC with an electrospray ionization ion-trap mass spectrometry detector was used to identify pyrene metabolites. SULT and UGT activities were measured using liver cytosols and microsomes, respectively, and kinetic parameters were determined. We could detect seven and six metabolites each in bird and rat excreta, respectively. The ratio of each metabolite differed greatly between birds and rats. Interestingly, one of the seven metabolites, pyrenediol diglucronide, was identified as a unique metabolite in bird species. In bird plasma, pyrene-1-sulfate (PYOS) was a dominant metabolite, on the other hand, bird excreta contained a relatively large amount of other various metabolites. The ratio of glucuronidated conjugates in bird excreta was larger than in rat excreta. Moreover, UGT activity in rats was significantly higher than in birds. This implied that the kidneys have the ability to metabolite pyrene or that the excretion tendency differs depending on each metabolite because of substrate affinity.

In conclusion, this study showed great differences in the metabolism of birds compared to rats, and even among bird species. This suggests that the xenobiotic metabolism of birds has unique characteristics. These species differences may influence the chemical sensitivity of birds.

Reproductive characteristics of feral, female raccoons (*Procyon lotor*) in Hokkaido, based on megascopic and histological observation of genitals

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The aim of this study was to reveal the reproductive characteristics of feral, female raccoons (*Procyon lotor*), an invasive species in Hokkaido, mainly by histological observation of the ovaries. We attempted to elucidate factors effecting the fecundity of feral, female raccoons in order to better understand population dynamics.

We examined the morphological measurements, reproductive status (pregnant or non-pregnant, and whether they had delivered or not in the latest breeding season), and observed the ovaries of 511 feral, female raccoons, which were captured from March 2008 through September 2012. We also compared reproductive characteristics between yearlings and adults (> 2 years old).

In 2011, the mean pregnancy rate was 59.2% among yearlings and 94.3% among adults. In 2011, the litter size ranged from 1 to 7 offspring, and the mean was 3.8 among yearlings and 4.2 in adults. We examined the pregnancy rate and the litter size only in 2011. From 2008 through 2012, the number of embryos or fetuses ranged from 1 to 7, and the mean was 3.6 among yearlings and 3.6 among adults. From 2008 through 2012, the number of ovulations ranged from 2 to 7 in pregnant females, and the mean was 4.2 among yearlings and 4.5 among adults. From 2008 through 2012, the implantation rate varied from 33.3% to 100%, and the mean was 85.6% among yearlings and 79.2% among adults. The pregnancy rate among yearlings was significantly lower than that in adults ($P < 0.001$); however, the litter size, number of embryos or fetuses, number of ovulations, and the implantation rate were not significantly different between yearlings and adults. This suggests that the fecundity of yearlings was equivalent to adults. From 2008 through 2012, the body size of non-reproductive yearlings that were non-pregnant and nulliparous was smaller than that of yearlings that were pregnant or had delivered in the latest breeding season. This suggests that non-reproductive yearlings had not sufficiently developed to reproduce. Concerning the mating season, we found that female raccoons could mate from January through June, and the peak of the mating season was from late February through early March. Fecundity of yearlings was equivalent to that of adults and the long mating season is thought to be a factor allowing raccoons to rapidly increase in number in Hokkaido. Concerning histological changes of the ovaries, we found that the corpus luteum in female raccoons begins regression during the gestation period.

Seasonal modulation of energy metabolism in skeletal muscle of captive female Japanese black bears (*Ursus thibetanus japonicus*)

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Concerning seasonal changes in the energy metabolism of bears, considerable research on the liver and fat have been reported as functions of energy generation and energy storage, respectively; however, little has been studied concerning energy consumption. In this study, we focused on skeletal muscle, which is thought to require a large amount of energy and prevent significant muscle atrophy during hibernation. In order to clarify the seasonal change of energy metabolism and the unique mechanism of muscle atrophy prevention during hibernation, we measured plasma component levels and analyzed the gene expression of energy metabolism-related enzymes in the skeletal muscle of female, Japanese black bears with real-time RT PCR.

Plasma total protein, creatinine, total cholesterol, triglycerides and ketone body levels in March (during hibernation) were significantly higher than in June (during the active phase) and November (during hiperphagia) ($P < 0.05$). In contrast, plasma BUN in March was significantly lower than in June ($P < 0.05$). Plasma glucose levels in March were similar to June levels ($P < 0.05$).

In skeletal muscle, the mRNA expression of FAS and ACC1, related to lipid synthesis, was significantly decreased in March ($P < 0.05$) and that of AQP7, CD36 and UCP2, which is related to skeletal muscle intracellular uptake of lipid and fatty acid oxidation, was conversely and significantly increased in March ($P < 0.05$). On the other hand, the mRNA expression of PKM and PC, which is related to glycolysis, was significantly decreased in March ($P < 0.05$), but that of PDK4 and LDH-B was significantly increased in March ($P < 0.05$). Concerning ketone body metabolism, SCOT mRNA expression was significantly decreased in March ($P < 0.05$). In addition, the expression of ATG1, MuRF1 and GLUD1, which is related to muscle protein degradation, was significantly decreased in March ($P < 0.05$).

In skeletal muscle during hibernation, these results indicate that 1) lipid intake in skeletal muscle and fatty acid oxidation are upregulated, 2) glycolysis is suppressed and glucose recycle is upregulated, and 3) muscle protein degradation is suppressed.

Analysis of microglial activation state in brains of prion-infected mice

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Prion diseases are fatal neurodegenerative disorders that are characterized by the vacuolation of neurons and neuropils, reactive astrogliosis, microglial activation and accumulation of an abnormal isoform of prion protein (PrP^{Sc}) in the central nervous system. Microglia are multifunctional cells in the central nervous system that are involved in the surveillance of neuronal activity, phagocytosis, regulation of innate immunity, and so on, and may exert either neuroprotective or neurotoxic responses depending on their activation state. Although the activation of microglial cells is observed at the early stage of prion infection in brain regions where the accumulation of PrP^{Sc} can be detected, how they participate in the pathogenesis of prion diseases is not yet understood. In this study, the gene expression of CD11b-positive microglia isolated from the brains of prion-infected mice was analyzed to assess the activation state of microglia during disease progression. Microglia derived from prion-infected mice expressed a higher level of neurotrophic factors such as NGF and BDNF at the early stage (60 days post-inoculation, dpi) than microglia derived from uninfected mice, and temporal upregulation of CXCL10, which is a chemokine suggested to play a protective role in prion disease, was observed at the intermediate stage (90 dpi). On the other hand, gene expression of proinflammatory cytokines TNF-α, IL-12p40, and IL-1β increased markedly with progression of the disease, in particular after 90 dpi, and the gene expression of M2-type macrophage markers YM-1, MRC-1, FIZZ-1, and CD163 was decreased. These results suggest that microglia play a neuroprotective role by secreting neurotrophic factors at the early stage of prion infection, but the microglial activation state shifts to neurotoxic in the progression of prion disease. Further investigation of the relationship between the gene expression profile and functional change (i.e. phagocytosis, or neuronal damage) of microglia in prion-infected mice will reveal the role of microglia in the pathobiological mechanism of prion diseases.

Molecular epidemiological analysis of the causative agent of proliferative enteropathy *Lawsonia intracellularis* and investigation of the actual condition of infection in an outbreak of equine proliferative enteropathy at one breeding farm

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Molecular genetic information about *L. intracellularis*, a causative agent of proliferative enteropathy (PE), has not been well investigated. In this study, to obtain molecular genetic information about *L. intracellularis* present in pigs in Japan, the nucleotide sequences of 7 gene fragments of *L. intracellularis* (8028 bp) from intestinal mucosa or feces of pigs collected from various areas of Japan from 2000 to 2010 were amplified and analyzed. All the samples showed 100% identity, except for *L. intracelluaris* from a pig in Miyazaki that possessed a non-synonymous substitution in outer membrane protein 4 (Omp4) gene. This result suggests that *L. intracellularis* in pigs in Japan shares extremely high genomic similarity regardless of the place or time at which the samples were collected. On the other hand, there are subtle nucleotide sequence differences among *L. intracellularis* from pigs, rabbit and horses, albeit with high similarity. In particular, deduced amino acid sequences of Omp4 of *L. intracellularis* in pigs, rabbit and horses were different, suggesting that *L. intracellularis* strains vary according to host animals.

In 2011, an outbreak of equine PE (EPE) occurred in foals at one breeding farm. To investigate the cause of this outbreak, the presence of *L. intracellularis* in feces was analyzed by detecting the gene fragments with nested PCR and TaqMan assay. All feces from horses clinically diagnosed with EPE were positive for *L. intracellularis*-specific DNA fragment and more than half of the horses that did not show apparent clinical signs were found to be carriers. Analysis of the amount and duration of bacterial shedding into feces revealed that it was intermittent, with a small amount, even after recovery. This result implies that bacteria were not eliminated from the host completely and that some horses carry *L. intracellularis* for a long time after recovery. Considering indigenous contamination of a stable with *L. intracellularis*, foals born in 2012 were tested for *L. intracellulairs*. The positive rate was higher in foals born in the stable where horses diagnosed with EPE in 2011 were kept than in those born in a different stable with little contact with the diseased horses. This finding may suggest that the agent persists easily once an outbreak occurs.

The results of this study will provide useful data for analyzing the source of infection and the entry route of *L. intracellularis* and for taking preventive measures against PE in animals.

Attempt to isolate subfraction of mouse mesenchymal stem cells and production of mesenchymal stem cells expressing anti-PrP antibodies

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Prion diseases are fatal neurodegenerative disorders that are characterized by the vaculoation of neurons and neuropils, astrogliosis, and the accumulation of a disease-specific isoform of prion protein in the central nervous system. Effective treatment of the disease is not available. Transplantation of human bone marrow-derived mesenchymal stem cells (hMSCs) or administration of an anti-PrP monoclonal antibody prolonged the survival of mice infected with prions even when initiated after clinical onset. The hMSCs have the ability to migrate to brain lesions of prion diseases and a neuroprotective function, while anti-PrP antibodies have inhibitory effects against prion propagation; therefore, the combination of MSCs and anti-PrP mAb is expected to exhibit an additive effect on the treatment of prion diseases. In this study, a single-chain fragment variable antibody of anti-PrP monoclonal antibody 44B1 (44B1scFv) in hMSCs was expressed by lentiviral vectors and the production of 44B1scFv and their migration ability were analyzed. The hMSCs stably produced 44B1scFv for a long period and migrated to brain extracts from prion-infected mice, suggesting that hMSCs expressing scFv will be applicable as a therapeutic model of prion diseases with anti-prion effects and neuroprotective function. Additionally, in order to construct a better model for the treatment of prion diseases using autologous MSCs, mouse MSCs (mMSCs) were isolated from bone marrow, compact bone and adipose tissues, and the expressions of surface markers were analyzed. The mMSCs derived from adipose tissues and compact bone expressed Sca1, CD105, CD90.2, CD44 and CD106, which are reported to be MSCs-positive markers; however, those mMSCs consisted of mixed cell populations with positive and negative for several markers including CD105 so magnetic-activated cell sorting was attempted to isolate a homogeneous subfraction from mMSCs. The mMSCs fractionated from adipose tissues by magnetic-activated cell sorting of CD105-positive cells appeared homogenous for the expression of MSC-positive markers examined. Furthermore, serial passaging did not affect the expression of MSC-positive markers, and in vitro adipogenesis and osteogenesis demonstrated multipotency of the fractionated mMSCs. Applying this method to obtain various mMSC subfractions and their analyses will greatly contribute to the identification of MSC subfractions that are useful for regenerative medicine/cell therapy for prion diseases.