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INFORMATION

Hokkaido University confered the degree of Doctor of Philosophy (Ph.D.) in Veterinary Medicine on March 25, 2010 to 12 recipients.
The titles of theses and other information are as follows:

Novel insights into the postnatal and pregnancy-associated mammary gland development: effects of diet-induced obesity and roles of leptin

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The mammary gland develops after birth, especially during puberty and pregnancy, for post-partum lactation. It has been reported that diet-induced obesity results in impaired lobuloalveolar development during pregnancy and reduced lactation in mice, cows, and also in humans. However, mechanisms by which obesity impairs lactation remain obscure. To reveal the mechanisms, I examined the processes of the puberty- and pregnancy-associated mammary gland development in lean and obese mice fed a normal diet and a high-fat diet, respectively.

Pre-pregnant lean mice had the branched fundamental mammary ducts that spread over the subcutaneous adipose tissues. The fundamental ducts in lean mice were composed of luminal epithelia and basal myoepithelia, and were surrounded by a thin collagen layer and stromal cells such as adipocytes. In contrast, pre-pregnant obese mice had less frequently branched fundamental ducts in the hypertrophic adipose tissues. And the fundamental ducts in obese mice were composed of short luminal epithelia and an incomplete myoepithelial layer, and were covered with a thick collagen layer and enlarged adipocytes.

After the onset of pregnancy, lean mice showed sprouting of side branches from the fundamental ducts during early pregnancy, emergence of acini during mid-pregnancy, and maturing of milk-producing acini with the abundant expression of lactation-associated genes during late pregnancy. In contrast, obese mice exhibited less sprouting of side branches during early pregnancy and less matured milk-producing acini with the insufficient expression of the lactation-associated genes during late pregnancy. However the cellular composition of side branches and acini in pregnant obese mice was not different from that in pregnant lean mice. These results indicate that the abnormalities of the cellar composition during pre-pregnancy and the suppression of side-branch sprouting during early pregnancy might be the causes of retardation of subsequent acinar maturation in obese mice.

Side-branch sprouting during early pregnancy is regulated by reproductive hormones and local factors. However, there was no difference in the plasma levels of estrogen and progesterone between lean and obese mice in the timing of side-branch sprouting. On the other hand, the plasma level of leptin, which is secreted from adipocytes, was elevated in obese mice, concomitant with the increased expression of leptin mRNA in the mammary glands. Leptin

receptors were localized in the adjacent region of the fundamental ducts and side branches, but rarely in the surrounding region of acini. Leptin treatment at superphysiological concentration stimulated the production of type I collagen in cultured fibroblasts, and hyperleptinemia induced by leptin-expressing adenovirus enhanced collagen deposition in the mammary glands of pre-pregnant lean mice. In addition, leptin inhibited the proliferation of cultured mammary epithelial cells derived from bovine mammary glands. Therefore, it is likely that, in obese mammary gland, excess local leptin suppresses the side-branch formation during early pregnancy through the periductal fibrosis and epithelial growth arrest.

In conclusion, I have demonstrated the impairment of fundamental ductal formation and the suppression of subsequent side-branch formation, which may lead the lactation failure in obese animals. Moreover, I found potential roles of leptin in the regulation of mammary epithelial growth and fibrillar formation, which are probably linked with the obesity-induced impairment in the mammary gland development. These results also provide new insights into the roles of adipocytes in the mammary gland development.

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Studies of *in vivo* drug-induced QT interval prolongation in early-stage drug development

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The potential for non-cardiac drugs to induce QT interval prolongation accompanied by a rare but life-threatening lethal arrhythmia has generated intense interest and concern in the development of pharmaceuticals. Because inhibition of the hERG (human *ether-a-go-go* related gene) channel is considered the main cause for the QT interval prolongation, *in vitro* hERG inhibitory tests are generally conducted in early-stage drug development. However, hERG inhibitors do not necessarily cause QT prolongation and hERG tests are considered insufficient for actual QT risk assessment. In this study, in consideration of accurate QT risk evaluation in the early stages, *in vivo* assays using anesthetized guinea pigs, anesthetized dogs and conscious common marmosets were developed.

The monophasic action potential (MAP) was measured in anesthetized guinea pigs under a fixed heart rate using atrial pacing. Eight positive drugs and four negative drugs were intravenously administered to assess the effects on the MAP duration ($MAP_{90(pacing)}$: action potential duration at 90% repolarization level). All positive drugs showed dose-dependent $MAP_{90(pacing)}$ prolongation, whereas the negative drugs did not. There was a clear correlation between each estimated 5% $MAP_{90(pacing)}$ prolonging dose (ED_5: 5% effective dose) of the eight positive drugs and the clinical plasma concentration associated with QT prolongation previously reported.

The electrocardiogram (ECG) under atrial pacing conditions was measured in anesthetized dogs. Since QT interval adapts to changes in RR interval, a study original formula (QTcX = QT/

$RR^{0.3879}$) was established in this study. Two positive drugs and one negative drug were intravenously administered to assess the effects on the QTcX interval and QT interval under atrial pacing ($QT_{(pacing)}$). All of the positive drugs dose-dependently prolonged the QTcX interval and $QT_{(pacing)}$, whereas the negative drug failed to do so. The plasma concentrations of the positive drugs associated with QT prolongation were mostly consistent with those associated with previously reported clinical QT prolongation.

The ECG was measured under a conscious condition in common marmosets implanted with telemetry transmitters. In this study, an individual QT correction method ($QTci = RR_{ref}^{\beta} \times QT/RR^{\beta}$, where β is the individual correction coefficient) was established. Two positive drugs and two negative drugs were orally administered to assess the effects on the QTci interval. All positive drugs showed QTci interval prolongation, whereas the negative drugs did not. The plasma concentrations of the positive drugs associated with QT prolongation were mostly consistent with those associated with previously reported clinical QT prolongation.

All of the assays using anesthetized guinea pigs, anesthetized dogs and conscious marmosets developed in this study showed high sensitivity and high specificity. In addition, the quality of sensitivity was appropriate because the results of the positive compounds showed a clear correlation with clinical outcomes. Therefore, these assays would be useful for the assessment of drug-induced QT interval prolongation. Incorporation of these assays into early-stage drug development would provide a potentially accurate and more integrated assessment of QT risk and thus a more timely introduction of new pharmaceuticals into the clinic stages with fewer safety issues.

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Studies on the development of vaccine and molecular basis of pathogenicity of avian influenza viruses for chicken.

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Since 1997, outbreaks of highly pathogenic avian influenza caused by H5N1 viruses have occurred. The present study revealed that the antigenicities of H5HA of the isolates from water birds and the HPAI viruses were similar. Sakabe *et al*. (*Vaccine*., 26 2127–2134 (2008)) suggested that antigenicities of the H7HA of avian influenza viruses were also conserved. Based on these results, A/duck/Hokkaido/Vac-1/2004 (H5N1) [Vac-1/04 (H5N1)], A/duck/Hokkaido/Vac-3/2007 (H5N1), and A/duck/Hokkaido/Vac-2/2004 (H7N7) [Vac-2/04 (H7N7)] were generated from non-pathogenic avian influenza viruses isolated from migratory ducks as the vaccine strain candidates. Mice vaccinated subcutaneously with 100 μg of inactivated Vac-1/04 (H5N1) were protected from lethal infection with Vietnam/1194/2004 (H5N1). It was also reported that Vac-1/04 (H5N1) and Vac-2/04 (H7N7) were potent in giving protective immunity

against HPAI virus challenge to chickens and cynomologus macaques. These findings support the notion that non-pathogenic viruses isolated from water birds are useful for production of vaccine against infection with HPAI virus presently circulating in the world.

However, misuse of vaccine may lead to antigenic drift or shift of the HPAI viruses. Actually, HPAI viruses have been perpetuating and killing not only poultry but also humans in some countries doing vaccination procedure without testing and culling, fundamental countermeasures against HPAI. Therefore, examining the cross reactivities between epidemic strains and vaccine strain candidates in the present study should be continued and it is recommended that all of the countries where HPAI occurs should make a effort for the eradication of HPAI.

In addition to HPAI caused by H5 or H7 viruses, avian influenza caused by low pathogenic H9N2 viruses have occurred in poultry, resulting in serious economic losses in Asia and the Middle East. Its eradication is still difficult because of its low pathogenicity, frequently causing inapparent infection. It is important for the control of avian influenza to assess whether the H9N2 virus is capable of becoming pathogenic like H5 and H7 viruses. In the present study, H9 virus acquired high intravenous pathogenicity by introducing a pair of di-basic amino acid residues at the HA cleavage site as observed in H5 or H7 HPAI viruses and passaging in the air sac of chicks. Artificial mutation in H9HA cleavage site and amino acid mutations during consecutive passages in the air sac of chicks must have been involved in conferring high pathogenicity on Y55. On the other hand, chickens inoculated intranasally with rgY55sub-P10 (H9N2) did not show any clinical signs of disease while H5 viruses were readily acquired high intranasal pathogenicity. It is, thus, predicted that H5 viruses passaged in chicks exerted their intranasal pathogenicity with high levels of viremia caused by replication in vascular endothelial cells, leading to invasion of the brain of chickens. Further study including a pathological analysis is needed to assess this hypothesis. The present study demonstrated that H9N2 viruses can acquire further intravenous pathogenicity. Co-infection of rg Y55sub-P10 (H9N2) with bacteria may exacerbate not only intravenous pathogenicity but intranasal pathogenicity.

Taken together, the results shown in this thesis suggest that continuous surveillance in migratory birds and poultry is important to predict and prevent the emergence of pathogenic viruses, and to stock the viruses as vaccine strains.

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Studies on antitrypanosomal activity of medicinal plants

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Trypanosoma evansi is an animal-pathogenic flagellated protozoan parasite. The parasite infects a variety of large animals including equines, camels, cattle, buffaloes, goats, sheep, and pigs, causing the trypanosomiasis condition known as surra. The disease has a wide geographical distribution because the parasites are mechanically transmitted by biting arthropods, especially horse flies (*Tabanus* spp.) and stable flies (*Stomoxys* spp.), from one infected host to another. The disease causes great economical losses in areas of Africa, Asia, and South America, where thousands of animals die from *T. evansi* infections. Existing trypanocidal drugs have been associated with side effects, and the development of drug resistant trypanosomes has occurred in many regions. Therefore, research on new compounds for the treatment of surra, as well as sleeping sickness in man and nagana in cattle, is an urgent and important task.

Natural compounds in plants offer a valuable source for novel lead drug discovery. A medicinal plant, *Brucea javanica* contains quassinoids, which are the bitter principles found in various species of the Simaroubaceae in the tropics. In Chapter I, the content of quassinoids in *B. javanica* was analyzed by fractionation with column chromatography, ultra performance liquid chromatography (UPLC), electrospray ionization triple quadrupole mass spectrometry (MS/MS) and nuclear magnetic resonance. In the previous studies, C-20 types of quassinoids such as bruceine A, bruceine B, bruceine C, bruceine D, bruceantinol, bruceantinol B, bruceine J, and yadanziolide A were isolated and purified from the organic layer of the Indonesian plant materials. In addition, brusatol, bruceantin, dehydrobruceine A, dehydrobruceine B, dehydrobrusatol, bruceoside A, and yadanzioside G were isolated from the organic layer of the Chinese plant materials. In this study, the dried fruits of Indonesian *B. javanica* were extracted with 70% aqueous methanol and partitioned using ethyl acetate to yield aqueous and organic layers. From the water-soluble fraction, bruceine D was obtained. A rapid UPLC-MS/MS method for the quantitative analysis of quassinoids was developed. The peak area in multiple reaction monitoring chromatograms for each quassinoid can be used for estimation for the amounts of some quassinoids in crude methanol extracts from *B. javanica* in different origins. As expected, the amounts of quassinoids in the plant materials were different with different countries and places, suggesting that the quantity and composition of quassinoids in the same plant species depend on geographic factors. Rapid and accurate quantification method developed in this study will be useful for the screening of the detection and quantification of quassinoids or other bioactive compounds from crude plant extracts. Further studies including the development of simple and low-priced method for isolation of active ingredients in a large quantity are required.

Quassinoid compounds are known to exhibit inhibitory activities on protozoan parasites such as *Plasmodium falciparum*, *Entamoeba histolytica*, *Giardia intestinalis*, *Toxoplasma gondii* and *Babesia gibsoni*. In Chapter II, the antitrypanosomal activity of quassinoid compounds against *T. evansi in vitro* was

evaluated and the structure-activity relationship was discussed. Cytotoxic activity of quassinoids against human lung diploid fibroblast (MRC-5) cells was also examined. Among fifteen C-20 quassinoids, bruceine A, bruceantinol, bruceine C, brusatol, and bruceine B showed strong *in vitro* antitrypanosomal activities with IC_{50} values in the range of 2.9–17.8 nM, which compared well with the standard trypanocidal drugs diminazene aceturate with IC_{50} of 8.8 nM and suramin with IC_{50} of 43.2 nM. However, dehydrobruceine A, dehydrobruceine B, and dehydrobrusatol were about 2100, 900, and 1200 times less active, respectively, than bruceine A, bruceine B, and brusatol. The relationship of the structure and antitrypanosomal activity of these quassinoid compounds suggested that the presence of a diosphenol moiety in ring A and the nature of the C-15 side chain are important for their activities against *T. evansi*. Bruceine A, B, C, and D had relative low cytotoxicity with selectivity index (SI) values in the range of 1900–4200 against MRC-5 cells.

The findings of antibabesial and antitrypanosomal activities quassinoids isolated from the fruits of a medicinal plant, *Brucea javanica*, suggest a promise use of medicinal plant extracts for protozoan diseases in livestock as well. In Chapter III, it was attempted to discover antitrypanosomal medicinal plants from Myanmar. A total of 55 fresh and 16 dry medicinal plant specimens from 60 plant species were extracted with 70% ethanol and 70% methanol, respectively. The *in vitro* antitrypanosomal activity against trypomastigotes of *T. evansi* and cytotoxic activity against MRC-5 cells were determined. Three of 55 fresh samples and 6 of 16 dry samples showed IC_{50} values with < 100 μg/ml against *T. evansi*. *Eucalyptus globulus* and *Jatropha podagrica* showed higher antitrypanosomal activities with IC_{50} values of 51.1 and 52.3 μg/ml, respectively, and higher selectivity indexes of 12.2 and 12.5, respectively, than other fresh samples. Among dry samples, *Vitis repens* showed the highest antitrypanosomal activity with IC_{50} of 8.6 μg/ml and SI of 24.4. In conclusion, some medicinal plants used in Myanmar for the treatment of various ailments such as malaria, dysentery, tumor, and pulmonary diseases may offer a potential use for the treatment of *T. evansi* infection.

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Genetic analysis and transcriptome profile characterizing pathogenesis of host response to Sendai virus infection in mice

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Pathology of lethal peripartum broad ligament hematoma in mares

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The broad ligament is a double peritoneal membrane connecting the abdominal wall and the uterus, and the blood vessels pass through the broad ligament to supply the uterus. In aged mares, broad ligament hematoma that often leads to the death by blood loss can occur in perinatal period. This study aims to elucidate the etiology and pathogenesis of broad ligament hematoma in mares.

Thirty-six mares that died of broad ligament hematoma peripartum were examined pathologically for bleeding sites. The mean age of the affected mares was 17.15 ± 3.43 years (S. D.), and the parity was 9.86 ± 2.85 times (S. D.). At necropsy, arterial injuries were identified in 31 of 36 mares, and were commonly observed in the uterine artery (24 mares), internal pudendal artery (5 mares), caudal mesenteric artery (1 mare), and internal iliac artery (1 mare). Among these, the proximal uterine artery that lies near the bifurcation from the iliac artery was the most frequent site of rupture. The arterial ruptures preferably occurred at the sites subjected to severe hemodynamic stress, such as the bifurcations, lateral part of curvatures and abrupt flexures of the artery.

Histologically, fibrosis of the tunica media and thickened tunica intima were observed adjacent to the ruptured proximal uterine artery. These changes were observed commonly on both sides of the ruptured and apparently intact arterial walls, and might be related with aging and multiparity. The middle to distal uterine artery had abundant longitudinal smooth muscle fasicles in tunica adventitia. This structures might make the middle to distal uterine artery more resistant to mechanical forces such as tugging and increased blood pressure, and might contribute to lower incidence of arterial ruptures in those areas in compare with proximal uterine artery.

To confirm the aging changes, the uterine arteries of various ages without arterial rupture that caused broad ligament hematoma were examined histologically. The aging changes included an atrophy of smooth muscle cells with medial fibrosis. The other changes with aging were the disruption of internral elastic lamina and thickening of tunica intima due to an increase stroma and proliferation of smooth muscle cells. The results of morphometrical analysis also confirmed the aging change in arterial walls.

The present study revealed that arterial ruptures led to broad ligament hematoma in peripartum mares occurred most frequently at the proximal uterine artery, and atrophy of smooth muscle cells with fibrosis of arterial wall was one of the predisposing factors of broad ligament hematoma in aged and multiparous mares. These results may provide useful information for the early diagnosis by transrectal echography, surgical treatment such as hemostasis by laparoscope, and perhaps prevention of broad ligament hematoma of mares.

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Establishment and pathology of a murine model of influenza virus-associated encephalopathy

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Influenza virus-associated encephalopathy (IAE) is a highly lethal neural complication of influenza virus infection that mostly affects children aged 2–4 years. The characteristic clinical finding of IAE is acute symmetric brain edema after the onset of flu-related fever. Histopathological analysis has revealed that the encephalopathy is accompanied by blood-brain barrier (BBB) dysfunction due to an impairment of cerebral vasculatures. Therefore, the essential component of IAE is considered to be a rapid onset of vascular disorder. Elevated inflammatory cytokines, such as TNF-α, IL-1β, and IL-6, have been observed in the serum of IAE patients, which has led to the suggestion that hypercytokinemia is the cause of the encephalopathy.

In this study, neonatal ICR mice treated with a combination of influenza A virus (IAV) and lipopolysaccharide (LPS) were investigated as a candidate experimental model of IAE. The mice inoculated with IAV and LPS (IAV + LPS mice) showed exacerbated neuropathogenicity and increased BBB permeability, compared to the mice inoculated with each IAV or LPS alone. In addition, IAV infection enhanced LPS-induced production of inflammatory cytokines in the blood. Cerebral microscopic changes in IAV + LPS mice were characterized by scattered microhemorrhage, dilation of perivascular spaces, and hyaline droplet formation in the peripheries of blood vessels, which suggested increased BBB permeability due to the development of cerebral vascular damage. Plasma levels of TNF-α and IL-6 were significantly increased in IAV + LPS mice, which was also consistent with IAE in humans. Thus, IAV + LPS mice showed an IAE-like encephalopathy with respect to CNS histopathology and the dynamics of plasma inflammatory cytokines.

To investigate the underlying mechanism of BBB breakdown in IAV + LPS mice, apoptosis of BBB component cells and changes in the expression level of the tight junction (TJ) proteins were analyzed. In the results, apoptosis of vascular endothelial cells and astrocytes was elevated in brains of IAV + LPS mice as compared to mice treated with IAV or LPS alone. Since these cells contribute to the integrity and function of the BBB, apoptotic damage of these cells would likely affect BBB permeability. Western blot analysis demonstrated that the expression levels of TJ proteins were unaltered. These results suggested that impairment of the BBB and the resultant IAE-like encephalopathy in IAV + LPS mice is caused by apoptosis of cerebral vascular endothelial cells and astrocytes in the brain. Previous reports have suggested that apoptosis of CNS resident cells and vascular endothelial cells plays an important role in the development of IAE. Thus, the pathological mechanism of increased BBB permeability in IAV + LPS mice appears to be similar to that of IAE in humans.

In conclusion, the encephalopathy in IAV + LPS mice shared similar characteristics with IAE in terms of CNS lesions and the dynamics of inflammatory cytokines. Moreover, apoptosis of vascular endothelial cells and astrocytes may contribute to the IAE-like encephalopathy in IAV + LPS mice, which suggests that both encephalopathies in IAE and

in our murine model induced by IAV + LPS treatment have a similar pathological mechanism of increased BBB permeability. The murine model established in the current study will be useful in the development of strategies for early diagnosis and treatment of IAE.

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Cryopreservation of zebrafish (*Danio rerio*) primordial germ cells by embryo vitrification

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Studies on the natural transmission cycle of West Nile virus and the antibody survey in birds

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In this thesis, the effect of the glycosylation of E protein of Wes Nile (WN) virus New York (NY) strain on the virus multiplication was examined and antibody survey of WN virus was performed among wild birds in Far Eastern Russia.

Many of the WN virus isolates associated with significant human outbreaks, including the recent North American epidemic, possess the glycosylation site on the E protein. In experimental infection in mice, the glycosylated variants caused higher mortality than the non-glycosylated variants, which suggests that E protein glycosylation is a molecular determinant of neuroinvasiveness of the NY strain of WN virus. In the chapter I, the author examined the effect of E protein glycosylation on the interaction between WN virus and avian hosts and mosquito vectors. Using a young chick infection model, the author examined whether the glycosylated (LP) and non-glycosylated (SP) variants exhibit differences in virulence and viremic level in the birds. The survival rate of chicks inoculated with LP variant was 0–20%. And pathology in these chicks included severe necrosis in the liver and heart. In contrast, the LP variant exhibited low virulence in young chicks. The viremia titers of the LP variant in chicks were ten or more times greater than those of the SP variant. However, the

glycosylation status of the variants did not affect viral multiplication and dissemination in *Culex* mosquitoes. The author then examined the multiplication characteristics of the variants *in vitro* tissue culture cells of mammalian, avian and mosquito origin to establish how E protein glycosylation affects WN virus multiplication in these different cell types. LP variants showed more heat stable propagation than SP variants in mammalian (BHK) and avian (QT6) cells, but not in mosquito (C6/36) cells. These results suggested that high viremic titer in avian host was related with glycosylation of E protein. Viremic titer in avian host is known to be crucial for the WN virus transmissibility to the mosquitoes. Therefore, N-linked glycosylation of E protein may be essential for an efficient transmission of WN virus NY strain in nature.

In areas where Japanese encephalitis (JE) virus is endemic, discrimination between WN and JE viruses is critical for the detection of WN virus invasion. However, the JE serocomplex flaviviruses are antigenically cross reactive and are thus not readily differentiated by serological methods. In the chapter II, the author evaluated neutralization test (NT) in young chicks inoculated with JE and WN viruses. After the single virus infection, only the specific neutralizing antibody to the homologous virus was detected in chicks. In order to investigate the effect of heterologous virus infection, a double infection experiment was conducted. Two-day-old chicks were inoculated with JE or WN virus, and challenged with the other virus after 3 weeks. Regardless of which virus was inoculated first, booster immune responses to both homologous and heterologous virus were observed after challenge inoculation. However, it was difficult to judge which virus infected first or how many times the chicks were exposed to the viruses based on the NT. The information about the extent of WN virus infection in Russia still remains very limited. Therefore, a seroepidemiological survey of WN virus infection was performed among wild birds in Far Eastern Russia using NT. One hundred forty five wild birds were captured in Far Eastern Russia and serum samples were examined for WN and JE viruses by the NT. Twenty one out of 145 sera showed positive neutralizing antibodies to WN virus and most of these showed specific neutralizing antibody titers to WN virus. These results suggest that WN virus is prevalent among wild birds in Far Eastern region of Russia.

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Skull morphology and genetic variation of the Kuril harbor seal (*Phoca vitulina stejnegeri*) and the spotted seal (*Phoca largha*) around Hokkaido, Japan

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Five species of seals inhabit and/or migrate to coastal regions around Hokkaido, Japan. The

major species are the Kuril harbor seal and the spotted seal. Because the Kuril harbor seal has been as an endangered species by the Ministry of the Environment and the spotted seal is under the control of the Hokkaido Government, these differences will affect the management infrastructure. To clarify the differences between the two sibling species around Hokkaido, the skull morphology and genetic variations were investigated.

There is a paucity of information regarding the morphological characteristics of the skulls of seal pups and their associated developmental morphological changes. We measured 29 metric and 6 non-metric cranial characteristics of the two species. Sexual dimorphism in Kuril harbor seal pups, subadults, and adults were detected. Although interspecies differences were detected in each growth class, Kuril harbor seals were larger and more massive than spotted seals; this feature was already detectable in pups. Using 6 non-metric cranial characteristics, we identified significant interspecies differences with regard to the shape of the temporozygomatic suture and the extent of the nasal-incisive suture; the shape of the temporozygomatic suture and the shape of the nares were indicators of growth class in Kuril harbor seals.

The variations of mitochondrial DNA (mtDNA) cytochrome *b* sequences in the two species were studied. Fifteen haplotypes were observed in 39 Kuril harbor seals and 23 were observed in 31 spotted seals. The phylogenetic trees showed two Kuril harbor seal lineages: Group I primarily contained haplotypes from Erimo, and Group II contained haplotypes from Akkeshi and Nosappu. Because Erimo population had fewer haplotypes and less nucleotide diversity than Akkeshi and Nosappu populations, it was considered to be isolated from the others. In contrast, the genetic variance within populations of spotted seals (97.3%) showed far higher than the variance among populations (2.7%) by analysis of molecular variance. There was no significant difference between spotted seal populations, indicating that the absence of lineage throughout Hokkaido. The differences in the genetic population structures between the two species could have been generated by their ecological differences.

To determine the hybridization between Kuril harbor seals and spotted seals in the wild, mtDNA and SRY gene were studied. However Kuril harbor seals and spotted seals had the same SRY haplotype, and it failed to identify their paternal species and the hybrids. Those were from only Erimo that three seals showed their mtDNA haplotype differed from species by their pelage. The probability of hybridization was higher in the Erimo individuals than others.

This study provided basic and meaningful information, but the task of conservation and management of both fishery and seals remains a challenge. It is especially necessary to emphasize the isolation of the Erimo Kuril harbor seal population.

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Studies on the mechanism of regulation for seasonal changes in testicular activity in the Japanese black bear (*Ursus thibetanus japonicus*)

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Many wildlife species mate only during certain times of the year to bear and raise their young successfully in an optimal season for survival. The Japanese black bear (*Ursus thibetanus japonicus*) is a long-day breeder, and their gonadal activity largely varies by season in both males and females. Seasonal changes in spermatogenesis and peripheral testosterone concentrations in male bears are well known; however, a large part of the biological mechanism of their variation remains to be elucidated. Hence, the objective of the present study is to reveal a part of the mechanism of seasonal change in testicular activity in Japanese black bears. The change in gonadal activity is controlled mainly by the endocrine system centering on the hypothalamus-pituitary-gonadal (HPG) axis, and also by sex steroid hormones released from the gonads. This study was carried out particularly focusing on gonadotropins, namely follicle stimulating hormone (FSH) and luteinizing hormone (LH), released from the anterior pituitary gland, and on steroidogenic enzymes which contribute to testicular steroidogenesis.

Blood and testicular tissue were sampled from captive, adult, male bears, 2 to 3 times during testicular regressive and active phases, and once a month during the testicular recrudescent phase.

Firstly, variations in the concentration of plasma testosterone, FSH, LH, inhibin and gonadotropin receptor mRNA expressions in the testis were investigated. Plasma testosterone and inhibin were increased significantly during recrudescent and active phases, and FSH concentration had a tendency to increase during the recrudescent phase. These hormonal changes were synchronized with testicular enlargement and the resumption of spermatogenesis. FSH receptor mRNA expression was recognized in the basal portion of the seminiferous tubules for all phases. These results suggested that the change in FSH secretion affected testicular recrudescence. On the other hand, LH receptor mRNA expression, which was observed in the interstitium, and plasma LH concentration did not vary in this study. Variations in plasma inhibin concentration were synchronized with the fluctuation in testosterone concentration. This indicates that increases in inhibin concentration during the recrudescent phase resulted from the elevation of testicular activity.

Secondly, mRNA nucleotide of steroidogenic enzymes was sequenced, and localization of mRNA expression in the bear testis was examined by using DNA probes based on the sequence, together with protein immunolocalization. Sex steroid hormones are synthesized from cholesterol catalyzed by 5 kinds of steroidogenic enzymes (P450scc, 3βHSD, P450c17, 17βHSD3 and P450arom). P450scc, 3βHSD and P450c17 expression in the interstitial tissue indicates that the conversion from cholesterol into androstenedione occurs in this region. Meanwhile, testosterone may be synthesized in the basal portion of the seminiferous tubules as well as in the

interstitium, because 17βHSD3 mRNA signals were observed in both locations. Estradiol-17β may be synthesized in elongated spermatids and Leydig cells, judging from the expression of P450arom.

Thirdly, to determine factors responsible for seasonal change in sex steroid hormone synthesis in the testis, variation in gene expression of 5 steroidogenic enzymes and steroidogenic acute regulatory protein (StAR) were examined by real-time PCR. The results showed that 3βHSD gene expression in the active phase of the testis was significantly higher than in the regressive phase. Changes in expression levels of this enzyme were synchronized with those in testosterone concentration; therefore, seasonal variations in testosterone synthesis in male bear testes may be mainly regulated by 3βHSD gene expression. Expression of P450arom mRNA was also significantly decreased during the regressive phase. This suggests that estrogen synthesis in testes may also fluctuate seasonally, though plasma estradiol-17β concentration did not vary.

Lastly, androgen receptor expression was examined immunohistologicaly. Immunolocalization of androgen receptors was observed in testicular somatic cells. This suggests that testosterone activates spermatogenesis through the function of Sertoli cells.

From these studies, I demonstrated a part of the mechanism in reproductive physiology involving in seasonal change in testicular function in male Japanese black bears, which exhibit definite variation in gonadal activity. Further studies are needed to fully reveal the mechanism of testicular seasonality. The present study provides interesting and valuable information for further investigation of the mechanism of seasonal breeding, especially from a perspective of comparative reproductive biology.

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The development of novel therapeutic targets for diabetic nephropathy: hyperinsulinemia, HIF-1*α*, and megsin

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Diabetic nephropathy is the most common cause of end-stage renal failure in developed countries. Strict glycemic control and/or blood pressure control of diabetic patients with nephropathy result in delay of dialysis onset. However, there is no therapeutic agent at the moment to regress the kidney injury in diabetic nephropathy. Thus, the development of novel therapeutics for diabetic nephropathy is an intensively investigated topic. In the present study, the mechanisms for the progression of diabetic nephropathy were investigated, using type II diabetic nephropathy rat model SHR/NDmcr-*cp* and *in vitro* cultured renal cells.

First, the renoprotective effects achieved by an insulin-sensitizer, pioglitazone, or by insulin were compared in SHR/NDmcr-*cp*. Pioglitazone markedly attenuated hyperinsulinemia and renal dysfunction in contrast with insulin treatment. Although renal accumulation of pentosidine and the local oxidative stress were both reduced to a similar extent by pioglitazone

and insulin, the enhanced TGF-β expression was suppressed only by pioglitazone, suggesting that hyperinsulinemia up-regulated TGF-β expression. These data suggest that hyperinsulinemia together with a stimulated TGF-β expression significantly contributes to the incidence and deterioration of diabetic nephropathy.

Second, the renoprotective effects of cobalt chloride, which enhances a key defensive factor against hypoxia, i.e. HIF, were evaluated in SHR/NDmcr-*cp*. The renal expression of TGF-β and CTGF, renal accumulation of pentosidine, and the local oxidative stress were reduced, and significant renoprotection was observed by cobalt treatment. Cobalt treatment up-regulated renal HIF-1α expression, increased the expression of HIF-regulated genes, e.g. erythropoietin, VEGF, and HO-1, and protected peritubular capillaries. Thus, cobalt achieved renal protection via the activation of HIF-1α signals, and its effect was attributed to prevention of renal hypoxia.

Third, the role of megsin, a member of the serine protease inhibitor superfamily, in the decreased activities of MMP-2, MMP-9, and plasmin was investigated. High-glucose up-regulated megsin RNA expression in the kidney of SHR/NDmcr-*cp* as well as *in vitro* in cultured mesangial cells (RMC). The functional studies in RMC showed that megsin potentially inhibits total enzymatic activities of MMP-2, MMP-9, and plasmin, indicating decreased degradation of mesangial matrix. An anti-megsin neutralizing antibody restored the reduced activities of MMP-2 and MMP-9 by megsin in RMC. These data suggest that hyperglycemia induces up-regulation of megsin which, in turn, inhibits activities of plasmin, MMP-2, and MMP-9, potentially contributing to mesangial matrix accumulation in diabetic nephropathy.

In conclusion, this study shows that the etiology of diabetic nephropathy involves hyperinsulinemia due to the increased expression of TGF-β that occurs through several distinct pathways including activation of HIF-1α signals and up-regulation of megsin. These findings provide new insights into searching and identifing therapeutic targets for diavetic nephropathy.

The full text of this thesis (PDF) appears at http://eprints.lib.hokudai.ac.jp/dspace/handle/2115/42817

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