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INFORMATION

Hokkaido University confered the degree of Doctor of Philosophy on March 24, 2011 to 10 recipients.
The titles of theses and other information are as follows:

Clarification of molecular network during nephrogenesis in mice –Hepatocyte nuclear factor 4 alpha plays a central role in nephrogenesis–

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Kidney development is started by invasion of the uretelic bud (UB) arising from the Wöllfian duct into the metanephric mesenchyme (MM). Then the MM concentrates around the UB tips and forms the condensed mesenchyme (CM). The CM transforms sequentially to the renal vesicles, comma-shaped body (CSB), S-shaped body (SSB) and finally the nephron. In these processes, MM cells turn to nephron resulting by the mesenchymal-epithelial transition (MET). Although the biological mechanism of nephrogenesis and MET have been clarified gradually, a lot of uncertain points still remain, owing to the complexity of kidney structure.

Hepatocyte nuclear factor 4 alpha (Hnf4α) protein is one of the Zinc finger type-nuclear transcription factors expressing in the liver, intestine, pancreatic beta cells and kidney. Hnf4α has a relationship among large amount of gene regulation in hepatocytes and pancreatic beta cells, playing a key role in fat metabolism, insulin secretion and maintenance of the homeostasis. However, since disruption of the *Hnf4a* gene results in embryonic lethality due to emblyonal endodermal cell death and failure of gastrulation before kidney development, its renal function is still unknown.

In this research, to clarify the dynamics and the function of Hnf4α during nephrogenesis, following analyses were performed. In chapter 1, the localization of Hnf4α in several organs and the dynamics of this gene during nephrogenesis were investigated. In chapter 2, to assess the function during nephrogenesis, gene-silencing of *Hnf4a* was performed in organ-cultured kidney by RNAi method. Additionally, micro-environmental gene expression of Hnf family was investigated. In chapter 3, to elucidate the details of MET during nephrogenesis, the embryonic kidneys were analyzed by electron microscopy, immunohistochemistry, and molecular biology. In addition, cellular model inducing the expression of *Hnf4a* gene into fibroblast cell line was produced and analyzed, in order to assess the relationship between Hnf4α and MET.

In chapter 1, it was clarified that *Hnf4aP1* or *P2* was expressed dominantly in kidney and in stomach and pancreas respectively, while both types were expressed in liver, small intestine and large intestine. In immunohistochemical analysis, Hnf4α protein was located only in the segment of the proximal tubules in kidney, but not in the other segment. In the developing kidney, Hnf4α was detected first at the epithelial cell nuclei in part of the CSB/SSB, distributed widely throughout the developing nephron and finally

restricted to proximal tubules. Interestingly, it was noted that *Hnf4aP1* and *P2* were detected from stomach, pancreas and kidney tissues in embryonic periods. Additionally, Hnf4α was expressed earlier than Hnf1α in the same tubule, giving the impression that Hnf4α played the more fundamental role in Hnf network during nephrogenesis.

In chapter 2, as a result of gene-silencing, the cellular organization in the CM fell into disorder and many apoptotic cells appeared in cultured kidney. LMD-RT-PCR revealed that both of *Hnf4aP1* and *P2* were expressed in the CM and only *P1* was expressed in the CSB/SSB, while both were not detected in the MM. Additionally, only *Hnf1b* which are up-stream genes of *Hnf4a* was expressed in the CM and *Hnf1a* and *Hnf1b* were expressed in the CSB/SSB. These results suggested that Hnf4α played an important role during cell survival in the CM and this gene expression was induced by Hnf1β.

In chapter 3, the TEM observation and immunohistochemistry in developing kidney showed that the intercellular junction, but not the basal lamina, was present in the CM. Additionally, immediately after *Hnf4a* gene expression, the expression of epithelial genes (*Krt8*, *Tjp1*, and *Cdh1*) increased, and those of mesenchymal genes (*Acta2* and *Vim*) decreased, in the CM compared to the MM. In the cellular model analysis, it was noted that Hnf4α induced increasing epithelial and decreasing mesenchymal gene expression. In this analysis, up-regulation of *Pvrl1*, *-2*, and *Mllt4* genes which mediate the formation of apico-basal polarity, were found. These results suggested that the cells in the CM showed the intermediated forms between epithelial and mesenchymal and Hnf4α proteins induced the MET in typical fibroblast.

In conclusion, following dynamics model was provided; Hnf4α appears in the CM stage and in the CSB/SSB stage. In the CM stage, both of *P1* and *P2* induced by Hnf1β relate to the cellular survival and the initiation of MET via activating Nectin-Afadin. In the CSB/SSB stage, only *P1* is expressed and up-regulates the expression of *Hnf1a*. Hnf1α and Hnf4α activate the gene expression each other and relate to the characterization of proximal tubules.

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Epidemiological studies of avian influenza

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Recent outbreaks of highly pathogenic avian influenza (HPAI) have spread from southeast Asia to 62 Eurasian and African countries. H5N1 viruses isolated from dead water birds in Mongolia on the way back to their nesting areas in Siberia in April to May in 2005, 2006, 2008 and 2009 were genetically closely related to those isolated from birds in China, and some southeast Asian countries. It is now a serious concern that these HPAI viruses may perpetuate in the lakes where migratory water birds nest in summer.

Therefore, virological surveillance of avian

influenza virus in the migratory birds that fly from their nesting lakes in Siberia to Hokkaido, Japan was carried out in autumn every year. During 2008–2009, 62 influenza viruses of 21 different combinations of hemagglutinin (HA) and neuraminidase (NA) subtypes were isolated. Up to September 2010, no HPAI virus has been found, indicating that H5N1 HPAI virus has not perpetuated at least dominantly in the lakes where ducks nest in summer in Siberia.

It is reported that the PB2 protein is a component of the viral polymerase complex that plays an important role in virus replication, and is a determinant of host range and pathogenicity of influenza viruses. The PB2 genes of 57 influenza viruses out of 283 influenza viruses isolated in Hokkaido in 2000–2009 were phylogenetically analysed. None of the genes showed close relation to those of H5N1 HPAI viruses that were detected in wild birds found dead in Eurasia on the way back to their northern territory in spring.

Avian influenza virus strains isolated from migratory ducks in the global surveillance have been stored for the use for vaccines and diag nosis. Vaccine is used to reduce virus shedding into the environment and as an optional measure in cases where the disease spreads widely, in addition to stamping out. In the present study, the efficacy of the vaccine was comparable to that prepared from genetically modified HPAI virus strain ΔRRRRK rg-A/whooper swan/Mongolia/3/2005 (H5N1), which is more antigenically related to the challenge virus strain, in chickens. Inactivated influenza virus vaccine prepared from a non-pathogenic inflenza virus strain A/duck/Hokkaido/Vac-1/2004 (H5N1) from the virus library conferred protective immunity to chickens against the challenge of antigenically drifted HPAI virus, A/whooper swan/Hokkaido/1/2008 (H5N1).

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Development of molecular diagnostic tools for canine taeniosis

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Taeniid cestodes are important zoonotic helminths responsible for human illness as well as severe economic losses in livestock industry around the world. Since canids often harbor several taeniid species and evacuate egg-contaminated feces, it is important to monitor these parasites prevalence for controlling the disease. Therefore, this study aimed to develop two hybridization assays targeting the mitochondrial gene NADH dehydrogenase subunit 1 (*nad1*) for differential diagnosis of taeniid eggs present in canid feces.

At the beginning of this study, partial sequences of the cestode *nad1* gene were aligned and new primers were designed based on conserved regions. Species-specific oligonucleotide probes for canine taeniid cestodes were then designed manually based on the variable region

between the conserved primers. Specifically, species-specific probes were designed for the *Taenia crassiceps*, *T. hydatigena*, *T. multiceps*, *T. ovis*, *T. taeniaeformis*, *Echinococcus granulosus*, *E. multilocularis* and *E. vogeli*. In PCR/dot blot assay, each probe showed high specificity as no cross-hybridization with any amplified *nad1* fragment was observed. Then, the assay was further evaluated using 49 taeniid egg-positive samples collected from dogs in Zambia. DNA from 5 to 10 eggs was extracted in each sample. Using the PCR/dot blot assay, the probes successfully detected PCR products from *T. hydatigena* in 42 samples, *T. multiceps* in 3 samples, and both species (mixed infection) in the remaining 4 samples.

Thereafter, one *Echinococcus* genus-specific and a taeniid cestode common oligonucleotide probe were designed. Using these probes in addition to 8 species-specific probes and *nad1* primers above mentioned, PCR/reverse line blot assay was carried out for the 8 species of Taeniid cestodes. In the result, all species-specific oligonucleotide probes specifically hybridized with PCR products from their targets, meanwhile the *Echinococcus* genus-specific probe and the common probe for taeniid cestodes hybridized with all from *Echinococcus* spp. and all from cestodes (including non-taeniid cestodes) used in this study, respectively.

In conclusion, the results indicate that both hybridization assays developed in this study are relatively rapid, simple and reliable alternative method to be used with field samples in laboratories where no DNA sequencer is available.

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Genetic analysis of the hooded phenotype in the rat

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The hooded phenotype showing non-pigmented hairs in the abdominal skin is one of the coat color phenotypes seen peculiarly in the laboratory rat. The hooded locus showing autosomal recessive inheritance has been mapped on Chr 14 and that the hooded phenotype receives modification by hooded-modifier gene showing linkage to the hooded locus. Thus, I have conducted genetic studies to identify a gene responsible for the hooded locus and genes responsible for hooded-modifiers.

In part 1 of this study, I narrowed critical region of the hooded locus and revealed that only *Kit* gene, known as a marker of melanocyte and one of coat color genes, exists in this region through genetic fine mapping using backcrosses from feral rat-derived inbred strain IS and hooded phenotype strain, LEA. Although a G to C transversion was observed in exon 2 of the *Kit* gene, it was synonymous substitution. Further, the expressions of *Kit* mRNA were not different in fetal neural tubes and neonatal and adult skins between IS and LEA rats. Furthermore, Kit-positive cells were observed in LEA rat abdominal skin in spite of the absence of melanin in this region. These results suggest that the synthesis of melanin is impaired possibly due to the malfunction of *Kit*-expressed melanocytes residing in the non-pigmented hair follicles of hooded phenotype rats. However, substantial

mutation of the *Kit* gene and the mechanisms by which Kit impairs the function of melanocytes in non-pigmented hair follicle remain unknown.

In part 2 of this study, I carried out genetic linkage studies using BN and LEA rats to clarify genetic control in the extent of the hooded phenotype. A genome-wide scan was conducted on 152 F_2 rats for linkage with ratio of pigmented coat area for the dorsal, ventral, and total regions. The result indicated that a major QTL was mapped to *D14Got40*, which is the microsatellite marker closely present to the hooded locus. In addition, another QTL, *D17Rat2* showing highly significant linkage was also detected on Chr 17 in dorsal region phenotype as well as a QTL showing suggestive linkage on Chr15 in ventral region phenotype. I further investigated a genome-wide scan for epistatic interactions and detected significant interactions between *D14Got40* and *D20Mit1*, and between *D14Got40* and *D17Rat2* in dorsal region phenotype. These results suggest that the hooded locus regulates the extent of the hooded phenotype with some modifier genes.

This study proposes that *Kit* is a strong candidate gene responsible for the hooded locus and some genetic loci modify the extent of the hooded phenotype, although the precise mechanisms of them are still unclear. Thus, further study is necessary to understand the mechanisms by which the hooded phenotype appears with various extent of pigmented ratio in the rat.

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Study on the mechanism of resistance to flaviviruses of oligoadenylate synthetase 1b (Oas1b) in mice

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The effects of a vitamin D deficient diet on chronic cadmium exposure in rats

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Itai-itai disease (IID) of humans is one of the most severe forms of chronic cadmium (Cd) intoxication occurring mainly in post-menopausal women and is characterized by osteoporosis with osteomalacia, renal tubular disorder, and renal anemia. Some researchers insist the major cause

of IID is not Cd but malnutrition, especially hypovitaminosis D. This prompted the present study in which the effects of vitamin D deficiency on Cd intoxication were investigated in ovariectomized (OX) female rats.

The effects of vitamin D deficiency on Cd intoxication were investigated in OX female rats. We administrated a low concentration of Cd chloride intravenously to OX female rats that were fed a vitamin D deficient diet (< 0.3 IU/g) or a normal diet (3 IU/g) 5 days a week for up to 50 weeks. The vitamin D deficient diet decreased serum concentrations of active and inactive forms of vitamin D, but did not affect the metabolism of the kidney or bone. Cd treatment alone induced a decrease in serum concentrations of vitamin D as well as renal dysfunction, renal anemia and abnormal bone metabolism. Osteoporosis with osteomalacia, tubular nephropathy, fibrous osteitis and bone marrow hyperplasia occurred following Cd treatment. In rats treated with Cd and administered a vitamin D deficient diet, the toxic effects of Cd on kidney, bone and hematopoiesis were enhanced in comparison to rats treated with the Cd and a normal diet. The present experiment demonstrated that hypovitaminosis D did not evoke IID by itself, but enhanced the toxicity of Cd in regard to osteoporosis with osteomalacia, renal tubular nephropathy and renal anemia.

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Study on evaluation methods for nutritional and reproductive conditions of the Japanese black bear (*Ursus thibetanus japonicus*) using samples from carcasses

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The Japanese black bear (*Ursus thibetanus japonicus*) is a subspecies of the Asiatic black bear (*Ursus thibetanus*), which is classified as a vulnerable species in the IUCN red list. In the Japanese red list, six local populations of the Japanese black bear are designated as threatened local populations, although the subspecies as a whole is not listed as threatened. The Japanese black bear is one of the largest mammals in forest ecosystems in Japan, and efforts in their conservation could contribute to the health of the forest ecosystems. On the other hand, as the distribution of the Japanese black bear has been expanding recently in most regions of Japan, intrusions of bears into human residential areas have become a social issue. Although solutions desirable for both humans and bears are being sought, a primary countermeasure, at present, is killing bears under permission by local authorities.

Biological information of the target species is basis for effective wildlife management. The necessary information varies depending on the goal of management, but nutritional and reproductive statuses are of the most essential pieces of information. There are a few approaches to studying nutritional and reproductive conditions of Japanese black bears. One is the

direct observation of wild bears. Another approach deals with captive bears. The third approach is research on killed bears. These approaches are not mutually exclusive, but complement each other. Bear carcasses are precious sources of biological information. Exploring their effective uses should not be left behind in research of the Japanese black bear. The purpose of this study is to establish evaluation methods for nutritional and reproductive conditions of the Japanese black bear using samples from carcasses. I also kept in mind that the evaluation methods should fit the sample collection through administrative damage control kills, and contribute to population monitoring and better understanding of intrusion mechanisms.

I employed three body fat indices as indicators of nutritional conditions: femur marrow fat (FMF), modified kidney fat index (mKFI), and abdominal subcutaneous fat (ASF). I measured these indices on samples from bears killed in Gifu Prefecture, 2006, and revealed the age dependency, sex dependency and seasonality of the indices. All the 3 body fat indices of cubs were significantly lower than those of the other age classes. mKFI and ASF in late fall (November and December) were significantly higher than those in summer (July-September). ASF of females was significantly lower than that of males. I examined the preferential order of catabolism among different fat depots, and found that low FMF suggests a poor nutritional condition. Based on these results, I statistically analyzed the yearly change in the body fat indices of bears killed in Gifu and Fukushima prefectures during the summers (July-September) of 2005–2007, and investigated their relation to the number of control-killed bears. Since a negative correlation has been reported between food abundance in bear habitat and the number of control-killed bears, I hypothesized that the nutritional condition of killed bears in years with more killed bears is poorer than in years with fewer killed bears. However, I found that the number of killed bears was higher in one year with better nutritional conditions of bears. This suggests that a poorer nutritional condition does not necessarily lead to more intrusions of bears into human residential areas.

To establish a method for reproductive evaluation, I observed corpora lutea, corpora albicantia and placental scars in females killed in several prefectures on Honshu Island from 2001 to 2009. I examined the postpartum durations of placental scars and corpora albicantia, and found that placental scars remained at least until November, and that, under the present study's method of histological examination, corpora albicantia were $> 95\%$ detectable until August and became undetectable around February of the next year. From these results, I developed a method for determining the parturition history of females in the year of their capture, which constituted a foundation for new reproductive evaluation methods. To assess the mating season and the age of first ovulation in the wild, I examined the monthly and age-specific changes in the proportion of females with corpora lutea. I found that most females had finished ovulation by August, and that the age at which most females began to ovulate (standard age of first ovulation) was 4 years, with some precocious ovulations at as early as 2 years of age. I introduced two parameters for new reproductive evaluation: the success rate of ovulation (SRO; the probability that solitary females over the standard age of first ovulation actually succeed in ovulation) and early litter loss rate (ELLR; the probability that parenting females lose all of their cubs before the mating season). I established methods for estimating those parameters from the presence or absence of placental scars, corpora albicantia and corpora lutea. The estimates of those parameters were SRO $= 0.93$ and ELLR $= 0.29$. I also examined the age dependency of ovulation success (SRO $\times$ ovulation rate), and found it almost constant among females ≥ 4 years old. The ovulation success of females < 4 years old was found to be

lower than that of females ≥ 4 years old, but ovulation rates were similar.

In the present study, I provided an essential guideline for the evaluation of nutritional conditions of the Japanese black bear, and established new methods for reproductive evaluation such as the determination of parturition history and new reproductive parameters of SRO and ELLR. I believe that these achievements will bring a new dimension to research and management of the Japanese black bear.

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Study on C-type lectin-mediated entry of filoviruses into cells

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The glycoprotein (GP) of filoviruses is responsible for virus entry into cells. Cellular C-type lectins have been reported to recognize glycans on GP and to enhance filovirus infection into cells. Since primary target cells of filoviruses, such as macrophages, dendritic cells, and hepatocytes, express C-type lectins, C-type lectin-mediated entry is thought to be a possible determinant of virus tropism and pathogenesis.

However, the role of C-type lectins during filovirus entry is not clearly known. In the first chapter of this thesis, whether interaction between C-type lectins and GP mediates all the steps of virus entry (i.e., attachment, internalization, and membrane fusion) was studied. Vesicular stomatitis viruses (VSVs) pseudotyped with mutant GPs that have impaired structures of the putative receptor binding regions were generated to analyze virus entry independent from putative receptor (s) but not independent from C-type lectins. The mutant GPs reduced ability of the viruses to infect the monkey kidney cells that are routinely used for virus propagation. Infectivities of viruses with the mutant GPs dropped in C-type lectin-expressing cells, parallel with those in the monkey kidney cells, whereas binding activities of these GPs to the C-type lectins were not correlated with the reduced infectivities. The C-type lectins could not mediate all the steps of virus entry independently and therefore, C-type lectin-mediated entry of filoviruses requires other cellular molecule (s) that may be involved in virion internalization or membrane fusion.

In the second chapter of the present thesis, possible correlation between C-type lectin-mediated entry and pathogenesis of filovirus was discussed. Using VSV pseudotype system, the efficiencies of C-type lectin-mediated entry of Marburg virus strains, Angola and Musoke, were compared. VSV pseudotyped with Angola GP (VSV-Angola) infected cells expressing the C-type lectin, hMGL or DC-SIGN, more efficiently than VSV pseudotyped with Musoke GP (VSV-Musoke). Unexpectedly, the binding affinity of the C-type lectins to the carbohydrate on GPs did not correlated with the different efficiency of C-type lectin-mediated entry. Site-directed mutagenesis identified the amino acid at position 547, which switched the efficiency of C-type

lectin-mediated entry. In a three-dimensional model of GP, this amino acid was in close proximity to the putative site of cathepsin processing. Interestingly, the cathepsin inhibitors reduced the infectivity of VSV-Angola less efficiently than that of VSV-Musoke in the C-type lectin-expressing cells, whereas only a limited difference was found in control cells. The amino acid at position 547 was critical for the different effects of the inhibitors on the virus infectivities. Thus, the possible contribution of cellular proteases to the efficiency of C-type lectin-mediated entry was suggested.

In conclusion, the present study showed that the efficiency of C-type lectin-mediated entry of filoviruses is controlled not only by binding affinity between C-type lectins and GP but also by mechanisms underlying endosomal entry such as proteolytic processing by the cathepsins. The contribution of C-type lectin-mediated entry to filovirus pathogenicity in vivo should be demonstrated.

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Studies on MHC class I-mediated entry of equine herpesvirus-1 into cells

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Equine herpesvirus 1 (EHV-1) is an endemic virus that affects horse populations worldwide, causing respiratory disease, abortion and a serious neurologic disease known as encephalomyelitis. Outbreaks of EHV-1 encephalomyelitis can have a severe economic impact on farms, racetracks and veterinary hospitals, and currently available vaccines are not effective to protect against EHV-1 encephalomyelitis. Histological analyses of affected horses indicate that viral infection of vascular endothelial cells leads to damage of the central nervous system due to the resulting shortage of blood supply. Although infection of these cells is closely associated with the development of encephalomyelitis, the molecular mechanisms underlying this association remain poorly understood.

Alphaherpesviruses enter target cells through sequential multistep processes. Following the initial attachment of the viruses to the cell surface, binding of viral glycoproteins to cell surface receptors triggers fusion of the viral envelope with the cell membrane, resulting in the release of viral capsid (containing viral genome) into the cytoplasm.

EHV-1 attaches to cell surface using an interaction between viral glycoprotein C (gC) and cell surface heparan sulfate. Although the role of gC is important for effective infection, it does not trigger viral entry into cells. Glycoprotein D (gD) of EHV-1 is known to be essential for EHV-1 entry into cells. However, functional gD receptor that mediates EHV-1 entry into equine central nervous system (CNS) endothelial cells remains unidentified.

In chapter 1, I performed functional cloning using an equine brain microvascular endothelial cell cDNA library and identified that equine major histocompatibility complex (MHC) class I

heavy chain conferred susceptibility to EHV-1 infection in mouse NIH3T3 cells, which are resistant to EHV-1 infection. I investigated the role of equine MHC class I in EHV-1 infection and revealed that equine MHC class I acts as an entry receptor for EHV-1 through its binding to EHV-1 gD. I further investigated the role of MHC class I in EHV-1 entry into different types of equine cells.

In chapter 2, I constructed equine MHC class I heavy chain specific-RNA probe and performed Northern hybridization to examine the level of equine MHC class I gene expression in the major organs of the adult horse body. The localization of equine MHC class I mRNA in brain tissue was investigated by *in situ* hybridization.

In this thesis, I demonstrated equine MHC class I is a bona fide receptor of EHV-1 entry into equine cells. This study provides new insights into the mechanism of EHV-1 entry into host cells and a potential way to treat and prevent this infectious disease.

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Requirement of Siva-1 for replication of influenza A virus through apoptosis induction

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Influenza A virus is an enveloped RNA virus belonging to the *Orthomyxoviridae*, and its genome consists of eight segmented negative-sense RNAs which encode viral specific proteins. An RNA polymerase complex of the influenza A virus consists of PA, PB1, and PB2, and is responsible for the transcription of viral RNA, viral mRNA, and viral complementary RNA. Previous reports using the highly pathogenic H5N1 subtype avian influenza A virus, which was isolated from patients in Hong Kong in 1997 during the outbreak of H5N1 avian influenza A virus epidemic, demonstrated that mutation of the PB2 protein is correlated with the effective replication of the avian influenza viruses in mammalian cells.

To find host-cellular molecules which bind to the influenza A virus PB2, a screening using an antibody array was performed, and Siva-1 was identified as a candidate for the PB2 binding protein. The result of a co-immunoprecipitation assay indicates that the PB2 actually binds to Siva-1 in HEK293T cells. In addition, the subcellular localization of Siva-1 in the A549 cells which were infected with A/Puerto Rico/8/34 (PR8; H1N1) strain of influenza A virus was mainly observed in the nucleus overlapped with that of PB2 nearly completely.

Siva-1 is known to be involved in induction of various types of apoptosis. To investigate whether Siva-1 expression affects the apoptosis induced by infection with the influenza A virus, a cell line which stably expresses Siva-1 was established by Flp-in system using the A549 cells. The results of phase-contrast microscopy and the trypan blue exclusion assay indicate that the cells which stably express Siva-1 clearly showed a higher susceptibility to cell death caused by the infection with influenza A virus than the control cells. The results obtained by

the DNA fragmentation assay show that the expression of Siva-1 significantly enhanced the DNA fragmentation, suggesting that Siva-1 mediated cell death is caused by the progression of apoptosis.

Next, to investigate the function of endogenously expressed Siva-1 on cells infected with influenza A virus, retrovirus vectors for expression of short hairpin RNAs (shRNAs) to effectively silence the Siva-1 mRNA were constructed. The influenza A virus PR8 strain was infected to the Siva-1 knockdown A549 cells, and cell viability was measured by the trypan blue exclusion assay. The results show that these cells were not subject to apoptosis induced by influenza A virus infection by the knockdown of Siva-1 gene expression. Interestingly, the virus titer in the cells was significantly decreased by the knockdown of the Siva-1 gene expression. However, the results of real-time RT-PCR analysis indicated that transcription of influenza A virus RNAs was not significantly affected by the Siva-1 gene knockdown. It has been reported that activation of caspase-3 is involved in the effective export of the viral ribonucleotide protein complex from the nucleus to the cytoplasm. Therefore, the effect of caspase inhibition on the Siva-1 function for the virus replication was investigated using a pan-caspase inhibitor, Z-VAD-FMK. The results show that the difference in the viral replication between the control and the Siva-1 knockdown cells disappeared by the treatment of Z-VAD-FMK.

In conclusion, the results of the present study demonstrated that Siva-1, identified as a PB2 binding protein, is crucial for the induction of apoptosis caused by the infection with influenza A virus, and is also important for viral replication though the activation of caspase.

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