RESEARCH ACTIVITIES

Department of Microbiology and Immunology

Division of Host-Parasite Interaction
宿主寄生体学

Professor	Hideo Iba, Ph.D.	教　授　理学博士	伊　庭　英　夫
Assistant Professor	Takeshi Haraguchi, Ph.D.	助　教　理学博士	原　口　　健
Assistant Professor	Kyousuke Kobayashi, D.V.M., Ph.D.	助　教　獣医学博士	小　林　郷　介
Assistant Professor	Hiroaki Ito, Ph.D.	助　教　理学博士	伊　藤　寛　明

The goal of our Department is to elucidate the cellular defense system and the counteracting viral strategy at the level of gene regulation and to establish new approaches for suppressing cancer and pathogenic viruses and also for modulating human immunological response. We have been studying epigenetical regulation of human and virus genomes by analyzing regulatory networks formed among microRNAs, chromatine remodeling factor, SWI/SNF complex and such important transcriptional factors as AP-1 and NF-kappaB to explore the cause of human diseases. We have developed and are continuously improving retrovirus/lentivirus vectors that express efficient inhibitory RNAs targeting specific miRNAs (designated as Tough Decoy RNAs) and low molecular inhibitors of miRNA (designated synthetic TuD; S-TuD) for tools of human gene therapy and basic researches.

1. Regulatory networks formed among miRNA, transcription factors and Brm type-SWI/SNF complex.

The SWI/SNF chromatin remodeling complex plays important roles in the epigenetic regulation of many organisms and regulates a wide variety of genes. In mammals, this complex is an assembly of about nine polypeptides, and each complex contains a single molecule of either Brm or BRG1, but not both. These two proteins are the catalytic subunits and drive the remodeling of nucleosomes via their ATP-dependent helicase activity. Evidence has now accumulated that Brm and BRG1 regulate a set of target promoters that is not fully overlapping. Indeed, Brm and BRG1 show clear differences in their biological activities; Brm, but not BRG1, is essential for the maintenance of gene expression driven by LTRs of murine leukemia virus (MLV) and HIV as we have previously reported. This year, we described detailed mechanisms involved in the elongation steps of short HIV transcripts (a).

In 2001, we showed that BAF60a, a subunit of the SWI/SNF chromatin remodeling complex, is a determinant of the transactivation potential of Fos/Jun dimers. BAF60a binds to a specific subset of Fos/Jun heterodimers using two different interfaces for c-Fos and c-Jun, respectively, explaining why a specific subset of Fos/Jun dimers (containing c-Fos and/or c-Jun) recruits SWI/SNF complex to AP-1 binding sites present in many gene promoters via BAF60a to drastically induce their transcription simultaneously. Like AP-1, NFκB plays crucial roles in such physiological processes as development, cell proliferation viral replication, apoptosis and innate and adaptive immune functions. The NFκB family is composed of five different proteins; RelA (p65), RelB, c-Rel, p50 (which is processed its precursor p105) and p52 (which is processed its precursor p100). These proteins form active transcription factors as homodimers or heterodimers. Importantly, some NFκB target genes stimulated by these cytokines or growth factors were often suggested to require SWI/SNF complexes for their optimum induc-

tion. However, the underlying molecular mechanisms and factors involved in this process are largely unknown, mainly because no direct interaction between NFκB submits and SWI/SNF components has been reported. In 2011, we present evidence that DPF2 (Requiem) protein, which belongs to the d4-family of proteins, is a specific adaptor protein that links RelB/p52 with Brm-type SWI/SNF complexes and thereby plays pivotal roles in the most downstream non-canonical NFκB pathway. Using highly sensitive assay cell lines that harbor two NF-κB binding sites and a minimal promoter just upstream of the reporter gene, we further found that DPF1, DPF2, DPF3a, DPF3b and PHF10 potentially activates three representative NF-κB dimmers, RelA/p50, RelB/p52 and c-Rel/p50 transactivation significantly, when they are exogenously expressed at very high levels. We finally showed that among them, DPF3a and DPF3b are most critical component for NF-κB RelA/p50 heterodimer transactivation induced by TNF-α stimulation (canonical pathway).

We further found however that a functional *Brm* gene is present and transcribed actively in all of the Brm-deficient human cancer cell lines in nuclear run-on transcription assays. This indicated that post-transcriptional gene silencing suppresses *Brm* in these human cancer cells. In 2011, we demonstrated that *Brm* mRNA is a target of miR-199a-5p and miR-199a-3p, both of which are processed from pre-miR-199a. By promoter analysis of the *miR-199a-2* gene, which was found to be the main contributor to the production of pri-miR-199a in these cell lines, Egr1 was identified as a major transcriptional activator at this gene locus. Our analysis further showed that the expression patterns of mature miR-199a-5p and -3p, and of the Brm protein, are mutually exclusive in many human tumor cell lines originated from epithelial cells. We final showed that these miRNAs, miR-199a-5p/-3p, Brm and Egr1 form double-negative feedback loops in a wide variety of human cancer cell lines, allowing them to be categolized into two cancer cell types: [Brm (+)/Egr1(−)1/miR-199 (−)] cells and [Brm (−)/Egr1 (+)/miR-199a (+)] cells, which are denoted hereafter as Type 1 and Type 2 cells, respectively. This year, we have analyzed basic biological properties that discuminate these two cell types and revealed that the robust regulatory network formed by Brm/miR-199a axis is very important to understand molecular mechanisms involved in either cancer development (b) or virus replication (c).

(a) 7SK small nuclear ribonucleoprotein complex is recruited to the HIV-1 promoter via short viral transcripts

Taketoshi Mizutani[1,2], Aya Ishizaka[1,2], Yutaka Suzuki[3], and Hideo Iba[1]: [1]Division of Host-Parasite Interaction, Institute of Medical Science, The University of Tokyo, [2]Laboratory of Basic Science, Institute of Microbial Chemistry; BIKAKEN, [3]Department of Medical Genome Sciences, Graduate School of Frontier Sciences, The University of Tokyo

We here demonstrate that the 7SK small nuclear ribonucleoprotein (snRNP) complex is recruited to the HIV-1 promoter via HIV-1 nascent transcripts (short transcripts) in an hnRNP A1-dependent manner and negatively regulates transcriptional elongation. Our deep-sequence analysis showed these short transcripts were mainly arrested at approximately +50 to +70 nucleotides from the transcriptional start site in HIV-1 latent model, U1 cells. TNF-α treatment promptly disrupted the 7SK snRNP complex on the nascent transcripts and viral elongated transcripts were increased. This report provides insight into how 7SK snRNP complex is recruited to HIV-1 promoter in the absence of Tat.

(b) Characterization of two cancer cell types that utilize distinct regulatory loops formed by miR-199a and Brm.

Kazuyoshi Kobayashi, Kouhei Sakurai, Kyosuke Kobayashi, Hiroaki Hiramatsu, Ken-ichi Inada[4], Kazuya Shiogama[4], Aya Ishizaka, Jessica Chang, Takeshi Haraguchi, Yutaka Tsutsumi[4], and Hideo Iba. First Department of Pathology, Faculty of Medicine, Fujita Health University, Aichi

We here evaluated the biological properties and gene expression patterns specific to Type 1 and 2 cell lines originated from human cancer cell lines. We found that Type 1 cells specifically exhibited efficient anchorage-independent growth and expressed high levels of the miR-199a-3p target, *CD44*, and such inflammatory cytokine genes as IL-6 and IL-8, all of which are transactivated through NF-κB in a Brm type SWI/SNF complex-dependent manner. These results indicate that regulatory loops formed among Brm/miR-199a axis function as molecular switches to establish the specific gene expression patterns in Type 1 and 2 cancer cells. We have evidence that these Type 1 specific proteins contribute for efficient anchorage-independent growth of Type 1 cells. Therefore, the typing according to expression status of Brm and miR-199a will be useful for cancer diagnosis and for guiding the development of distinct therapeutic strategies in each case.

(c) miR-199a plays important roles in host cell competency for virus replication.

Kyosuke Kobayashi, Shinya Nakamura, Fumiko Suemasa, Kazuyoshi Kobayashi, Hiroaki Hira-

matsu, Takeshi Haraguchi, and Hideo Iba

It was previously reported that using global miRNA expression analysis, the miR-199a/miR-214 cluster manifests antiviral properties in mouse and human cells, and further that these molecules confer broad inhibitory potential against multiple viruses. Since in human cancer cell lines originated from epitherial cells, miR-199a/miR-214 expressing cells lacks Brm expression (Type 2 cells) as described above, our previous report that Type 2 cells (expressing miR-199a/miR-214 at high levels) do not stably express lenti/retrovirus is consistent with their report.

Using human cancer cell lines infected with HSV-1, as a model system, we have confirmed that high level exogenous expression of miR-199a-3p in Type 1 cells suppresses HSV-1 replication, whereas suppression of miR-199a-3p in Type 2 cells activated HSV-1 replication. Considering specific gene expression pattern of either type 1 and type 2 cells, we are now analyzing cellular regulatory networks involved in the determination of cellular competency for virus replication.

2. Development new regulatable expression vectors for decoy RNAs (TuD) which strongly inhibit function of specific miRNAs.

The development of reagents that strongly suppress specific miRNAs will be important for both basic miRNA research and also as a possible therapeutic strategy. To achieve the long-term suppression of a specific miRNA, we developed specialized plasmid- and virus- vectors carrying expression units for inhibitory RNA molecules, which we have termed Tough Decoy (TuD) RNA. This year, we have developed several regulatable promoters designed for TuD production (d).

TuD RNA is a single RNA molecule with a complex secondary structure composed of four elements: a stem of 18bp in length (Stem 1), two miRNA binding sites (MBSs) that have a sequence complementary to that of a mature miRNA of interest, a stem-loop structure which connects two MBSs (Stem 2), four linkers with three nucleotides connecting the two MBSs, and the flanking stems. These elements provide efficient nuclear export, binding to the target miRNA, resistance to cellular nucleases and enhancement of the MBS accessibility to the target miRNA, respectively. By screening several alternative MBS sequences to optimize the decoy activity, we have further identified a highly potent TuD, the MBS of which has a 4 nucleotide insertion between positions 10 and 11 from the 3' end of the perfectly complementary sequence to the entire mature miRNA of interest, where the Ago2-containing RISC cleaves target mRNAs. When these TuD RNAs were expressed by lentivirus vectors, they were shown to be efficiently transported to the cytoplasm and exhibit strong inhibitory effects for more than one month. To determine whether constitutive expression of a miRNA is required for its specific function or not, it is very important to develop inducible promoters. This year, we have started to develop such regulatable promoters driven by RNA polymerase II or III (a).

(a) Development of Regulatable vectors for TuD RNA expression.

Takeshi Haraguchi, Masayuki Kondo, Hideo Iba

Since RNA polymerase II promoters are regulatable through several regulatory elements that are either activated or suppressed in stimuli and tissued specific manners. We first constructed Pol II driven CAG promoter as a model system. From the promoter, a transcript containing several tandem repeats of TuD RNA were designed to be produced. Whereas 3 tandem repeats of TuD have strongest inhibitory effect among them, it was still weaker than that of TuD RNA produced from Pol III 7SK promoters. We have next developed Tet-ON regulatory Pol III promoter system for TuD RNA production by constructing composite promoters between modified 7SK promoter and several Tet operator responsive elements. We finally obtained highly inducible a Pol III Tet- ON promoter, which would be useful tools to analyze whether continuous expression of a certain miRNA is required for its specific function.

Publications

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Department of Microbiology and Immunology

Division of Virology
ウイルス感染分野

Professor	Yoshihiro Kawaoka, D.V.M., Ph.D.	教　授	獣医学博士	河　岡　義　裕
Associate Professor	Takeshi Noda, D.V.M., Ph.D.	准教授	獣医学博士	野　田　岳　志
Assistant Professor	Kiyoko Iwatsuki-Horimoto, D.V.M., Ph.D.	助　教	獣医学博士	岩附(堀本)研子
Assistant Professor	Shinya Yamada, Ph.D.	助　教	医学博士	山　田　晋　弥
Project Assistant Professor	Maki Kiso, D.V.M., Ph.D.	特任助教	医学博士	木　曽　真　紀
Project Assistant Professor	Seiya Yamayoshi, D.V.M., Ph.D.	特任助教	医学博士	山　吉　誠　也
Research Associate	Yuko Sakai-Tagawa, Ph.D.	助　手	医学博士	坂井(田川)優子

Viruses can cause devastating diseases. The long-term goal of our research is to understand the molecular pathogenesis of viral diseases, using influenza and Ebola virus infections as models. Interactions between viral and host gene products during viral replication cycles determine the consequences of infection (i.e., the characteristics of disease manifestation, whether limited or widespread); hence, our research has centered on such interactions in theses viral infections.

1. Virulence determinants of pandemic A(H1N1) 2009 virus in a mouse model.

Uraki R, Kiso M, Shinya K[1], Goto H, Takano R, Iwatsuki-Horimoto K, Takahashi K, Daniels RS[2], Hungnes O[3], Watanabe T[4], Kawaoka Y: [1]Division of Zoonosis Department of Microbiology and Infectious Diseases, Graduate School of Medicine, Kobe University, Japan, [2]Virology Division, MRCNational Institute for Medical Research, United Kingdom, [3]Department of Virology, Norwegian Institute of Public Health, Norway. [4]ERATO KAWAOKA Infection-Induced Host Responses Project, Japan Science and Technology, Japan.

A novel swine-origin H1N1 influenza virus [A(H1N1)pdm09 virus] caused the 2009 influenza pandemic. Most patients exhibited mild symptoms similar to seasonal influenza, but some experienced severe clinical signs and, in the worst cases, died. Such differences in symptoms are generally associated with preexisting medical conditions, but recent reports indicate the possible involvement of viral factors in clinical severity. To better understand the mechanism of pathogenicity of the A(H1N1)pdm09 virus, here, we compared five viruses that are genetically similar but were isolated from patients with either severe or mild symptoms. In a mouse model, A/Norway/3487/2009 (Norway3487) virus exhibited greater pathogenicity than did A/Osaka/164/2009 (Osaka164) virus. By exploiting reassortant viruses between these two viruses, we found that viruses possessing the hemagglutinin (HA) gene of Norway3487 in the genetic background of Osaka164 were more pathogenic in mice than other reassortant viruses, indicating a role for HA in the high virulence of Norway3487 virus. Intriguingly, a virus possessing HA, NA, and NS derived from Norway3487 exhibited greater pathogenicity in mice in concert with PB2 and PB1 derived from Osaka164 than did the parental Norway3487 virus. These findings demonstrate that reassortment between A(H1N1)pdm09 viruses can lead to increased pathogenicity and highlight the need for continued surveillance of A(H1N1)pdm09 viruses.

2. Identification of novel influenza A virus proteins translated from PA mRNA.

Muramoto Y, Noda T, Kawakami E, Akkina R[5], Kawaoka Y: [5]Department of Microbiology, Immunology and Pathology, Colorado State University, USA.

Many replication events are involved in the influenza A virus life cycle, and they are accomplished by different virus proteins with specific functions. However, because the size of the influenza virus genome is limited, the virus uses different mechanisms to express multiple viral proteins from a single gene segment. The M2 and NS2 proteins are produced by splicing, and several novel influenza A virus proteins, such as PB1-F2, PB1-N40, and PA-X, have recently been identified. Here, we identified novel PA-related proteins in influenza A virus-infected cells. These newly identified proteins are translated from the 11th and 13th inframe AUG codons in the PA mRNA and are, therefore, N-terminally truncated forms of PA, which we named PA-N155 and PAN182, respectively. The 11th and 13th AUG codons are highly conserved among influenza A viruses, and the PA-N155 and PAN182 proteins were detected in cells infected with various influenza A viruses isolated from different host species, suggesting the expression of these N-truncated PAs is universal in nature among influenza A viruses. These N-truncated PAs did not show polymerase activity when expressed together with PB1 and PB2; however, mutant viruses lacking the N-truncated PAs replicated more slowly in cell culture and had lower pathogenicity in mice than did wild-type virus. These results suggest that these novel PA-related proteins likely possess important functions in the replication cycle of influenza A virus.

3. 1918 HA and the viral RNA polymerase complex enhance viral pathogenicity, but only HA induces aberrant host responses in mice.

Watanabe T[4], Tisoncik-Go J[6], Tchitchek N[6], Watanabe S[4], Benecke AG[6,7], Katze MG[6], Kawaoka Y: [6]Department of Microbiology, School of Medicine, University of Washington, USA, [7]Université Pierre et Marie Curie, Centre National de la Recherche Scientifique, France.

The 1918 pandemic influenza virus was the most devastating infectious agent in human history, causing fatal pneumonia and an estimated 20 to 50 million deaths worldwide. Previous studies indicated a prominent role of the hemagglutinin (HA) gene in efficient replication and high virulence of the 1918 virus in mice. It is, however, still unclear whether the high replication ability or the 1918 influenza virus HA gene is required for 1918 virus to exhibit high virulence in mice. Here, we examined the biological properties of reassortant viruses between the 1918 virus and a contemporary human H1N1 virus (A/Kawasaki/173/2001 [K173]) in a mouse model. In addition to the 1918 influenza virus HA, we demonstrated the role of the viral RNA replication complex in efficient replication of viruses in mouse lungs, whereas only the HA gene is responsible for lethality in mice. Global gene expression profiling of infected mouse lungs revealed that the 1918 influenza virus HA was sufficient to induce transcriptional changes similar to those induced by the 1918 virus, despite difference in lymphocyte gene expression. Increased expression of genes associated with the acute-phase response and the protein ubiquitination pathway were enriched during infections with the 1918 and 1918HA/K173 viruses, whereas reassortant viruses bearing the 1918 viral RNA polymerase complex induced transcriptional changes similar to those seen with the K173 virus. Taken together, these data suggest that HA and the viral RNA polymerase complex are critical determinants of Spanish influenza pathogenesis, but only HA, and not the viral RNA polymerase complex and NP, is responsible for extreme host responses observed in mice infected with the 1918 influenza virus.

4. Protective efficacy of orally administered, heat-killed Lactobacillus pentosus b240 against influenza A virus.

Kiso M, Takano R, Sakabe S, Katsura H, Shinya K[1], Uraki R, Watanabe S[4], Saito H[8], Toba M[8], Kohda N[8], Kawaoka Y: [8]Otsu Nutraceuticals Research Institute, Nutraceuticals Division, Otsuka Pharmaceutical Co., Ltd., Japan.

Influenza A(H1N1)pdm virus caused the first human pandemic of the 21st century. Although various probiotic Lactobacillus species have been shown to have anti-microbial effects against pneumonia-inducing pathogens, the prophylactic efficacy and mechanisms behind their protection remain largely unknown. Here, we evaluated the prophylactic efficacy of heat-killed Lactobacillus pentosus b240 against lethal influenza A(H1N1)pdm virus infection in a mouse model. To further define the protective responses induced by b240, we performed virologic, histopathologic, and transcriptomic analyses on the mouse lungs. Although we did not observe an appreciable effect of b240 on virus growth, cytokine production, or histopathology, gene expressional analysis revealed that oral administration of b240 differentially regulates antiviral gene expression in mouse lungs. Our results unveil the possible mechanisms behind the protection mediated by b240 against influenza virus infection and provide new insights into probiotic therapy.

5. A novel bivalent vaccine based on a PB2-knockout influenza virus protects mice from pandemic H1N1 and highly pathogenic H5N1 virus challenges.

Uraki R, Kiso M, Iwatsuki-Horimoto K, Fukuyama S[4], Takashita E[9], Ozawa M[10,11], Kawaoka Y: [9]Influenza Virus Research Center, National Institute of Infectious Diseases, Japan, [10]Laboratory of Animal Hygiene, Joint Faculty of Veterinary Medicine, Kagoshima University, Japan, [11]Transboundary Animal Diseases Center, Joint Faculty of Veterinary Medicine, Kagoshima University, Japan.

Vaccination is an effective means to protect against influenza virus. Although inactivated and live-attenuated vaccines are currently available, each vaccine has disadvantages (e.g., immunogenicity and safety issues). To overcome these problems, we previously developed a replication-incompetent PB2-knockout (PB2-KO) influenza virus that replicates only in PB2 protein-expressing cells. Here, we generated two PB2-KO viruses whose PB2-coding regions were replaced with the HA genes of either A/California/04/2009 (H1N1pdm09) or A/Vietnam/1203/2004 (H5N1). The resultant viruses comparably, or in some cases more efficiently, induced virus-specific antibodies in the serum, nasal wash, and bronchoalveolar lavage fluid of mice relative to a conventional formalin-inactivated vaccine. Furthermore, mice immunized with these PB2-KO viruses were protected from lethal challenges with not only the backbone virus strain but also strains from which their foreign HAs originated, indicating that PB2-KO viruses with antigenically different HAs could serve as bivalent influenza vaccines.

6. Characterization of H7N9 influenza A viruses isolated from humans.

Watanabe T[4], Kiso M, Fukuyama S[4], Nakajima N[12], Imai M[9], Yamada S, Murakami S, Yamayoshi S, Iwatsuki-Horimoto K, Sakoda Y[13], Takashita E[9], McBride R[14], Noda T, Hatta M[15], Imai H[15], Zhao D[4], Kishida N[9], Shirakura M[9], de Vries RP[14], Shichinohe S[13], Okamatsu M[13], Tamura T[13], Tomita Y[4], Fujimoto N[4], Goto K[4], Katsura H, Kawakami E, Ishikawa I[4], Watanabe S[4], Ito M, Sakai-Tagawa Y, Sugita Y, Uraki R, Yamaji R, Eisfeld AJ[15], Zhong G[15], Fan S[15], Ping J[15], Maher EA[15], Hanson A[15], Uchida Y[16], Saito T[16], Ozawa M[10,11], Neumann[15], Kida H[13,16], Odagiri T[9], Paulson JC[14], Hasegawa H[12], Tashiro M[9], Kawaoka Y: [12]Department of Pathology, National Institute of Infectious Diseases, Japan, [13]Laboratory of Microbiology, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Japan, [14]The Scripps Research Institute, USA, [15]Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin-Madison, USA, [16]Influenza and Prion Disease Research Center, National Institute of Animal Health, Japan, [17]Research Center for Zoonosis Control, Hokkaido University, Japan.

Avian influenza A viruses rarely infect humans; however, when human infection and subsequent human-to-human transmission occurs, worldwide outbreaks (pandemics) can result. The recent sporadic infections of humans in China with a previously unrecognized avian influenza Avirus of the H7N9 subtype (A(H7N9)) have caused concern owing to the appreciable case fatality rate associated with these infections (more than 25%), potential instances of human-to-human transmission1, and the lack of pre-existing immunity among humans to viruses of this subtype. Here we characterize two early human A(H7N9) isolates, A/Anhui/1/2013 (H7 N9) and A/Shanghai/1/2013 (H7N9); hereafter referred to as Anhui/1 and Shanghai/1, respectively. In mice, Anhui/1 and Shanghai/1 were more pathogenic than a control avian H7N9 virus (A/duck/Gunma/466/2011 (H7N9);Dk/GM466) and a representative pandemic 2009 H1N1 virus (A/California/4/2009 (H1N1pdm09); CA04). Anhui/1, Shanghai/1 and Dk/GM466 replicated well in the nasal turbinates of ferrets. In nonhuman primates, Anhui/1 and Dk/GM466 replicated efficiently in the upper and lower respiratory tracts, whereas the replicative ability of conventional human influenza viruses is typically restricted to the upper respiratory tract of infected primates. By contrast, Anhui/1 did not replicate well in miniature pigs after intranasal inoculation. Critically, Anhui/1 transmitted through respiratory droplets in one of three pairs of ferrets. Glycan arrays showed that Anhui/1, Shanghai/1 and A/Hangzhou/1/2013 (H7N9) (a third human A (H7N9) virus tested in this assay) bind to human virus-type receptors, a property that may be critical for virus transmissibility in ferrets. Anhui/1 was found to be less sensitive in mice to neuraminidase inhibitors than a pandemic H1N1 2009 virus, although both viruses were equally susceptible to an experimental antiviral polymerase inhibitor. The robust replicative ability in mice, ferrets and nonhuman primates and the limited transmissibility in ferrets of Anhui/1 suggest that A(H7N9) viruses have pandemic potential.

7. The configuration of viral ribonucleoprotein complexes within the influenza A virion.

Sugita Y, Sagara H[17], Noda T, Kawaoka Y: [17]Medical Proteomics Laboratory, Institute of Medical Science, University of Tokyo, Japan.

The influenza A virus possesses an eight-seg-

mented, negative-sense, single-stranded RNA genome (vRNA). Each vRNA segment binds to multiple copies of viral nucleoproteins and a small number of heterotrimeric polymerase complexes to form a rod-like ribonucleoprotein complex (RNP), which is essential for the transcription and replication of the vRNAs. However, how the RNPs are organized within the progeny virion is not fully understood. Here, by focusing on polymerase complexes, we analyzed the fine structure of purified RNPs and their configuration within virions by using various electron microscopies (EM). We confirmed that the individual RNPs possess a single polymerase complex at one end of the rod-like structure and that, as determined using immune EM, some RNPs are incorporated into budding virions with their polymerase-binding ends at the budding tip, whereas others align with their polymerase-binding ends at the bottom of the virion. These data further our understanding of influenza virus virion morphogenesis.

8. A comprehensive map of the influenza A virus replication cycle.

Matsuoka Y[4,18], Matsumae H[19], Katoh M[4], Eisfeld AJ[15], Neumann G[15], Hase T[18], Ghosh S[18], Shoemaker JE[4], Lopes TJ[4], Watanabe T[4], Watanabe S[4], Fukuyama S[4], Kitano H[4,18,20,21], Kawaoka Y: [18]The Systems Biology Institute, Japan, [19]Department of Bioinformatics, Medical Research Institute, Tokyo Medical and Dental University, Japan. [20]Sony Computer Science Laboratories, Inc., Japan., [21]Okinawa Institute of Science and Technology Graduate University, Japan.

Background: Influenza is a common infectious disease caused by influenza viruses. Annual epidemics cause severe illnesses, deaths, and economic loss around the world. To better defend against influenza viral infection, it is essential to understand its mechanisms and associated host responses. Many studies have been conducted to elucidate these mechanisms, however, the overall picture remains incompletely understood. A systematic understanding of influenza viral infection in host cells is needed to facilitate the identification of influential host response mechanisms and potential drug targets. Description: We constructed a comprehensive map of the influenza A virus ('IAV') life cycle ('FluMap') by undertaking a literature-based, manual curation approach. Based on information obtained from publicly available pathway databases, updated with literature-based information and input from expert virologists and immunologists, FluMap is currently composed of 960 factors (i.e., proteins, mRNAs etc.) and 456 reactions, and is annotated with ~500 papers and curation comments. In addition to detailing the type of molecular interactions, isolate/strain specific data are also available. The FluMap was built with the pathway editor CellDesigner in standard SBML (Systems Biology Markup Language) format and visualized as an SBGN (Systems Biology Graphical Notation) diagram. It is also available as a web service (online map) based on the iPathways+ system to enable community discussion by influenza researchers. We also demonstrate computational network analyses to identify targets using the FluMap. Conclusion: The FluMap is a comprehensive pathway map that can serve as a graphically presented knowledgebase and as a platform to analyze functional interactions between IAV and host factors. Publicly available webtools will allow continuous updating to ensure the most reliable representation of the host-virus interaction network. The FluMap is available at http://www.influenza-x.org/flumap/.

9. A replication-incompetent influenza virus bearing the HN glycoprotein of human parainfluenza virus as a bivalent vaccine.

Kobayashi H, Iwatsuki-Horimoto K, Kiso M, Uraki R, Ichiko Y, Takimoto T[22], Kawaoka Y: [22]Department of Microbiology and Immunology, University of Rochester Medical Center, U.S.A.

Influenza virus and human parainfluenza virus (HPIV) are major etiologic agents of acute respiratory illness in young children. Inactivated and live attenuated influenza vaccines are approved in several countries, yet no vaccine is licensed for HPIV. We previously showed that a replication-incompetent PB2-knockout (PB2-KO) virus that possesses a reporter gene in the coding region of the PB2 segment can serve as a platform for a bivalent vaccine. To develop a bivalent vaccine against influenza and parainfluenza virus, here, we generated a PB2-KO virus possessing the hemagglutinin-neuraminidase (HN) glycoprotein of HPIV type 3 (HPIV3), a major surface antigen of HPIV, in its PB2 segment. We confirmed that this virus replicated only in PB2-expressing cells and expressed HN. We then examined the efficacy of this virus as a bivalent vaccine in a hamster model. High levels of virus-specific IgG antibodies in sera and IgA, IgG, and IgM antibodies in bronchoalveolar lavage fluids against both influenza virus and HPIV3 were detected from hamsters immunized with this virus. The neutralizing capability of these serum antibodies was also confirmed. Moreover, the immunized hamsters were completely protected from virus challenge with influenza virus or HPIV3. These results indicate that PB2-KO virus expressing the HN of HPIV3 has the potential to be a novel bivalent vaccine against influenza and human parainfluenza viruses.

10. Virulence-affecting amino acid changes in the PA protein of H7N9 influenza A viruses.

Yamayoshi S, Yamada S, Fukuyama S[4], Murakami S, Zhao D[4], Uraki R, Watanabe T[4], Tomita Y[4], Macken C[23], Neumann G[15], Kawaoka Y: [23]Theoretical Division, Los Alamos National Laboratory, USA.

Novel avian-origin influenza A(H7N9) viruses were first reported to infect humans in March 2013. To date, 143 human cases, including 45 deaths, have been recorded. By using sequence comparisons and phylogenetic and ancestral inference analyses, we identified several distinct amino acids in the A(H7N9) polymerase PA protein, some of which may be mammalian adapting. Mutant viruses possessing some of these amino acid changes, singly or in combination, were assessed for their polymerase activities and growth kinetics in mammalian and avian cells and for their virulence in mice. We identified several mutants that were slightly more virulent in mice than the wild-type A (H7N9) virus, A/Anhui/1/2013. These mutants also exhibited increased polymerase activity in human cells but not in avian cells. Our findings indicate that the PA protein of A(H7N9) viruses has several amino acid substitutions that are attenuating in mammals.

Publications

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Department of Microbiology and Immunology

Division of Infectious Genetics
感染遺伝学分野

Professor	Kensuke Miyake, M.D., Ph.D.	教　授	医学博士　三　宅　健　介
Assistant Professor	Sachiko Akashi -Takamura, M.D., Ph.D.	助　教	医学博士　高村(赤司)祥子
Assistant Professor	Shin-Ichiroh Saitoh, Ph.D.	助　教	医学博士　齋　藤　伸一郎
Assistant Professor	Ryutaro Fukui, Ph.D.	助　教	医学博士　福　井　竜太郎

Immune cells express multiple Toll-like receptors (TLRs) that are concomitantly activated by a variety of pathogen products derived from microbes and viruses. TLRs also sense host derived products such as RNAs and DNAs. Recent reports have indicated that losing the balance of TLRs responses result in autoimmune diseases. Hence, there must exist regulatory mechanisms coordinating the expression and the function of TLRs to avoid excessive immune responses. Our research focuses on molecular mechanisms controlling pathogenic and endogenous ligand recognition by TLRs.

1. The Arf-like GTPase Arl8b drives TLR7 into type I interferon-mediated inflammation.

Shin-Ichiroh Saitoh[1], Kenji Kontani[3], Ryutaro Fukui[1], Fumiko Abe[3], Atsuo Kanno[1], Mabel Chan[1], Masahiro Onji[1], Yuji Motoi[1], Takuma Shibata[1,2], Katsuaki Sato[4], Toshiaki Katada[3], and Kensuke Miyake[1,2]: [1]Division of Infectious Genetics, Department of Microbiology and Immunology, [2]Laboratory of Innate Immunity, The Institute of Medical Science, The University of Tokyo, [3]Department of physiological Chemistry, Graduate School of Pharmaceutical Sciences, The University of Tokyo, [4]Division of Immunology, Department of Infectious Diseases, Faculty of Medicine, University of Miyazaki

Toll-like receptor 7 (TLR7) and 9, innate immune sensors for microbial RNA or DNA, erroneously respond to self RNA/DNA. RNA-sensing TLR7 exacerbates systemic lupus erythematosus (SLE), a systemic autoimmune disease mediated by type I interferon (IFN-I), whereas DNA-sensing TLR9 regulates disease progression. We here show that TLR7-unique risk of IFN-I-mediated autoimmunity is due to its link with Arl8b, the GTPase required for lysosomal trafficking. Arl8b is associated with Unc93B1, which transports TLR7/9 to DNA/RNA-sensing endolysosomes. TLR7 constitutively formed a complex with Unc93B1 and Arl8b in plasmacytoid dendritic cells (pDCs). Arl8b was required for TLR7-dependent IFN-I production by pDCs. Arl8b$^{-/-}$ mice were impaired in TLR7-, and IFN-I-dependent inflammatory responses. Arl8b reveals a link between TLR7 and type I interferon.

2. Collapse of TLR7/TLR9 balance develops strain-dependent lethal phenotypes.

Ryutaro Fukui[1], Shin-Ichiroh Saitoh[1], Atsuo Kanno[1], Masahiro Onji[1], Takuma Shibata[1,2], Ikuo Miura[5], Shigeharu Wakana[5], Mitsuru Matsumoto[4], Nobuaki Yoshida[3], and Kensuke Miyake[1,2]: [1]Division of Infectious Genetics, Department of Microbiology and Immunology, [2]Laboratory of Innate Immunity, [3]Laboratory of Developmental Genetics, Center for Experimental Medicine and Systems Biology, [4]Division of Molecular Immunology, Institute for Enzyme Research, University of Tokushima, [5]Technology and Development Team for

Mouse Phenotype Analysis, Japan Mouse Clinic, Bio Resource Center, RIKEN

We found that the responses of TLR7 (Toll-like receptor 7) and TLR9 are controlled reciprocally by Unc93B1 (Unc93 homolog B1). This function depends on 34th aspartic acid from N-terminal of Unc93B1 (D34), and alanine mutant of the aspartic acid (D34A) up-regulates TLR7 response and down-regulates TLR9 response (Fukui et al., *J. Exp. Med.* 2009). To investigate the significance of reciprocal TLR7/TLR9 balance *in vivo*, we generated *Unc93b1*$^{D34A/D34A}$ mice and observed their phenotypes. As results, over half of *Unc93b1*$^{D34A/D34A}$ mice died within one year by various phenotypes, and not only inntate immune cells, but also B cells and T cells were activated (Fukui et al, *Immunity* 2011).

These study was performed by using 129 background mice, so we generated C57BL/6 (B6), BALB/c (BALB), C3H/HeN, DBA1/J, and NOD background *Unc93b1*$^{D34A/D34A}$ mice to analyze the effect of genetical background on the phenotypes of *Unc93b1*$^{D34A/D34A}$ mice. The difference of phenotypes among the background was observed, for example, over half of B6 background of *Unc93b1*$^{D34A/D34A}$ mice died within one year but few of BALB background of *Unc93b1*$^{D34A/D34A}$ mice died. As a phenotype of B6 *Unc93b1*$^{D34A/D34A}$ mice, most all of mice developed thrombocytopenia at four month old but no BALB *Unc93b1*$^{D34A/D34A}$ mice developed at same age.

Whereas the phenotypes were different between B6 and BALB background, collapse of TLR7/TLR9 balance were strain-independent. In the cells from both of strain, TLR7 was hyper-response and TLR9 was hypo-resonse. From these data, we hypothesized that some modifier genes link TLR7 inducing-inflammation to phenotypes, and started to search the factor by cytokine analysis, cell analysis and linkage analysis. We found that a locus seems to contribute for the different phenotype, and we are proceeding fine mapping of the genome.

3. An essential role for the N-terminal fragment of Toll-like receptor 9 in DNA sensing.

Masahiro Onji[1], Atsuo Kanno[1], Shin-Ichiroh Saitoh[1], Ryutaro Fukui[1], Yuji Motoi[1], Takuma Shibata[1,2], and Kensuke Miyake[1,2]: [1]Division of Infectious Genetics, Department of Microbiology and Immunology, [2]Laboratory of Innate Immunity, The Institute of Medical Science, The University of Tokyo

Toll-like receptor 9 (TLR9) is an innate immune sensor for microbial DNA that erroneously responds to self DNA in autoimmune disease. To prevent autoimmune responses, Toll-like receptor 9 is excluded from the cell surface and silenced until the N-terminal half of the ectodomain (TLR9N) is cleaved off in the endolysosome. Truncated Toll-like receptor 9 (TLR9C) senses ingested microbial DNA, although the precise role of the truncation remains controversial. Here we show that TLR9 is expressed on the surface of splenic dendritic cells. Following the cleavage of TLR9 in the endolysosome, N-terminal half of the ectodomain remains associated with truncated TLR9, forming the complex TLR9N + C. The TLR9-dependent cytokine production by TLR9(−/−) dendritic cells is rescued by a combination of TLR9N and TLR9C, but not by TLR9C alone. These results demonstrate that the TLR9N + C complex is a bona fide DNA sensor.

4. Influence on the immune response by lipid associated molecules

Sachiko Akashi-Takamura[1], Natsuko Yamakawa[1], Takuma Shibata[1,2], Umeharu Ohto[2], Hiroki Nakanishi[4], Toshiyuki Shimizu[3], and Kensuke Miyake[1,2]: [1]Division of Infectious Genetics, Department of Microbiology and Immunology, [2]Laboratory of Innate Immunity, The Institute of Medical Science, The University of Tokyo, [3]Graduate School of Pharmaceutical Science, The University of Tokyo, [4]Research Center for Biosignal, Akita University

TLR is not only indispensable sensor as an infection protective mechanism, but it is a regulator for disease development of symptoms. RP105 is a molecule which makes the mature B cell avoid from the radiation or steroid induced apoptosis, and induces a strong proliferative reaction. RP105 is also one of the important TLR molecules from follower reason. 1) RP105 resembles TLR4 structurally. 2) As TLR4 associates with secretion protein called MD-2, RP105 associates with MD-1. 3) RP105/MD-1 reinforces the B cell activation and the antibody production through TLR4/MD-2.

Recently the structural analysis of MD molecules is reported that MD-2 binds to LPS (Lipopolysaccharide), and MD-1 binds to Phosphatidyl choline (PC) or Phosphatidyl ethanolamine (PE). We recognized that MD-1 also binds to the negative-charged phospholipids by using purified MD-1 or immune-precipitation technique.

In order to examine a meaning in the living body about MD-1, we established MD-1-deficient SLE model mice. Compared with the control mice, splenomegaly and lymph nodes swelling was reinforced in MD-1 deficient SLE model mice. Furthermore, the serum antibody titer against phospholipids was higher in MD-1 deficient SLE model mice than control mice. It was possible that MD-1 is a molecule which has on the survival extinction and the immune response against lipid of B cells.

5. The linkage of TLR4 activation and antigen presentation, in the light of molecular trafficking

Natsuko Tanimura and Kensuke Miyake

The bacterial compounds, such as lipopolysaccharide (LPS), lipoproteins, DNAs and RNAs, have played critical roles in the vaccinations as immunomodulators since early times. Considering clinical applications, the vaccine adjuvant must be reliable and safe. The bacterial compounds are now well-identified ligands to activate the innate immune system through Toll-like receptors (TLRs). This activation results in both inflammation and the boosts of acquired immune responses. For instance, TLR4 can be activated via lipid A, the active center of LPS, which is well known as a robust immunostimulator. Despite our knowledge of the TLR activation mechanism, we are still unable to harness the excessive inflammations and the effective boosts of acquired immunity using TLR-ligands. Recent studies on innate immune cell biology figured out the activation platforms of TLR4 where the respective responses occur; plasma membrane for inflammation, endosome for interferon production and mitochondria for reactive oxygen species (ROS). To address this issue, we have focused on the dynamics of signaling molecules in the downstream of TLR4 and ligand transfer mechanisms before TLR4 initiation. The signaling pathways are modulated by the fine structure of the ligands. We have found one interesting derivative, which induces low inflammations and enough antigen presentations, showing unique molecular rearrangements and extraordinary TLR4 initiation means. Currently we are analyzing these TLR4-activation/initiation events as a key of the antigen presentations triggering.

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Department of Microbiology and Immunology

Division of Mucosal Immunology
炎症免疫学分野

Professor	Hiroshi Kiyono, D.D.S., Ph.D.	教　授	医学博士	清　野	宏
Assistant Professor	Yoshikazu Yuki, M.B.A., Ph.D.	助　教	医学博士	幸	義　和
Assistant Professor	Shintaro Sato, Ph.D.	助　教	医学博士	佐　藤	慎太郎
Assistant Professor	Yosuke Kurashima, Ph.D.	助　教	医学博士	倉　島	洋　介
Project Assistant Professor	Eun Jeong Park, Ph.D.	特任助教	医学博士	朴	恩　正

The mucosal immune system not only plays an important role as the first line of immunological defense for preventing the host from invasion of harmful microorganisms, but also contributes to the establishment and maintenance of mucosal homeostasis. Our major focus is the elucidation and understanding of molecular and cellular nature of the mucosal immune system for the development of mucosal vaccine against infectious diseases and mucosal immune therapy for mucosa-associated diseases, such as food allergy and inflammatory bowel diseases.

1. MucoRice for New Generation of Oral Vaccine

Yoshikazu Yuki[1], Mio Mejima[1], Koji Kashima[1], Masaharu Kuroda[2], Natsumi Takeyama[1], Shiho Kurokawa[1], Yuji Suzuki[1], Tatsuhiko Azegami[1], Hiroshi Kiyono[1]: [1]Division of Mucosal Immunology, Department of Microbiology and Immunology, The Institute of Medical Science, The University of Tokyo, [2]Crop Development Division, NARO Agriculture Research Center.

We previously developed a molecularly uniform rice-based oral cholera vaccine (MucoRice-CTB) by using an overexpression system for modified cholera toxin B-subunit, CTB (N4Q) with RNAi to suppress production of the major rice endogenous storage proteins. To establish MucoRice-CTB for human use, here we developed hygromycin phosphotransferase (HPT) selection marker-free MucoRice-CTB by using two different *Agrobacterium tumefaciens*, each carrying a distinct T-DNA for co-transformation. We produced six HPT selection marker-free MucoRice-CTB lines from 95 co-transformants by segregation in the seed progeny. From among them, we selected a line with very high CTB expression, line 51A, which we advanced to the T6 generation by self-pollination to obtain a homozygous line without down-regulation of CTB expression. Southern blot analysis showed that three copies of the CTB gene was inserted into the rice genome of MucoRice-CTB line 51A. By whole genome resequencing, we showed that the transgenes in this line were inserted into intergenic regions in chromosome 3 and chromosome 12. We determined that two full-length copies, each containing the CTB and RNAi expression cassettes, were inserted in a tandem reverted orientation into chromosome 3. An additional copy of the CTB over-expression cassette with a truncated RNAi cassette was inserted into chromosome 12. These findings provide useful information for the establishment of a seed bank of marker-free MucoRice-CTB for human use and demonstrate that whole genome resequencing analysis is a powerful tool to assess the genetic structure and function of transgenic rice.

2. New Generation Nasal Vaccine

Yoshikazu Yuki[1], Yoshiko Fukuyama[1], Yuko Katakai[2], Mio Mejima[1], Shiho Kurokawa[1], Eun

Jeong Park[1], Sunyi Joo[1], Hiroaki Shibata[2], Haruko Takahashi[3], Shinichi Sawada[3], Kazunari Akiyoshi[3], Hiroshi Kiyono[1]: [1]Division of Mucosal Immunology, Department of Microbiology and Immunology, The Institute of Medical Science, The University of Tokyo, [2]Tsukuba Primate Research Center, National Institute of Biomedical Inovation, [3]Department of Polymer Chemistry, Kyoto University Graduate School of Engineering

We previously demonstrated a cationic type of cholesteryl group-bearing pullulan based nanogel containing pneumococcal surface protein A (PspA-nanogel) induced both protective Th2-mediated Ag-specific systemic and mucosal antibody (Ab) responses and Th17 cell-mediated immunity. In this study, we examined whether PspA-nanogel nasal vaccine could induce PspA-specific protective immunity and cytokines-related miRNA expression in nonhuman primates. When cynomolgus macaques were nasally immunized with 25 μg of PspA-nanogel/dose, increased levels of Th2 cell dependent PspA-specific serum and broncho alveolar lavage fluid (BALF) IgG, and nasal wash (NW) secretory IgA (SIgA) Ab responses with elevated Th17 cell immunity were seen when compared with control macaques nasally immunized with 25 μg of PspA alone or PBS. MicroRNA (miRNA) analysis of serum and nasal tissues from these PspA-nanogel immunized macaques revealed specific elevation of miR-181a and miR-326 which have been shown to corresponding to Th2 and Th17 responses, respectively. These results suggest that these two miRNAs are also associated with non-human primate Th2 and Th17 cell responses after nasal vaccination with PspA-nanogel vaccine which accounted for the induction of protective immunity against pneumonia.

3. Oral antibody therapy for gut infectious disease

Yoshikazu Yuki[1], Daisuke Tokuhara[1], Mio Mejima[1], Shiho Kurokawa[1], Harold Marcotte[2], Masaharu Kuroda[3], Miren Iturriza-Gómara[4], Leon Frenken[5], Lennart Hammarström[3], Hiroshi Kiyono[1]: [1]Division of Mucosal Immunology, Department of Microbiology and Immunology, The Institute of Medical Science, The University of Tokyo, Japan, [2]Division of Clinical Immunology, Karolinska Institutet at Karolinska University, Sweden, [3]Rice Physiology Research Team, National Agriculture Research Center, Japan, [4]Enteric Virus Unit, Virus Reference Department, Health Protection Agency, UK, [5]Unilever Research & Development, the Netherlands.

Rotavirus-induced diarrhea is a life-threatening disease in children in developing countries and in immunocompromised individuals. We have developed a novel system for prophylaxis and therapy against rotavirus disease using transgenic rice expressing the neutralizing variable domain of a rotavirus-specific llama heavy-chain antibody fragment (MucoRice-ARP1). MucoRice-ARP1 was produced at high levels in rice seeds using an overexpression system and RNAi technology to suppress the production of major rice endogenous storage proteins. Orally administered MucoRice-ARP1 markedly decreased the viral load in immunocompetent as well as immunodeficient mice. MucoRice-ARP1 retained *in vitro* neutralizing activity after long-term storage (>1 yr) and boiling, and conferred protection in mice even after heat treatment at 94 oC for 30 min. High-yield, water-soluble and purification-free MucoRice-ARP1 thus forms the basis for orally administered prophylaxis and therapy against rotavirus infections.

4. Identification of Oral Vaccination-Induced MicroRNA Biomarkers and Targets Revealing the Potential Applicable for Developing New Adjuvant

Eun Jeong Park[1], Sunyi Joo[1], Juneyoung Lee[1], Kyohei Naruse[1], Shiho Kurokawa[1], Mio Mejima[1], Koji Kashima[1], Yoshikazu Yuki[1], Hiroshi Kiyono[1,2]: [1]Division of Mucosal Immunology, Department of Microbiology and Immunology, The Institute of Medical Science, The University of Tokyo, [2]International Research and Development Center for Mucosal Vaccine, The Institute of Medical Science, The University of Tokyo

MicroRNAs (miRNAs) are 22-nucleotide regulatory RNA molecules that alter the activity of specific mRNA targets and involve in a wide range of biological processes. Accumulating evidence demonstrates an essential role of several miRNAs in inducing both innate and adaptive immune responses. But, little is known about the mechanisms by which the miRNAs tissue-selectively up-regulated upon administration with oral vaccine engage in induction and maintenance of humoral and protective immunity. Here, we hypothesize that certain mucosa-specific miRNAs involve in the production of vaccine-specific fecal immunoglobulin A (IgA) (and serum IgG) that is supposed to be critical for enabling host to obtain protective immunity. Our study design in current project represents as follows: 1) to discover blood-borne miRNA biomarkers in the orally immunized mice that possess high titers of vaccine-specific fecal IgA and serum IgG; 2) to investigate miRNA expressions in the tissues at different number of oral immunization; 3) to identify targets or relevant molecules for the miRNAs and to examine their molecular cross-talk or intervention for vaccine-specific antibody production; and 4) to exploit our results to developing

novel adjuvants. For example, once the targets specific for miRNA biomarkers are identified, we test if treating activated B or plasma cells with either mimics or inhibitors specific for the target genes positively influences on promotion of Ig class switch or Ab production. After thorough verification of our hypothesis both *in vivo* and *in vitro*, we will exploit these biomaterial candidates to develop novel types of adjuvant that possesses the capacity to elevate mucosal immunity to orally administered vaccine. Together, our current study aims in not only explicitly elucidating the mechanisms by which miRNAs and their targets contribute to mediating humoral immunity to orally administered vaccine, but providing the proof-of-concept for the development of a rice-based oral vaccine, MucoRice, by thoroughly optimizing its safety and efficacy.

5. Regulation of Mast Cells in the Allergic and non-Allergic Diseases

Yosuke Kurashima[1-4], Takeaki Amiya[1,2,4,5], Kumiko Fujisawa[1,2], Naoko Shibata[1,2,4,5], Yuji Suzuki[1], Yuta Kogure[1,4,5], Eri Hashimoto[1,4], Atsushi Otsuka[6], Kenji Kabashima[6], Shintaro Sato[1,2], Takeshi Sato[1,4,5], Yoshihiro Takasato[1], Masato Kubo[7,8], Shizuo Akira[9], Kensuke Miyake[3], Jun Kunisawa[1,2,5,9-11], and Hiroshi Kiyono[1,2,5,10]: [1]Division of Mucosal Immunology, Department of Microbiology and Immunology, The Institute of Medical Science, The University of Tokyo, [2]CREST, JST, [3]Division of Innate Immunity, Department of Microbiology and Immunology, The Institute of Medical Science, The University of Tokyo, [4]Laboratory of Vaccine Materials, National Institute of Biomedical Innovation, [5]Department of Medical Genome Science, Graduate School of Frontier Science, The University of Tokyo, [6]Department of Dermatology, Kyoto University Graduate School of Medicine, [7]Laboratory for Cytokine Regulation, Research Center for Integrative Medical Science, RIKEN Yokohama Institute, [8]Division of Molecular Pathology, Research Institute for Biological Sciences, Tokyo University of Sciences, [9]Laboratory of Host Defense, WPI Immunology Frontier Research Center, Osaka University, [10]International Research and Development Center for Mucosal Vaccines, The Institute of Medical Science, The University of Tokyo, [11]Graduate School of Pharmaceutical Sciences, Osaka University.

Mast cells (MCs) mature locally, thus possessing tissue-dependent phenotypes for their critical roles in both protective immunity against pathogens and the development of allergic or inflammatory responses. We previously reported that MCs highly express P2X7, a receptor for extracellular ATP, in the colon but not in the skin. An ATP-P2X7 pathway induced activation of MCs and accelerates inflammatory symptom. Here, we identified the unique mechanisms by which P2X7 expression on MCs is reduced by fibroblasts in the skin but not in other tissues. The retinoic-acid-degrading enzyme Cyp26b1 is highly expressed in skin fibroblasts, and its inhibition resulted in upregulation of P2X7 on MCs. We also noted increased expression of P2X7 on skin MCs and consequent P2X7- and MC-dependent dermatitis (so-called retinoid dermatitis) in the presence of excessive amounts of retinoic acid. These results demonstrate a unique skin-barrier homeostatic network operating through Cyp26b1-mediated inhibition of ATP-dependent MC activation by fibroblasts.

6. Runx2-I Isoform Contributes to Fetal Bone Formation Even in the Absence of Specific N-terminus Amino Acids

Hideaki Okura[1,2], Shintaro Sato[1,3], Sari Kishikawa[1], Satoshi Kaneto[1,4], Tomoki Nakashima[5], Nobuaki Yoshida[6], Hiroshi Takayanagi[7], and Hiroshi Kiyono[1-4,8]: [1]Division of Mucosal Immunology, Department of Microbiology and Immunology, [6]Laboratory of Developmental Genetics, and [8]International Research and Development Center for Mucosal Vaccine, The Institute of Medical Science, The University of Tokyo, [2]Department of Medical Genome Science, Graduate School of Frontier Science, The University of Tokyo, [3]CREST, JST, [4]Graduate School of Medicine, The University of Tokyo, [5]Department of Cell Signaling, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, and [7]Department of Immunology, Graduate School of Medicine and Faculty of Medicine, The University of Tokyo.

The Runt-related transcription factor 2 gene (*Runx2*) encodes the transcription factor Runx2, which is the master regulator of osteoblast development; insufficiency of this protein causes disorders of bone development such as cleidocranial dysplasia. Runx2 has two isoforms (Runx2-II and Runx2-I) in which transcription is controlled by unique distal (P1) and proximal (P2) promoter, respectively. Although several studies have focused on differences and similarities between the two Runx2 isoforms, their definitive roles in bone formation have not yet been determined conclusively, partly because a *Runx2-I*-targeted mouse model is not available. In this study, we established a novel *Runx2*-manipulated mouse model in which the first ATG of *Runx2-I* was replaced with TGA (stop codon), and a neomycin-resistant gene (*neo*) cassette was inserted at the first intron of *Runx2-I*. These homozygous *Runx2-I*$^{neo/neo}$ mice showed severe reduction of the expression of *Runx2-I* variant, and *Runx2-II* expression was largely retained. *Runx2-I*$^{neo/neo}$ mice

showed neonatal lethality, and intramembranous ossification was more severely defective than endochondral ossification, presumably because of the greater involvement of *Runx2-I* in intramembranous ossification. Interestingly, the depletion of *neo* rescued above phenotypes, indicating that specific N-terminal region of Runx2-I is not functionally essential for bone development. Taken together, our results provide the novel clue for understanding the roles of Runx2 isoforms in osteoblast development.

Surprisingly, when *Runx2-I*$^{neo/+}$ mice were crossed with Col2a1-Cre transgenic mice, which express Cre in chondrocytes specifically, the survival of Cre-*Runx2-I*$^{neo/neo}$ mice was comparable to that of other littermates, indicating that fetal inability to breath was partly due to defective chondrogenesis rather than osteogenesis. The amount of *Runx2-I* transcript in tissues other than bone (e.g., spleen) of Cre-*Runx2-I*$^{neo/neo}$ mice was still significantly lower than that of wild-type mice. Because *Runx2-I*, but not *Runx2-II*, is expressed in nonskeletal tissues such as lung and brain, these mice are useful for the investigation of the *in vivo* role of Runx2 in nonskeletal tissues.

7. Functional roles of Marcks-like protein expressed by murine Peyer's patch M cells

Satoshi Kaneto[1,2], Shintaro Sato[1,3], Sari Kishikawa[1], Nobuaki Yoshida[4], and Hiroshi Kiyono[1-3,5]: [1]Division of Mucosal Immunology, Department of Microbiology and Immunology, [4]Laboratory of Developmental Genetics, and [5]International Research and Development Center for Mucosal Vaccine, The Institute of Medical Science, The University of Tokyo, [2]Graduate School of Medicine, The University of Tokyo, and [3]CREST, JST.

Microfold cells (M cells), which are located in the apical surface of gut-associated lymphoid tissues such as Peyer's patch (PP), are thought to be an important portal site of antigens and the subsequent induction of antigen-specific immune responses. To investigate the molecular biology of PP M cells, we previously established M-cell-specific monoclonal antibody, and identified specific molecules: Marcks-like protein (MLP) and Gp2. In recent years, it has been known that GP2 acts as the scaffold receptor for the fimbrial protein of *E. coli* or *Salmonella* Typhimurium on M cells. On the other hand, it is still unknown about the biological role of MLP in M cells. To address this issue, we generated intestinal epithelial cell-specific MLP conditional knockout (MLP^{IEC-KO}) mice. The expression of MLP was completely abolished in the apical epithelial surface of PPs in these mice. Matured M cells, which defined as the GP2 expression and the unique ultra-architectures of shorter microvilli and pocket-like formation, could be found in MLP^{IEC-KO} mice, indicating that MLP is not involved in the development and differentiation of M cells. When the sampling ability of M cells in MLP^{IEC-KO} mice were examined, orally-administered fluorescence-nanoparticles were equally taken up into PPs. However, uptake of some gut-specific bacteria was markedly lower in MLP^{IEC-KO} mice than in wild-type mice, suggesting that MLP might be one of key molecules for the translocation of gut invading bacteria. Although further studies are required about the antigen sampling mechanism of M cells, our findings could shed light on the possibility that MLP could be one of novel targets for mucosal vaccine.

8. Innate lymphoid cells govern intestinal epithelial fucosylation

Yoshiyuki Goto[1,2,4], Takashi Obata[1], Jun Kunisawa[1], Shintaro Sato[1,2], Ivaylo I. Ivanov[3], Aayam Lamichhane[1], Natsumi Takeyama[1], Mariko Kamioka[1], Mitsuo Sakamoto[4], Takahiro Matsuki[5], Hiromi Setoyama[5], Akemi Imaoka[5], Satoshi Uematsu[6], Shizuo Akira[7], Steven E. Domino[8], Paulina Kulig[9], Burkhard Becher[9], Jean-Christophe Renauld[10], Chihiro Sasakawa[11], Yoshinori Umesaki[5], Yoshimi Benno[4], and Hiroshi Kiyono[1,2]: [1]Division of Mucosal Immunology, Department of Microbiology and Immunology, The Institute of Medical Science, The University of Tokyo, [2]CREST, JST, [3]Department of Microbiology and Immunology, Columbia University Medical Center, [4]Microbe Division/Japan Collection of Microorganisms, RIKEN BioResource Center, [5]Yakult Central Institute for Microbiological Research, [6]Division of Innate immune regulation, International Research and Development Center for Mucosal Vaccine, Institute for Medical Science, The University of Tokyo, [7]Laboratory of Host Defense, WPI Immunology Frontier Research Center, Osaka University, [8]Department of Obstetrics and Gynecology, Cellular and Molecular Biology Program, University of Michigan Medical Center, [9]Institute of Experimental Immunology, University of Zürich, [10]Ludwig Institute for Cancer Research and Universite catholique de Louvain, and [11]Division of Bacterial Infection, The Institute of Medical Science, The University of Tokyo.

Fucosylation of intestinal epithelial cells, catalyzed by fucosyltransferase 2 (Fut2), represents a major mechanism of host?microbiota symbiosis. Commensal bacteria induce epithelial fucosylation and epithelial fucose is utilized as a dietary carbohydrate by many of the bacteria. However, the molecular and cellular mechanisms of the induction of epithelial fucosylation remain unknown. Here, we show that type 3 innate lymphoid cells (RORγt + ILC3) are critical inducers of intestinal epithelial

Fut2 expression and fucosylation and that this induction is mediated by the production of interleukin 22 and Lymphotoxin in a commensal bacteria-dependent and -independent manner, respectively. Disruption of intestinal fucosylation leads to susceptibility to infection by Salmonella typhimurium. Our data unveil a novel role of ILC3 in creating the appropriate gut microenvironment through regulating the epithelial glycosylation.

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Department of Microbiology and Immunology

Division of Molecular Virology
ウイルス病態制御分野

Professor	Yasushi Kawaguchi, D.V.M., Ph.D.	教　授	獣医学博士	川　口　　寧
Assistant professor	Akihisa Kato, Ph.D.	助　教	医学博士	加　藤　哲　久
Assistant professor	Jun Arii, Ph.D.	助　教	獣医学博士	有　井　　潤

To date, approximately 250 herpesviruses have been identified, affecting most animal species. These viruses are associated with a variety of diseases such as encephalitis, malignancy and mucocutaneous diseases in human and animals. The objective of our research is to understand the mechanisms by which herpesviruses replicate in cells and manifest diseases in their hosts. Our goal is to apply our fundamental findings for the development of anti-herpetic drugs and vaccines for the control of these viral infections.

1. Roles of p53 in Herpes Simplex Virus 1 Replication

Yuhei Maruzuru, Hikaru Fujii, Masaaki Oyama[1], Hiroko Kozuka-Hata[1], Akihisa Kato and Yasushi Kawaguchi: [1]Medical Proteomics Laboratory, The Institute of Medical Science, The University of Tokyo, Minato-ku, Tokyo 108-8639, Japan

p53 is a critical factor in the cellular response to a broad range of stress factors through its ability to regulate various cellular pathways. In this study, tandem affinity purification of transiently expressed herpes simplex virus 1 (HSV-1) regulatory protein ICP22 coupled with mass spectrometry-based proteomics technology and subsequent analyses showed that ICP22 interacted with p53 in HSV-1-infected cells. In p53−/− cells, replication of wild-type HSV-1 was reduced compared to that in parental p53+/+ cells, indicating that p53 had a positive effect on HSV-1 replication. In contrast, the level of viral replication of an ICP22 null mutant virus was similar in both p53−/− and p53+/+ cells. At 2 h post-infection, the level of expression of ICP 27, an essential viral regulatory protein, in p53−/− cells infected with wild-type HSV-1 or the ICP22 null mutant virus was lower than in p53+/+ cells. In contrast, at 18 h post-infection, the level of expression of ICP0, a critical viral regulatory protein, in p53−/− cells infected with the ICP22-null mutant virus was higher than in p53+/+ cells, although the level of ICP0 expression in p53−/− and p53+/+ cells infected with wild-type HSV-1 was almost identical. These results suggested that p53 overall promoted HSV-1 replication and that p53 played both positive and negative roles in HSV-1 replication: up-regulating ICP27 expression very early in infection and down-regulating ICP0 expression later in infection, which was antagonized by ICP22.

2. Herpes Simplex Virus 1 Protein Kinase Us3 Phosphorylates Viral dUTPase and Regulates Its Catalytic Activity in Infected Cells

Akihisa Kato, Shumpei Tsuda, Zhuoming Liu, Hiroko Kozuka-Hata[1], Masaaki Oyama[1] and Yasushi Kawaguchi: [1]Medical Proteomics Laboratory, The Institute of Medical Science, The University of Tokyo, Minato-ku, Tokyo 108-8639, Japan

Us3 is a serine-threonine protein kinase encoded by herpes simplex virus 1 (HSV-1). In this study, a large scale phosphoproteomic analysis of titanium

dioxide affinity chromatography-enriched phosphopeptides from HSV-1-infected cells using high-accuracy mass spectrometry (MS) and subsequent analyses showed that Us3 phosphorylated HSV-1-encoded dUTPase (vdUTPase) at serine 187 (Ser-187) in HSV-1-infected cells. Thus, (i) in *in vitro* kinase assays, Ser-187 in the vdUTPase domain was specifically phosphorylated by Us3. (ii) Phosphorylation of vdUTPase Ser-187 in HSV-1-infected cells was detected by phosphate-affinity polyacrylamide gel electrophoresis analyses and was dependent on the kinase activity of Us3. (iii) Substitution of Ser-187 with alanine (S187A) in vdUTPase as well as an amino acid substitution in Us3 that inactivated its kinase activity significantly down-regulated the enzymatic activity of vdUTPase in HSV-1-infected cells, whereas a phosphomimetic substitution at vdUTPase Ser-187 restored the wild-type enzymatic activity of vdUTPase. (iv) The vdUTPase S187A mutation as well as the kinase-dead mutation in Us3 significantly reduced HSV-1 replication in human neuroblastoma SK-N-SH cells at a multiplicity of infection (MOI) of 5, but not at an MOI of 0.01, whereas the phosphomimetic substitution at vdUTPase Ser-187 restored the wild-type viral replication at an MOI of 5. In contrast, these mutations had no effect on HSV-1 replication in Vero and HEp-2 cells. Collectively, our results suggested that Us3 phosphorylation of vdUTPase Ser-187 promoted HSV-1 replication in a manner dependent on cell types and MOIs by regulating optimal enzymatic activity of vdUTPase.

3. Phosphorylation of a herpes simplex virus 1 dUTPase by a viral protein kinase Us3 dictates viral pathogenicity in the central nervous system but not at the periphery

Akihisa Kato, Keiko Shindo, Yuhei Maruzuru, and Yasushi Kawaguchi

Herpes simplex virus 1 (HSV-1) encodes Us3 protein kinase, which is critical for viral pathogenicity in both mouse peripheral sites (e.g., eyes and vaginas) and in the central nervous system (CNS) of mice following intracranial and peripheral inoculations, respectively. Whereas some Us3 substrates involved in Us3 pathogenicity in peripheral sites have been reported, those involved in Us3 pathogenicity in the CNS remain to be identified. We recently reported that Us3 phosphorylated HSV-1 dUTPase (vdUTPase) at serine 187 (Ser-187) in infected cells and this phosphorylation promoted viral replication by regulating optimal enzymatic activity of vdUTPase. In the present study, we showed that the replacement of vdUTPase Ser-187 by alanine (S187A) significantly reduced viral replication and virulence in the CNS of mice following intracranial inoculation and that the phosphomimetic substitution at vdUTPase Ser-187 in part restored the wild-type viral replication and virulence. Interestingly, the S187A mutation in vdUTPase had no effect on viral replication and pathogenic effects in the eyes and vaginas of mice following ocular and vaginal inoculation, respectively. Similarly, the enzyme-dead mutation in vdUTPase significantly reduced viral replication and virulence in the CNS of mice following intracranial inoculation, whereas the mutation had no effect on viral replication and pathogenic effects in the eyes and vaginas of mice following ocular and vaginal inoculation, respectively. These observations suggested that vdUTPase was one of the Us3 substrates responsible for Us3 pathogenicity in the CNS and that the CNS-specific virulence of HSV-1 involved strict regulation of vdUTPase activity by Us3 phosphorylation.

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