Laboratory Animal Research Center
実験動物研究施設

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Our major research interests are to elucidate molecular mechanisms of pathogenicity and species specificity of negative and single strand RNA viruses (Mononegavirales), and to control viral diseases. For these purposes, we are studying virus replication and identifying viral and host factors important for the expression of pathogenicity using a novel reverse genetics technique. We are also developing new virus vaccines and virus vectors by genetic engineering. In the animal research center, more than 30,000 mice, mainly transgenic or knockout, are kept for research of IMSUT, and the technical staff support their breeding, frozen storage of eggs and microbiological cleaning.

A measles virus selectively blind to signaling lymphocytic activation molecule shows anti-tumor activity against lung cancer cells.

Fujiyuki, T., Yoneda, M., Amagai, Y., Obayashi, K., Ikeda, F., Shoji, K., Murakami, Y[1]., Sato, H. and Kai, C.: [1]Division of Molecular Pathology, The Institute of Medical Science, The University of Tokyo.

Lung cancer cells, particularly those of non-small-cell lung cancer, are known to express Nectin-4. We previously generated a recombinant measles virus (MV) that uses Nectin-4 as its receptor but cannot bind its original principal receptor, signaling lymphocyte activation molecule (SLAM). This virus (rMV-SLAMblind) infects and kills breast cancer cells in vitro and in a subcutaneous xenograft model. However, it has yet to be determined whether rMV-SLAMblind is effective against other cancer types and in other tumor models that more closely represent disease. In this study, we analyzed the anti-tumor activity of this virus towards lung cancer cells using a modified variant that encodes green fluorescent protein (rMV-EGFP-SLAMblind). We found that rMV-EGFP-SLAMblind efficiently infected nine, human, lung cancer cell lines, and its infection resulted in reduced cell viability of six cell lines. Administration of the virus into subcutaneous tumors of xenotransplanted mice suppressed tumor growth. In addition, rMV-EGFP-SLAMblind could target scattered tumor masses grown in the lungs of xenotransplanted mice. These results suggest that rMV-SLAMblind is oncolytic for lung cancer and that it represents a promising tool for the treatment of this disease.

Measles virus infection inactivates cellular protein phosphatase 5 with consequent suppression of Sp1 and c-Myc activities.

Sato, H., Yoneda, M., Honma, R.[2], Ikeda, F., Watanabe, S[2]. and Kai, C.: [2]Clinical Informatics, Tokyo Medical and Dental University.

MV causes several unique syndromes, including transient immunosuppression. To clarify the cellular responses to MV infection, we previously analyzed a MV-infected epithelial cell line and a lymphoid cell line by microarray and showed that the expression of numerous genes was up- or down-regulated in the epithelial cells. In particular, there was a characteristic comprehensive downregulation of housekeeping genes during late stage infection.

To identify the mechanism underlying this phenomenon, we examined the phosphorylation status of transcription factors and kinase/phosphatase activities in epithelial cells after infection. MV infection inactivated cellular protein phosphatase 5 (PP 5) that consequently inactivated DNA-dependent protein kinase, which reduced Sp1 phosphorylation levels, and c-Myc degradation, both of which downregulated the expression of many housekeeping genes. In addition, intracellular accumulation of viral nucleocapsid inactivated PP5 and subsequent downstream responses. These findings demonstrate a novel strategy of MV during infection, which causes the collapse of host cellular functions.

Transcribed enhancers lead waves of coordinated transcription in transitioning mammalian cells.

Arner, E.[3], The FANTOM Consortium (Kai, C., Sato, H., Yoneda, M. et al).: [3]RIKEN Omics Science Center.

Although it is generally accepted that cellular differentiation requires changes to transcriptional networks, dynamic regulation of promoters and enhancers at specific sets of genes has not been previously studied en masse. Exploiting the fact that active promoters and enhancers are transcribed, we simultaneously measured their activity in 19 human and 14 mouse time courses covering a wide range of cell types and biological stimuli. Enhancer RNAs, then messenger RNAs encoding transcription factors, dominated the earliest responses. Binding sites for key lineage transcription factors were simultaneously overrepresented in enhancers and promoters active in each cellular system. Our data support a highly generalizable model in which enhancer transcription is the earliest event in successive waves of transcriptional change during cellular differentiation or activation.

Publications

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Amami Laboratory of Injurious Animals
奄美病害動物研究施設

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The Amami Laboratory of Injurious Animals was established in 1965 at Setouchi-cho in Amami-oshima Island in order to study on endemic diseases involving parasite, arthropods, and venomous snakes in the tropics or subtropics.
The Amami-oshima Island belongs to the Nansei (Southwest) Islands and the fauna is quite different from that in other islands of Japan. Since establishment of the laboratory, trials have been carried out to utilize small mammals found unique in the Amami islands as experimental animals in addition to studies on prevention of Habu bites. As well known, successful eradication of filariasis from this island is one of the monumental works of the laboratory. Our present works are as follows:

1. Research on the Habu control

Shosaku Hattori, Takeshi Kuraishi, Shinichi Yokota, Motonori Ohno[1], Naoko Oda-Ueda[2], Takahito Chijiwa[1], Aichi Yoshida[3], Yoshihiro Hayashi[4], and Tomohisa Ogawa[5]: [1]Department of Applied Life Science, Faculty of Bioscience, Sojo University, [2]Department of Biochemistry, Faculty of Pharmaceutical Science, Sojo University, [3]School of Health Science, Faculty of Medicine, Kagoshima University, [4]National Museum of Nature and Science, Tokyo, [5]Faculty of Agriculture, Tohoku University

Snake bites by the venomous snake Habu, *Protobothrops flavoviridis*, have been reported annually about 60 cases in the population of 100,000 in the Amami Islands. Moreover, there is no indication that the population of the Habu itself has decreased, despite a campaign for capture of snakes by the Kagoshima Prefectural Government. Rat-baited box traps have been introduced to catch the snakes and found to be quite effective. However, maintenance of live rats requires man power and its cost is expensive. Therefore, our effort has been focused on the development of attractant for Habu. The attractant extracted from rats seems ineffective if compared with use of live rats.

It was known that the Habu survived the injection of the Habu venom since early times, because some proteins in the serum of the Habu blood combine to the elements of the Habu venom. The research of these binding proteins has been initiated with an objective of clinical trials. Phospholipase A_2 and its isozymes isolated from Habu venom have myonecrotic activity and hemorrhagic activity, and metal protease has hemorahagic activity. The binding proteins isolated from serum of Habu inhibit myonecrotic activity of phospholipase A_2 and its isozymes. We found that protein-HSF and peptide-AHP isolated from the Habu serum effectively control the hemorrhage caused by venom of the Habu, *Ovophis okinavensis*, *Agkistrodon blomhoffi brevicaudus*, *Calloselasma rhodostoma*, *Bitis arietans*, *Bothrops asper*, and, *Trimeresurus stejnegeri*.

Further, a statistics analysis and the simulation were done with the snakes captured by the Government, and the analysis of population dynamics of Habu was attempted. As a result of investigating the individual measurement data of the captured Habu over 9 years, we were able to obtain the generous age composition of the Habu. From analyzing of the age pyramid of the Habu and the result of questionnaire surveys for the inhabitant in the Amami-oshima Island, the total population of the Habu which lives in this island was estimated at about 80,000. By the analysis of the measured data of last nine years, the snake sizes were miniatur-

ized, and the population of young snakes decreased. According to these investigations, the population of the Habu is expected to decrease in the near future.

These studies are supported by grants from the Ministry of Land, Infrastructure and Transport and the Kagoshima Prefectural Government.

2. Comparative analysis of interisland variegation of venom [Lys[49]]phospholipase A_2 isozyme genes in *Protobothrops genus snakes*

Hitomi Nakamura[6], Tatsuo Murakami[7], Shosaku Hattori, Yoshiyuki Sakaki[8], Takatoshi Ohkuri[6], Takahito Chijiwa, Motonori Ohno and Naoko Oda-Ueda: [6]Department of Biochemistry, Faculty of Pharmaceutical science, [7]Department of Applied Life Science, Faculty of Bioscience, Sojo University, [8]RIKEN Genomic Sciences center

Snake venoms contain phospholipase A_2 (PLA_2) isoforms as major toxic components. In especially, it is known that the myotoxic activity of venom PLA_2 is characterized by a Asp to Lys substitution of residue 49 ([Asp[49]]PLA_2 and [Lys[49]]PLA_2). We comparatively analyzed the PLA_2 isozymes between *Protobothrops tokarensis* (Takarajima and Kodakarajima) and *P. flavoviridis* (mainly Amami-Oshima, Tokunoshima, and Okinawa). Three [Asp[49]]PLA_2s and one [Lys[49]]PLA_2, named BPI, were identified from *P. tokarensis* (Kodakarajima island) venom. The cDNAs encoding three [Asp[49]]PLA_2s and BPI and the gene encoding BPI were cloned and sequenced. P. flavoviridis (Amami-Oshima island) venom have three [Lys[49]]PLA_2s, thus showing interisland variegation in their numbers. PtBPI gene has LINE-1 fragment inserted into its second intron, thus duplication of PtBPI gene appears to be restricted. The interisland variegation of venom [Asp[49]]PLA_2 isozyme genes in Protobothrops genus snakes in the southwestern islands of Japan is discussed.

3. Reproduction of squirrel monkeys and owl monekys.

Shosaku Hattori, Takeshi Kuraishi, Shinichi Yokota, Kumiko Ikeda, Hazuki Yoshimura and Chieko Kai

The squirrel monkey (*Saimiri boliviensis*) and the owl monkey (*Aotus lemurinus griseimenbra*) were widely distributed in the tropical rainforest in Central and South America. The advantage of using both species for medical researches resides in its small size and gentle behavior. In this laboratory, squirrel monkeys have a breeding season between winter and early spring. They are polygamy. Their puberty is 3-4 years old in females and 4-5 years old in males. Their gestation period is about 150 days. In contrast, the owl monkey is annual breeding animals. They are monogamy. Their puberty is 3 years old for both sex. Their gestation period is about 130 days. Nine newborns were given in reproductive groups of squirrel monkeys in 2015. Four of 9 newborns were nursed by laboratory staffs because of neglect of their mothers. On the other hand, 2 newborns were given in 3 female owl monkeys in 2015.

4. Intracytoplasmic sperm injection into oocytes matured in vitro and early embryonic development in the owl monkey (Aotus lemurinus)

Ken Takeshi Kusakabe[9], Takeshi Kuraishi, Shosaku Hattori, Yasuo Kiso[9], Chieko Kai, Midori Yoshizawa[10]: [9]Joint Faculty of Veterinary Science, Yamaguchi University, [10]Graduate School of Agricultural Science, Utsunomiya University

As mentioned above, breeding of owl monkey has been achieved at Amami laboratory of injurious animals, the number of animals produced has been small. We explored the possibility of employing intracytoplasmic sperm injection (ICSI), involving oocytes and sperm of owl monkeys. First, we estimated the morphology and normal parameters of owl monkey sperm and oocytes. As a result, because the motility and viability of owl monkey sperm are very poor, i.e. similar to those in some cases of male infertility in humans, embryo production by conventional in vitro fertilization is difficult in owl monkeys. Therefore, we decided that ICSI is required to produce embryos in vitro, and tried. As a result, we were able to produce two owl monkey embryos using ICSI of oocytes that matured to MII stage. Both embryos reached the 10-cell stage at 98 h after ICSI and showed signs of compaction, but failed to cleave further.

5. Pathological analysis of aquatic mycobacteria (*Mycobacterium marinum*) infection in squirrel monkey (*Saimiri boliviensis*)

Shinichi Yokota, Shosaku Hattori, Shiomi Yoshida[11], Takayuki Wada[12], Takeshi Kuraishi[13], and Chieko Kai: [11]National Hospital Organization Kinki Chuo Chest Medical Center, [12]Nagasaki University Graduate School of Biomedical Sciences, [13]Laboratory of Veterinary Pathology, Department of Veterinary Medicine, Gifu University

Mycobacterium marinum is an atypical *Mycobacterium* species, which causes opportunistic infections in humans by excessive exposure to an aquatic environment or marine animals. To determine the possibility for cross-species *M. marinum* transmission, we administrated *M. marinum* isolated from

Japanese Forest Green Tree Frogs (Rhacophorus arboreus) and from a human patient into squirrel monkeys, and investigated the clinical conditions. The monkey inoculated with *M. marinum* isolated from patient had local swelling at the injection site, and *M. marinum* was found in the axillary lymph nodes 4 weeks after the inoculation. On the other hand, the monkey inoculated with *M. marinum* isolated from Japanese Forest Green Tree Frogs did not show any clinical signs. The histopathological analysis is currently under consideration.

6. Establishment of the DFAT cells and application to cell sheet transplant in squirrel monkey (*Saimiri boliviensis*)

Shinichi Yokota, Takeshi Kuraishi, Shosaku Hattori, Akane Tanaka[14], Hiroshi Matsuda[14], and Chieko Kai: [14]Division of animal life science, Institute of agriculture, Tokyo University of Agriculture and Technology

Dedifferentiated fat cells (DFAT cells) are seemed to be a good candidate source of adult stem cells in regenerative medicine, because these cells exhibit multilineage potential as adipose tissue-derived stromal cells (ADSCs). We isolated squirrel monkey DFAT cells from a small amount of adipose tissue, and confirmed adipogenesis, osteogenic and chondrogenic differentiation in vitro. Furthermore, the DFAT cells derived from three different squirrel monkeys were expanded to sufficient numbers for transplantation as cell sheets. Before clinical application and transplantation of MSC-derived cells, the preclinical safety and efficacy studies should be preferably carried out in a non-human primate animal model, because mouse stem cells have proven to show great differences from the human. We are plan to apply to some wound healing model in squirrel monkey.

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服部正策．徳之島手々地区でのネットフェンスとハブの捕獲調査．平成26年度ハブとの共存に関わる総合調査事業報告書（鹿児島県）．pp. 10−14, 2015.

倉石武，服部正策，朝沼榎．ハブ咬傷者血清中のハブ毒特異的IgE抗体の測定．平成26年度奄美ハブ毒免疫機序応用研究報告書．（鹿児島県）．pp. 52−53, 2014.

Laboratory of Molecular Genetics
遺伝子解析施設

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This laboratory has three main activities: development and supply of novel adenovirus vectors useful for studies in various fields including gene therapy, study about hepatitis B virus and supporting the researchers by advising on recombinant DNA technology and on biohazards under the safety guidelines.

The purposes of our laboratory are concerned about not only research but also support for all researchers in this institute. Our supporting activity is involved in advising service on gene-manipulation experiments and on biohazards under the safety guidelines and laws. For the research part, we intend to develop novel methods or new experimental systems leading in the field of gene expression and its regulation. We are concentrating mainly on developing efficient adenovirus expression vectors useful for various fields including gene therapy. We are maintaining more than ten collaborations within and outside of this institute. In these collaborations, we can supply adenovirus vectors (AdVs) enabling strictly regulated gene expression and helper-dependent AdVs (HD AdVs) of high capacity up to 30 kilobases (kb). We have established a unique system producing HD AdVs using 293hde 12 cells. Our system is probably superior to currently available system, because in the latter HD AdVs are produced using cell lines expressing Cre, which is slightly toxic to cells when expressed in a large amount.

Previously we developed a system for construction of E1-deleted AdV, also called first-generation (FG) AdVs, using a full-length viral genome with intact viral termini (Fukuda. *et al*., Microbiol. Immunol. 50: 643-654, 2006). This cassette is available from Takara Bio and Nippon Gene. We have also developed a method for ON/OFF switching of gene expression in mammalian cells using a combination of adenovirus vector and Cre/*loxP* system (Kanegae *et al*., Nucleic Acids Res. 23: 3816-3821, 1995; Kanegae *et al*., Gene 181: 207-212, 1996) as well as FLP/frt system (Nakano *et al*., Nucleic Acids Res. 29: e40, 2001; Kondo *et al*., Nucleic Acids Res. 31: e 76, 2003; Kondo *et al*., Microbiol. Immunol., 50: 831-843, 2006; Kondo et al., J. Molec. Biol., 2009). These methods continuously promote studies of various fields of molecular biology and medicine.

There are two remarkable advances from our laboratory. We succeeded in developing new-generation AdVs that may replace current FG AdVs. The most important problem of AdV is severe immune responses *in vivo*. Firstly, we have identified adenovirus pIX gene as a main cause of inflammation: pIX gene is abnormally activated in AdV. Then we developed AdVs that do not express pIX protein. Transgene expression was lasted for six months in this new AdV (Nakai *et al*., Hum. Gene Ther. 18: 925-936, 2007). The AdV is now called the "low-inflammatory AdVs". For example, Cre-expressing AdV, AxCANCre, is replaced by the low-inflammatory Cre-expressing AdVs, AxEFNCre (Chiyo *et al*., Virus Res. 160: 89-97, 2011). Secondly, we have established a method for efficient production of AdVs lacking the genes of virus-associated (VA) RNAs that disturb cellular RNAi machinery (Maekawa *et al*., Sci. Rep. 2013) using 293hde12 cell line producing a large amount of the codon-human-

ized FLPe (hFLPe) recombinase (Kondo *et al.*, J. Molec. Biol., 2009). The VA-deleted AdVs possibly substitute for current FG AdVs.

The research activities in 2015 are shown below. They include the studies of hepatitis B virus (HBV) and the application of AdVs in the field of genome editing.

1. Efficient production of the replicating HBV genome using "HBV-AdV system"

Mariko Suzuki, Saki Kondo, Manabu Yamasaki[1], Masakatsu Shibasaki[1], Yumi Kanegae, and Izumu Saito: [1]Laboratory of Basic Biology, Institute of Microbial Chemistry (BIKAKEN), Tokyo

In most studies of HBV, both the transfection of 1.2 copy-HBV genome plasmids or the cell lines in which the HBV genome is integrated, have been used to elucidate the HBV replication mechanism. Previously, we reported a new method to detect the HBV genome replication using AdV, which named HBV-AdV system. Since AdV DNA doesn't remain on the cell surface (Pei et al., BBRC, 2012), the replicated HBV genomes are easily and precisely detected when during the use of the HBV-AdV system.

We applied the HBV-AdV system to HepG2 cells and human primary hepatocytes. In the HepG2 cells, where AdV showed 24-fold higher transduction efficiency than plasmid transfection, the HBV-AdV system was much superior to the plasmid transfection to detect replicating HBV genome and the produced products. Notably, the application of the HBV-AdV system to the primary hepatocytes was very efficient; it is valuable because, so far, the primary hepatocytes can efficiently be infected only HBV particles derived from sera from HBV carrier. Moreover, Because our HBV-AdV system abundantly provided the HBV pregenomic RNA (pgRNA) using CMV promoter, the HBV-AdV system enabled us to detect the replicating HBV genome only 4 days post-infection of AdV. Moreover, using HBV-AdV system the behaviors of the artificially-mutated HBV genomes can be investigated in various cell lines. Thus, the HBV-AdV system could widely be applied to the study of HBV replication and also to the screening of anti-HBV drug.

2. Multiplex guide RNAs of CRISPR/Cas9 system expressed from adenovirus vector efficiently disrupted the HBV genome

Aya Maekawa, Saki Kondo, Mariko Suzuki, Yumi Kanegae, and Izumu Saito

HBV infection increases the risk of developing liver cirrhosis and hepatocellular carcinoma. Current therapeutic treatments for HBV infection mostly use interferon alpha (IFN-α) and the nucleotide analogs such as lamivudine. However, these treatments cannot effectively eliminate the covalently closed circular DNA (cccDNA), which presents in the nuclei of the infected hepatocytes and continues to serve as the template for the pregenomic RNA (pg RNA). Consequently, complete clearance of HBV is difficult.

As a novel tool for genome editing, the CRISPR/Cas9 system directed by programable guide RNA (gRNA) to the target DNA is expected to offer antiviral activities against HBV. In the application of the CRISPR/Cas9 system to disrupt the HBV cccDNA, multiplex targeting could be superior to a single targeting for cccDNA disruption. For this reason we ligated in tandem the multiplex expression cassettes of the gRNA and were inserted into the AdV genome in order to express gRNAs simultaneously in a single cell. However, it is difficult to construct the tandemly-ligated expression cassettes of the gRNAs because of homologous recombination (HR) and subsequent deletion among the repeated sequences.

We succeeded in construction of AdVs expressing six multiplex gRNAs targeting the HBV genome. The expressed gRNAs together with Cas9-producing AdVs efficiently directed cleavages and cleavage-mediated mutations occurred in the cccDNA. These results suggest that AdV expressing multiplex gRNA targeting HBV genome might be used as an effective tool to treat chronic HBV infection.

Publications

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Maekawa, A., Kondo, S., Suzuki, M., Saito, I., and

Kanegae, Y. Multiplex guide RNAs targeting HBV genome expressed using adenovirus vector improve the efficiency of CRISPR/Cas9 system. 2015 International Meeting of Molecular Biology of Hepatitis B Viruses. Dolce Bad Nauheim, Germany.

Medical Proteomics Laboratory
疾患プロテオミクスラボラトリー

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The mission of our laboratory is to develop advanced technologies for integrative proteomic analyses from a physicochemical, structural and systems biology point of view. Currently, we mainly focus on functional protein-protein interaction networks related to a variety of diseases including cancer and infection. We are also engaged in collaborative researches regarding mass spectrometry and electron microscopy, which have made a substantial contribution to many scientific achievements.

<Group I>

1. Global characterization of the proteome and phosphoproteome in human glioblastoma initiating cells by high-resolution mass spectrometry

Hiroko Kozuka-Hata, Tomoko Hiroki, Ryo Koyama-Nasu[1], Kouhei Tsumoto, Jun-ichiro Inoue, Tetsu Akiyama[1] and Masaaki Oyama: [1]Laboratory of Molecular and Genetic Information, Institute of Molecular and Cellular Biosciences, University of Tokyo

Glioblastoma is one of the most common and aggressive brain tumors with the median survival of twelve months after diagnosis. Despite extensive studies of this malignant tumor, the outcomes of the treatment have not significantly improved over the past decade. To elucidate the underling mechanisms of its tumorigenicity, we performed parallel analyses of the comprehensive proteome and phosphoproteome in glioblastoma initiating cells that are widely recognized as key players in showing resistance to chemotherapy and radiation. Using high-resolution nanoflow LC-MS/MS (LTQ Orbitrap Velos) in combination with GELFREE™8100 fractionation system, we identified a total of 8,856 proteins and 6,073 phosphopeptides, respectively. Global protein network analysis revealed that the molecules belonging to ribosome, spliceosome and proteasome machineries were highly enriched at the proteome level. Our in-depth phosphoproteome analysis based on two fragmentation methodologies of CID and HCD detected various phosphorylation sites on neural stem cell markers such as nestin and vimentin, leading to identification of thirty-six phosphorylation sites including eleven novel sites of nestin protein. The SILAC-based quantitative analysis showed that 516 up-regulated and 275 down-regulated phosphorylation sites upon epidermal growth factor stimulation. Interestingly, the phosphorylation status of the molecules related to mTOR signaling pathway was dynamically changed upon EGF stimulation. More intriguingly, we also identified some novel phosphopeptides encoded by the undefined sequence regions of the human transcripts, which could be regulated upon external stimulation in glioblastoma initiating cells. Our result unveils an expanded diversity of the regulatory phophoproteome defined by the human transcriptome.

2. System-wide perturbation of the proteome and phosphoproteome dynamics in glioblastoma stem cells through mTOR signaling inihibition

Hiroko Kozuka-Hata, Tomoko Hiroki, Ryo Koyama-Nasu[1], Kouhei Tsumoto, Jun-ichiro Inoue, Tetsu Akiyama[1] and Masaaki Oyama.

As glioblastoma is the most common and aggressive brain tumor with poor prognosis, systematic elucidation of signaling networks causally linked to the tumorigenesis is very crucial for developing more effective treatments for this intractable cancer. In our previous study, we applied a high-resolution mass spectrometry-based proteomics technology in combination with SILAC quantitative methods to understand EGF-dependent phosphoproteome dynamics in patient-derived glioblastoma stem cells. We demonstrated that the phosphorylation levels of the representative mTOR signaling molecules such as RPS6 and PRAS40 were dramatically up-regulated upon EGF stimulation. As EGFR signaling has been reported to play a pivotal role in regulating the maintenance of cancer stem cells, we next carried out mTOR inhibitor-dependent signaling perturbations to unravel stemness-related pathways at the network level.

In the present study, we identified a total of 3,726 proteins including 49 up-regulated and 436 down-regulated factors by Torin 1 treatment. Interestingly, we found that one of the well-known cancer stem cell markers was significantly down-regulated through mTOR signaling inhibition. Our in-depth phosphoproteome analysis also led to identification of 6,250 unique phosphopeptides derived from 2,221 proteins and unveiled a variety of dynamic changes regarding phosphorylation levels of cancer and neural stem cell markers in a comprehensive manner. The integrative view of the mTOR inhibitor-dependent proteome and phosphoproteome dynamics in glioblastoma stem cells presents us with further prospects towards understanding previously unrecognized regulations at the system level.

3. Integrative network analysis based on high-accuracy quantitative phosphoproteomics reveals a novel regulator of glioblastoma stem cell differentiation

Yuta Narushima, Hiroko Kozuka-Hata, Ryo Koyama-Nasu[1], Kouhei Tsumoto, Jun-ichiro Inoue, Tetsu Akiyama[1] and Masaaki Oyama.

Glioblastoma is one of the most malignant brain tumors with poor prognosis and their development and progression are known to be driven by glioblastoma stem cells. Although glioblastoma stem cells lose their stem cell properties contributing to high tumorigenicity and resistance to the current chemo- and radio-therapies during the course of differentiation, little is known about the molecular mechanisms regulating signaling alteration in relation to stemness maintenance and differentiation. In order to elucidate the global phosphorylation-related signaling events, we performed a SILAC-based quantitative phosphoproteome analysis of serum-induced differentiation in glioblastoma stem cells established from the tumor tissues of the patient.

Among a total of 2,691 phosphorylation sites on 1,584 proteins identified in our analysis, 731 phosphorylation sites on 415 proteins were regulated through the differentiation. The integrative computational analyses based on the quantified phosphoproteome data revealed the relevant changes of phosphorylation levels regarding the proteins associated with cytoskeleton reorganization such as Rho family GTPase and Intermediate filament signaling, in addition to transforming growth factor-beta receptor type-2 (TGFBR2) as a prominent upstream regulator involved in the serum-induced phosphoproteome regulation. The functional association of TGFBR2 with glioblastoma stem cell differentiation was experimentally validated through signaling perturbation using the corresponding inhibitors, which indicated that TGFBR2 could play an important role as a novel cell fate determinant in glioblastoma stem cell regulation.

4. Integrative analysis of phosphoproteome and transcriptome dynamics defines drug-resistance properties of breast cancer

Masaaki Oyama, Takeshi Nagashima[2], Hiroko Kozuka-Hata, Noriko Yumoto[2], Yuichi Shiraishi[2], Kazuhiro Ikeda[3], Yoko Kuroki[2], Noriko Gotoh[4], Satoshi Inoue[3], Hiroaki Kitano[5] and Mariko Okada-Hatakeyama[2]: [2]RIKEN, [3]Research Center for Genomic Medicine, Saitama Medical University, [4]Division of Systems Biomedical Technology, IMSUT, [5]Sony Computer Science Laboratories, Inc.

Signal transduction system, in orchestration with subsequent transcriptional regulation, widely regulates complex biological events such as cell proliferation and differentiation. Therefore, a comprehensive and fine description of their dynamic behavior provides a fundamental platform for systematically analyzing the regulatory mechanisms that result in each biological effect. Here we developed an integrated framework for time-resolved description of phosphoproteome and transcriptome dynamics based on the SILAC-nanoLC-MS and GeneChip system. In this study, we analyzed cellular information networks mediated by estrogen receptor/ErbB2 pathways, which have long been implicated in drug response of breast cancer. Through shotgun identification and quantification of phosphorylated molecules in breast cancer MCF-7 cells, we obtained a global view of the dynamics regarding breast cancer-related signaling networks upon estrogen (E2)

or heregulin (HRG) stimulation. Comparative analysis of wild-type and tamoxifen-resistant MCF-7 cells revealed altered behaviors of signaling hub dynamics, indicating distinct signaling network properties between these two cell types. Pathway and motif activity analyses using the transcriptome data suggested that deregulated activation of GSK3β and MAPK1/3 signaling might be associated with altered activation of CREB and AP-1 transcription factors in tamoxifen-resistant MCF-7 cells. Thus, our integrative analysis of phosphoproteome and transcriptome in human breast cancer cells revealed distinct signal-transcription programs in tamoxifen-sensitive and insensitive tumor cells, which potentially defines drug-resistance properties against tamoxifen.

5. System-level analysis of CagA-dependent signaling network dynamics by Helicobacter pylori infection

Hiroko Kozuka-Hata, Masato Suzuki[6], Kotaro Kiga[6], Shinya Tasaki, Jun-ichiro Inoue, Tadashi Yamamoto[7], Chihiro Sasakawa[6] and Masaaki Oyama: [6]Division of Bacterial Infection, Department of Microbiology and Immunology, IMSUT, [7]Division of Oncology, Department of Cancer Biology, IMSUT

The signal transduction system within a cell regulates complex biological events in response to bacterial infection. The previous analyses of cell signaling in Helicobacter pylori-infected gastric epithelial cells have revealed that CagA, a major virulence factor of Helicobacter pylori, is delivered into cells via the type IV secretion system and perturbs signaling networks through the interaction with the key signaling molecules such as SHP-2, Grb2, Crk/Crk-L, Csk, Met, and ZO-1. Although the biological activity of tyrosine-phosphorylated CagA has intensively been studied, system-wide effects of the virulence factor on cellular signaling have yet to be analyzed. Here we performed time-resolved analyses of phosphoproteome and CagA-interactome in human gastric AGS cells by CagA-positive/negative Helicobacter pylori infection. Our highly sensitive nanoLC-MS/MS analyses in combination with the Stable Isotope Labeling by Amino acids in Cell culture (SILAC) technology defined CagA-dependent perturbation of signaling dynamics along with a subset of CagA-associated possible modulators on a network-wide scale. Our result indicated that the activation level of the phosphotyrosine-related signaling molecules in AGS cells was suppressed overall in the presence of CagA during Helicobacter pylori infection. As Helicobacter pylori infection plays pivotal roles in the progression of gastric diseases including carcinogenesis, a comprehensive and fine description of the signaling dynamics would serve as a fundamental platform to theoretically explore for the potential drug targets through analyzing the regulatory mechanisms at the system-level.

6. Mass spectrometry-based annotation of the human short ORFeome

Masaaki Oyama, Hiroko Kozuka-Hata, Sumio Sugano[8], Tadashi Yamamoto[7] and Jun-ichiro Inoue: [8]Laboratory of Functional Genomics, Department of Medical Genome Sciences, Graduate School of Frontier Sciences, The University of Tokyo

In parallel with the human genome projects, human full-length cDNA data has also been intensively accumulated. Large-scale analysis of their 5'-UTRs revealed that about half of these had a short ORF upstream of the coding region. Experimental verification as to whether such upstream ORFs are translated is essential to reconsider the generality of the classical scanning mechanism for initiation of translation and define the real outline of the human proteome. Our previous proteomics analysis of small proteins expressed in human K562 cells provided the first direct evidence of translation of upstream ORFs in human full-length cDNAs (Oyama et al., Genome Res, 14: 2048-2052, 2004). In order to grasp an expanded landscape of the human short ORFeome, we have performed an in-depth proteomics analysis of human K562 and HEK293 cells using a two-dimensional nanoLC-MS/MS system. The results led to the identification of eight protein-coding regions besides 197 small proteins with a theoretical mass less than 20 kDa that were already annotated coding sequences in the curated mRNA database. In addition to the upstream ORFs in the presumed 5'-untranslated regions of mRNAs, bioinformatics analysis based on accumulated 5'-end cDNA sequence data provided evidence of novel short coding regions that were likely to be translated from the upstream non-AUG start site or from the new short transcript variants generated by utilization of downstream alternative promoters. Protein expression analysis of the *GRINL1A* gene revealed that translation from the most upstream start site occurred on the minor alternative splicing transcript, whereas this initiation site was not utilized on the major mRNA, resulting in translation of the downstream ORF from the second initiation codon. These findings reveal a novel post-transcriptional system that can augment the human proteome via the alternative use of diverse translation start sites coupled with transcriptional regulation through alternative promoters or splicing, leading to increased complexity of short protein-coding regions defined by the human transcriptome (Oyama et al., Mol Cell Proteomics, 6: 1000-1006, 2007).

<Group II>

AIM: Life, as we understand it, requires of a concerted and complex set of interactions between different biological molecules, such as DNA, RNA, proteins, lipids, and carbohydrates. We sought to understand the nature of these interactions at the molecular and energetic level. Our dissecting tools are applied to study a broad range of biological phenomena, and to develop the next generation of therapeutic antibodies in the era of Bio-better and Bio-superior.

1. Structural features of interfacial tyrosine residue in ROBO1 fibronectin domain-antibody complex: Crystallographic, thermodynamic, and molecular dynamic analyses

Nakayama T, Mizohata E, Yamashita T, Nagatoishi S, Nakakido M, Iwanari H, Mochizuki Y, Kado Y, Yokota Y, Satoh R, Tsumoto K, Fujitani H, Kodama T, Hamakubo T, Inoue T.

ROBO1, fibronectin Type-III domain (Fn)-containing protein, is a novel immunotherapeutic target for hepatocellular carcinoma in humans. The crystal structure of the antigen-binding fragment (Fab) of B 2212A, the monoclonal antibody against the third Fn domain (Fn3) of ROBO1, was determined in pursuit of antibody drug for hepatocellular carcinoma. This effort was conducted in the presence or absence of the antigen, with the chemical features being investigated by determining the affinity of the antibody using molecular dynamics (MD) and thermodynamics. The structural comparison of B2212A Fab between the complex and the free form revealed that the interfacial Tyr(L) 50 (superscripts L, H, and F stand for the residues in the light chain, heavy chain, and Fn3, respectively) played important roles in Fn3 recognition. That is, the aromatic ring of Tyr(L) 50 pivoted toward Phe(F) 68, forming a CH/π interaction and a new hydrogen bond with the carbonyl O atom of Phe(F) 68. MD simulations predicted that the Tyr(L) 50-Phe(F) 68 interaction almost entirely dominated Fab-Fn3 binding, and Ala-substitution of Tyr(L) 50 led to a reduced binding of the resultant complex. On the contrary, isothermal titration calorimetry experiments underscored that Ala-substitution of Tyr(L) 50 caused an increase of the binding enthalpy between B2212A and Fn3, but importantly, it induced an increase of the binding entropy, resulting in a suppression of loss in the Gibbs free energy in total. These results suggest that mutation analysis considering the binding entropy as well as the binding enthalpy will aid in the development of novel antibody drugs for hepatocellular carcinoma.

2. pH responsiveness of fibrous assemblies of repeat-sequence amphipathic α-helix polypeptides

Takei T, Tsumoto K, Okonogi A, Kimura A, Kojima S, Yazaki K, Takei T, Ueda T, Miura K.

We reported previously that our designed polypeptide α3 (21 residues), which has three repeats of a seven-amino-acid sequence (LETLAKA)3, forms not only an amphipathic α-helix structure but also long fibrous assemblies in aqueous solution. To address the relationship between the electrical states of the polypeptide and its α-helix and fibrous assembly formation, we characterized mutated polypeptides in which charged amino acid residues of α3 were replaced with Ser. We prepared the following polypeptides: 2Sα3 (LSTLAKA)3, in which all Glu residues were replaced with Ser residues; 6Sα3 (LETLASA)3, in which all Lys residues were replaced with Ser; and 2S6Sα3 (LSTLASA)3; in which all Glu and Lys residues were replaced with Ser. In 0.1M KCl, 2Sα3 formed an α-helix under basic conditions and 6Sα3 formed an α-helix under acid conditions. In 1M KCl, they both formed α-helices under a wide pH range. In addition, 2Sα3 and 6Sα3 formed fibrous assemblies under the same buffer conditions in which they formed α-helices. α-Helix and fibrous assembly formation by these polypeptides was reversible in a pH-dependent manner. In contrast, 2S6Sα3 formed an α-helix under basic conditions in 1M KCl. Taken together, these findings reveal that the charge states of the charged amino acid residues and the charge state of the Leu residue located at the terminus play an important role in α-helix formation.

3. Structural basis for self-assembly of a cytolytic pore lined by protein and lipid

Tanaka K, Caaveiro JM, Morante K, González-Mañas JM, Tsumoto K.

Pore-forming toxins (PFT) are water-soluble proteins that possess the remarkable ability to self-assemble on the membrane of target cells, where they form pores causing cell damage. Here, we elucidate the mechanism of action of the haemolytic protein fragaceatoxin C (FraC), a α-barrel PFT, by determining the crystal structures of FraC at four different stages of the lytic mechanism, namely the water-soluble state, the monomeric lipid-bound form, an assembly intermediate and the fully assembled transmembrane pore. The structure of the transmembrane pore exhibits a unique architecture composed of both protein and lipids, with some of the lipids lining the pore wall, acting as assembly cofactors. The pore also exhibits lateral fenestrations that expose the hydrophobic core of the mem-

brane to the aqueous environment. The incorporation of lipids from the target membrane within the structure of the pore provides a membrane-specific trigger for the activation of a haemolytic toxin.

4. Click conjugation of a binuclear terbium(III) complex for real-time detection of tyrosine phosphorylation

Akiba H, Sumaoka J, Tsumoto K, Komiyama M.

Phosphorylation of proteins is closely associated with various diseases, and, therefore, its detection is vitally important in molecular biology and drug discovery. Previously, we developed a binuclear Tb(III) complex, which emits notable luminescence only in the presence of phosphotyrosine. In this study, we conjugated a newly synthesized binuclear Tb(III) complex to substrate peptides by using click chemistry. Using these conjugates, we were able to detect tyrosine phosphorylation in real time. These conjugates were superior to nonconjugated Tb(III) complexes for the detection of tyrosine phosphorylation, especially when the substrate peptides used were positively charged. Luminescence intensity upon phosphorylation was enhanced 10-fold, making the luminescence intensity of this system one of the largest among lanthanide luminescence-based systems. We also determined Michaelis-Menten parameters for the phosphorylation of various kinase/peptide combinations and quantitatively analyzed the effects of mutations in the peptide substrates. Furthermore, we successfully monitored the inhibition of enzymatic phosphorylation by inhibitors in real time. Advantageously, this system detects only the phosphorylation of tyrosine (phosphorylated serine and threonine are virtually silent) and is applicable to versatile peptide substrates. Our study thus demonstrates the applicability of this system for the analysis of kinase activity, which could lead to drug discovery.

5. A pore-forming toxin requires a specific residue for its activity in membranes with particular physicochemical properties

Morante K, Caaveiro JM, Tanaka K, González-Mañas JM, Tsumoto K.

The physicochemical landscape of the bilayer modulates membrane protein function. Actinoporins are a family of potent hemolytic proteins from sea anemones acting at the membrane level. This family of cytolysins preferentially binds to target membranes containing sphingomyelin, where they form lytic pores giving rise to cell death. Although the cytolytic activity of the actinoporin fragaceatoxin C (FraC) is sensitive to vesicles made of various lipid compositions, it is far from clear how this toxin adjusts its mechanism of action to a broad range of physiochemical landscapes. Herein, we show that the conserved residue Phe-16 of FraC is critical for pore formation in cholesterol-rich membranes such as those of red blood cells. The interaction of a panel of muteins of Phe-16 with model membranes composed of raft-like lipid domains is inactivated in cholesterol-rich membranes but not in cholesterol-depleted membranes. These results indicate that actinoporins recognize different membrane environments, resulting in a wider repertoire of susceptible target membranes (and preys) for sea anemones. In addition, this study has unveiled promising candidates for the development of protein-based biosensors highly sensitive to the concentration of cholesterol within the membrane.

6. The Atomic Structure of the HIV-1 gp41 Transmembrane Domain and Its Connection to the Immunogenic Membrane-proximal External Region

Apellániz B, Rujas E, Serrano S, Morante K, Tsumoto K, Caaveiro JM, Jiménez MÁ, Nieva JL.

The membrane-proximal external region (MPER) C-terminal segment and the transmembrane domain (TMD) of gp41 are involved in HIV-1 envelope glycoprotein-mediated fusion and modulation of immune responses during viral infection. However, the atomic structure of this functional region remains unsolved. Here, based on the high resolution NMR data obtained for peptides spanning the C-terminal segment of MPER and the TMD, we report two main findings: (i) the conformational variability of the TMD helix at a membrane-buried position; and (ii) the existence of an uninterrupted α-helix spanning MPER and the N-terminal region of the TMD. Thus, our structural data provide evidence for the bipartite organization of TMD predicted by previous molecular dynamics simulations and functional studies, but they do not support the breaking of the helix at Lys-683, as was suggested by some models to mark the initiation of the TMD anchor. Antibody binding energetics examined with isothermal titration calorimetry and humoral responses elicited in rabbits by peptide-based vaccines further support the relevance of a continuous MPER-TMD helix for immune recognition. We conclude that the transmembrane anchor of HIV-1 envelope is composed of two distinct subdomains: 1) an immunogenic helix at the N terminus also involved in promoting membrane fusion; and 2) an immunosuppressive helix at the C terminus, which might also contribute to the late stages of the fusion process. The unprecedented high resolution structural data reported here may guide future vaccine and inhibitor developments.

7. Protein stabilization by an amphiphilic short monodisperse oligo(ethylene glycol)

Sadhukhan N, Muraoka T, Ui M, Nagatoishi S, Tsumoto K, Kinbara K.

A short, monodisperse additive (octa(ethylene glycol) monophenyl ether) functions to suppress aggregation of thermally and chemically denatured lysozyme. Control studies with shorter and non-amphiphilic derivatives revealed that the amphiphilic structure is essential, and octa(ethylene glycol) is nearly the minimum chain length for amphiphilic poly(ethylene glycol)s to stabilize proteins.

8. Structural basis for binding of human IgG1 to its high-affinity human receptor FcγRI

Kiyoshi M, Caaveiro JM, Kawai T, Tashiro S, Ide T, Asaoka Y, Hatayama K, Tsumoto K.

Cell-surface Fcγ receptors mediate innate and adaptive immune responses. Human Fcγ receptor I (hFcγRI) binds IgGs with high affinity and is the only Fcγ receptor that can effectively capture monomeric IgGs. However, the molecular basis of hFcγRI's interaction with Fc has not been determined, limiting our understanding of this major immune receptor. Here we report the crystal structure of a complex between hFcγRI and human Fc, at 1.80 Å resolution, revealing an unique hydrophobic pocket at the surface of hFcγRI perfectly suited for residue Leu235 of Fc, which explains the high affinity of this complex. Structural, kinetic and thermodynamic data demonstrate that the binding mechanism is governed by a combination of non-covalent interactions, bridging water molecules and the dynamic features of Fc. In addition, the hinge region of hFcγRI-bound Fc adopts a straight conformation, potentially orienting the Fab moiety. These findings will stimulate the development of novel therapeutic strategies involving hFcγRI.

9. Thermodynamics of antibody-antigen interaction revealed by mutation analysis of antibody variable regions

Akiba H, Tsumoto K.

Antibodies (immunoglobulins) bind specific molecules (i.e. antigens) with high affinity and specificity. In order to understand their mechanisms of recognition, interaction analysis based on thermodynamic and kinetic parameters, as well as structure determination is crucial. In this review, we focus on mutational analysis which gives information about the role of each amino acid residue in antibody-antigen interaction. Taking anti-hen egg lysozyme antibodies and several anti-small molecule antibodies, the energetic contribution of hot-spot and non-hot-spot residues is discussed in terms of thermodynamics. Here, thermodynamics of the contribution from aromatic, charged and hydrogen bond-forming amino acids are discussed, and their different characteristics have been elucidated. The information gives fundamental understanding of the antibody-antigen interaction. Furthermore, the consequences of antibody engineering are analysed from thermodynamic viewpoints: humanization to reduce immunogenicity and rational design to improve affinity. Amino acid residues outside hot-spots in the interface play important roles in these cases, and thus thermodynamic and kinetic parameters give much information about the antigen recognition. Thermodynamic analysis of mutant antibodies thus should lead to advanced strategies to design and select antibodies with high affinity.

10. Osteomodulin regulates diameter and alters shape of collagen fibrils

Tashima T, Nagatoishi S, Sagara H, Ohnuma S, Tsumoto K.

Osteomodulin (OMD) is a member of the small leucine-rich repeat proteoglycan family, which is involved in the organization of the extracellular matrix. OMD is located in bone tissue and is reportedly important for bone mineralization. However, the details of OMD function in bone formation are poorly understood. Using the baculovirus expression system, we produced recombinant human OMD and analyzed its interaction with type I collagen, which is abundant in bone. In this result, OMD directly interacted with purified immobilized collagen and OMD suppressed collagen fibril formation in a turbidity assay. Morphological analysis of collagen in the presence or absence of OMD demonstrated that OMD reduces the diameter and changes the shape of collagen fibrils. We conclude that OMD regulates the extracellular matrix during bone formation.

11. Differential binding of prohibitin-2 to estrogen receptor α and to drug-resistant ERα mutants

Chigira T, Nagatoishi S, Tsumoto K.

Endocrine resistance is one of the most challenging problems in estrogen receptor alpha (ERα)-positive breast cancer. The transcriptional activity of ERα is controlled by several coregulators, including prohibitin-2 (PHB2). Because of its ability to repress the transcriptional activity of activated ERα, PHB2 is a promising antiproliferative agent. In this study,

were analyzed the interaction of PHB2 with ERα and three mutants (Y537S, D538G, and E380Q) that are frequently associated with a lack of sensitivity to hormonal treatments, to help advance novel drug discovery. PHB2 bound to ERα wild-type (WT), Y537S, and D538G, but did not bind to E380Q. The binding thermodynamics of Y537S and D538G to PHB2 were favorably altered entropically compared with those of WT to PHB2. Our results show that PHB2 binds to the ligand binding domain of ERα with a conformational change in the helix 12 of ERα.

12. Functional characterization of Val60, a key residue involved in the membrane-oligomerization of fragaceatoxin C, an actinoporin from Actinia fragacea

Morante K, Caaveiro JM, Viguera AR, Tsumoto K, González-Mañas JM.

Actinoporins are pore-forming toxins produced by different sea anemones that self-assemble within the membranes of their target cells and compromise their function as a permeability barrier. The recently published three-dimensional structures of two oligomeric complexes formed by fragaceatoxin C point to Val60 as a key residue involved in the oligomerization of the functional pore. To gain insight into the mechanism of toxin oligomerization, different point mutations have been introduced at this position. Functional characterization of the muteins suggests that Val60 represents a hot-spot where the introduction of mutations hinders protein assembly and reduces the overall affinity for membranes.

13. Thermodynamic characterization of the interaction between the human Y-box binding protein YB-1 and nucleic acids

Tanabe Y, Nagatoishi S, Tsumoto K.

Y-box binding protein 1 (YB-1) binds to both RNA and DNA to control transcription and translation for the regulation of various cellular systems. YB-1 is overexpressed in some cancer cells and is a potential target for treatment of cancer. Herein, we describe isothermal titration calorimetry analyses of the interaction between a number of recombinant YB-1 domains and nucleic acids to identify the RNA and DNA binding sites and their binding mechanisms. These results demonstrated that the C-terminal domain of the protein interacts with single-stranded DNA and RNA by exothermic and endothermic reactions, respectively. The highly conserved cold-shock domain (CSD) also bound to single-stranded RNA and DNA by exothermic and endothermic reactions, respectively. The specific binding manner for RNA is in the CSD, whereas DNA binds with the most affinity to the C-terminal region (amino acids 130-219). We found further that the C-terminal region (amino acids 220-324) regulates the binding stoichiometry of RNA. These quantitative thermodynamic results provide a preliminary indication on the molecular mechanism of binding of the multifunctional protein YB-1 to nucleic acids to regulate its biological function.

14. Structural and Thermodynamic Basis of Epitope Binding by Neutralizing and Nonneutralizing Forms of the Anti-HIV-1 Antibody 4E10

Rujas E, Gulzar N, Morante K, Tsumoto K, Scott JK, Nieva JL, Caaveiro JM.

The 4E10 antibody recognizes the membrane-proximal external region (MPER) of the HIV-1 Env glycoprotein gp41 transmembrane subunit, exhibiting one of the broadest neutralizing activities known to date. The neutralizing activity of 4E10 requires solvent-exposed hydrophobic residues at the apex of the complementarity-determining region (CDR) H3 loop, but the molecular basis for this requirement has not been clarified. Here, we report the cocrystal structures and the energetic parameters of binding of a peptide bearing the 4E10-epitope sequence (4E10ep) to nonneutralizing versions of the 4E10 Fab. Nonneutralizing Fabs were obtained by shortening and decreasing the hydrophobicity of the CDR-H3 loop (termed ΔLoop) or by substituting the two tryptophan residues of the CDR-H3 apex with Asp residues (termed WDWD), which also decreases hydrophobicity but preserves the length of the loop. The analysis was complemented by the first crystal structure of the 4E10 Fab in its ligand-free state. Collectively, the data ruled out major conformational changes of CDR-H3 at any stage during the binding process (equilibrium or transition state). Although these mutations did not impact the affinity of wild-type Fab for the 4E 10ep in solution, the two nonneutralizing versions of 4E10 were deficient in binding to MPER inserted in the plasma membrane (mimicking the environment faced by the antibody in vivo). The conclusions of our structure-function analysis strengthen the idea that to exert effective neutralization, the hydrophobic apex of the solvent-exposed CDR-H3 loop must recognize an antigenic structure more complex than just the linear α-helical epitope and likely constrained by the viral membrane lipids.

IMPORTANCE:

The broadly neutralizing anti-HIV-1 4E10 antibody blocks infection caused by nearly all viral strains and isolates examined thus far. However, 4E10 (or 4E10-like) antibodies are rarely found in HIV-1-infected individuals or elicited through vac-

cination. Impediments to the design of successful 4E10 immunogens are partly attributed to an incomplete understanding of the structural and binding characteristics of this class of antibodies. Since the broadly neutralizing activity of 4E10 is abrogated by mutations of the tip of the CDR-H3, we investigated their impact on binding of the MPER-epitope at the atomic and energetic levels. We conclude that the difference between neutralizing and nonneutralizing antibodies of 4E10 is neither structural nor energetic but is related to the capacity to recognize the HIV-1 gp41 epitope inserted in biological membranes. Our findings strengthen the idea that to elicit similar neutralizing antibodies, the suitable MPER vaccine must be "delivered" in a membrane environment.

15. Discovery and characterization of natural tropolones as inhibitors of the antibacterial target CapF from Staphylococcus aureus

Nakano K, Chigira T, Miyafusa T, Nagatoishi S, Caaveiro JM, Tsumoto K.

The rapid spread of antibiotic-resistance among pathogenic bacteria poses a serious risk for public health. The search for novel therapeutic strategies and antimicrobial compounds is needed to ameliorate this menace. The bifunctional metalloenzyme CapF is an antibacterial target produced by certain pathogenic bacteria essential in the biosynthetic route of capsular polysaccharide, a mucous layer on the surface of bacterium that facilitates immune evasion and infection. We report the first inhibitor of CapF from Staphylococcus aureus, which was identified by employing fragment-based methodologies. The hit compound 3-isopropenyl-tropolone inhibits the first reaction catalyzed by CapF, disrupting the synthesis of a key precursor of capsular polysaccharide. Isothermal titration calorimetry demonstrates that 3-isopropenyl-tropolone binds tightly (KD = 27 ± 7μM) to the cupin domain of CapF. In addition, the crystal structure of the enzyme-inhibitor complex shows that the compound engages the essential Zn(2 +) ion necessary for the first reaction catalyzed by the enzyme, explaining its inhibitory effect. Moreover, the tropolone compound alters the coordination sphere of the metal, leading to the overall destabilization of the enzyme. We propose 3-isopropenyl-tropolone as a precursor to develop stronger inhibitors for this family of enzymes to impair the synthesis of capsular polysaccharide in Staphylococcus aureus.

16. Structural analysis of Fc/FcγR complexes: a blueprint for antibody design

Caaveiro JM, Kiyoshi M, Tsumoto K.

The number of studies and the quality of the structural data of Fcγ receptors (FcγRs) has rapidly increased in the last few years. Upon critical examination of the literature, we have extracted general conclusions that could explain differences in affinity and selectivity of FcγRs for immunoglobulin G (IgG) based on structural considerations. FcγRs employ a little conserved asymmetric surface of domain D2 composed of two distinct subsites to recognize the well-conserved lower hinge region of IgG1-Fc. The extent of the contact interface with the antibody in subsite 1 of the receptor (but not in subsite 2), the geometrical complementarity between antibody and receptor, and the number of polar interactions contribute decisively toward strengthening the binding affinity of the antibody for the receptor. In addition, the uncertain role of the N-linked glycan of IgG for the binding and effector responses elicited by FcγRs is discussed. The available data suggest that not only the non-covalent interactions between IgG and FcγRs but also their dynamic features are essential for the immune response elicited through these receptors. We believe that the integration of structural, thermodynamic, and kinetic data will be critical for the design and validation of the next generation of therapeutic antibodies with enhanced effector capabilities.

17. Bidirectional Transformation of a Metamorphic Protein between the Water-Soluble and Transmembrane Native States

Tanaka K, Caaveiro JM, Tsumoto K.

The bidirectional transformation of a protein between its native water-soluble and integral transmembrane conformations is demonstrated for FraC, a hemolytic protein of the family of pore-forming toxins. In the presence of biological membranes, the water-soluble conformation of FraC undergoes a remarkable structural reorganization generating cytolytic transmembrane nanopores conducive to cell death. So far, the reverse transformation from the native transmembrane conformation to the native water-soluble conformation has not been reported. We describe the use of detergents with different physicochemical properties to achieve the spontaneous conversion of transmembrane pores of FraC back into the initial water-soluble state. Thermodynamic and kinetic stability data suggest that specific detergents cause an asymmetric change in the energy landscape of the protein, allowing the bidirectional transformation of a membrane protein.

18. Epiregulin Recognition Mechanisms by Anti-Epiregulin Antibody 9E5: Structural, Functional and Molecular Dynamics Simulation Analyses

Kado Y, Mizohata E, Nagatoishi S, Iijima M, Shinoda K, Miyafusa T, Nakayama T, Yoshizumi T, Sugiyama A, Kawamura T, Lee YH, Matsumura H, Doi H, Fujitani H, Kodama T, Shibasaki Y, Tsumoto K, Inoue T.

Epiregulin (EPR) is a ligand of the epidermal growth factor (EGF) family that upon binding to its epidermal growth factor receptor (EGFR) stimulates proliferative signaling, especially in colon cancer cells. Here, we describe the three-dimensional structure of the EPR antibody (the 9E5(Fab) fragment) in the presence and absence of EPR. Among the six complementarity-determining regions (CDRs), CDR1-3 in the light chain and CDR2 in the heavy chain predominantly recognize EPR. In particular, CDR3 in the heavy chain dramatically moves with cis-trans isomerization of Pro103. A molecular dynamics simulation and mutational analyses revealed that Arg40 in EPR is a key residue for the specific binding of 9E5 IgG. From ITC analysis, the dissociation constant is determined to be 6.5 nM. Surface plasmon resonance analysis revealed that the dissociation rate of 9E5 IgG is extremely slow. The superimposed structure of 9E5(Fab)-EPR on the known complex structure of EGF-EGFR showed that the 9E5(Fab) paratope overlaps with Domains I and III on the EGFR, which reveals that the 9E5 (Fab)-EPR complex could not bind to the EGFR. The 9E5 antibody will also be useful in medicine as a neutralizing antibody specific for colon cancer.

19. Rapid Heme Transfer Reactions between NEAr Transporter Domains of Staphylococcus aureus: A Theoretical Study Using QM/MM and MD Simulations

Moriwaki Y, Terada T, Tsumoto K, Shimizu K.

In vertebrates, most iron is present as heme or is chelated by proteins. Thus, Gram-positive pathogens such as Staphylococcus aureus have evolved an iron-regulated surface determinant (Isd) system that transports heme across thick cell walls into the cytoplasm. Recent studies have demonstrated that heme is rapidly transferred between the NEAr Transporter (NEAT) domains of the Isd system, despite its high affinity toward each domain, suggesting the presence of an intermediate NEAT•heme• NEAT complex. In the present study, we performed short restrained molecular dynamics (MD) simulations to dock the acceptor NEAT domain to the donor NEAT•heme complex and obtained models where the two NEAT domains were arranged with two-fold pseudo symmetry around the heme molecule. After turning off the restraints, complex structures were stably maintained during subsequent unrestrained MD simulations, except for the hydrogen bond between the propionate group of the heme molecule and the donor NEAT domain, potentially facilitating the transition of heme from the donor to the acceptor. Subsequent structural optimization using the quantum mechanics/molecular mechanics (QM/MM) method showed that two tyrosine residues, one from each NEAT domain, were simultaneously coordinated to the ferric heme iron in the intermediate complex only if they were deprotonated. Based on these results, we propose a reaction scheme for heme transfer between NEAT domains.

<Group III>

1. Development of new methods for analyzing the neural circuits in the retina

Neural circuits in the central nervous system are the basis of various high-order brain functions. It is also true in case of retina. In the retina, six main classes of neural cells connect systematically to make up complex neural circuits. Characteristics of the neural cell functions have been examined precisely by the electrophysiological methods and models of cell connectivity have been proposed. But morphological studies of the actual neural connection, which constitute the physiological properties of retinal neurons, are not enough. Until recently to collect ultrathin serial sections and observe in transmission electron microscope (TEM) were the only way to reveal the connectivity of actual neural cells morphologically. But the technical difficulties discouraged us from such a troublesome studies. Recent progress in scanning electron microscope (SEM) equipment allowed us to develop a new method to observe ultrathin TEM sections in SEM. Samples were specifically treated to enhance electron contrast and serial thin TEM sections were collected on the smooth conductive matrix. By using SEM to observe thin TEM sections, it became possible to analyze much wider areas than by using TEM. We have analyzed about 500 serial thin sections of zebrafish retinal outer plexiform layer by this method and succeeded in tracing thin processes of bipolar cells into the photoreceptor terminal. In the course of this study, it became obvious that thinner and larger numbers of section are needed to reveal the accurate connectivity especially for thinner processes. This year, we developed a new equipment to collect huge number of serial sections stably and efficiently. By using this equipment, more than 1000 serial sections of less than 30nm thickness were successfully collected and have been analyzing.

In parallel, methods to observe thick sections, which are difficult to observe because of their less conductivity, were developed and tested for use in counting actual cell number in tissues. In another work, 515 serial thin sections of a whole hepatic

cell were observed with the same method and reconstituted as a model for simulating the transduction of NF-kappa B signal in the real cell (ref. Ichikawa[1] et al) : [1]Division of Mathematical Oncology.

2. Collaborative and supportive works as electron microscope core-laboratory

This group is also engaged in collaborative researches using electron microscope. We offer supports for the research projects those need electron microscopic analysis. The services available in this group are the conventional thin section transmission electron microscopy, immuno-electron microscopy, negative staining techniques and scanning electron microscopy. By using these individual technique or combination of some of these we can offer direct visual evidence that cannot be acquired by other methods. This year, 23 projects in 13 laboratories were performed as core-laboratory works.

a. Thin section transmission electron microscopy

Thin section transmission electron microscopy is the most widely used technique to observe the inner structure of cells and tissues. In this method, samples are fixed and embedded in epoxy resin, thin sections with about 70nm thickness are cut and observed in the electron microscope. In case of immuno-electron microscopy, thin sections are obtained by similar procedure, and the antigen epitopes exposed on the surface of the sections are marked by sequentially reacted with appropriate primary antibodies and colloidal gold labeled secondary antibodies. This year, thin section electron microscopy combined with immuno-electron microscopy were used in many collaborative works.

a-1. Ultrastructural analysis of entry and assembly of Herpes Simplex Virus

We have been performing several studies with research groups in Dr. Kawaguchi[2]'s laboratory: [2]Division of Molecular Virology, Department of Microbiology and Immunology, regarding the infection/replication processes of herpes simplex virus (HSV). This year, thin section electron microscopy was used in 11 projects to analyze the function of viral proteins in trans-nuclear membrane processes of the newly forming viruses. Among these woks, investigatin of the type-specific differences between HSSV-1 and HSV-2 Us3 protein kinases (ref. Shindo[2] et al) and the work regarding the function of HSV-1 CD98 and beta-1 integrin in the denvelopment proces (ref. Hirohata[2] et al) were published.

a-2. Morphological analysis of the mouse Paneth cells and M cells

We have been performing several studies also with research groups in Dr. Kiyono[3]'s laboratory: [3]Division of Mucosal Immunology, Department of Microbiology and Immunology. This year, thin section transmission electron microscopy was used to analyze the change of Paneth cell structures in a certain gene knock out mouse. In another work, the structural changes of M cells were also analyzed electron microscopically.

Some other collaborative research works using thin section electron microscopy and/or immuno-electron microscopy were performed with Dr. Noda[4] et al in [4]Division of Virology, Department of Microbiology, regarding the structure of the influenza viruses and ebola virus, Dr. Yasuda[5]'s group, in [5]Department of Integrated Biosciences, Graduate School of Frontier Sciences regarding the fine structure of the developing central nervous system of Medaka fish (ref. Yasuda[5] et al).

b. Negative staining techniques

Negative staining techniques are simple and quick method to observe the morphology of the macro molecules. In the collaborative work with Dr. Noda[4] et al., this technique combined with thin section electron microscopy was used to analyze the morphology of the influenza virus ribonucleoprotein complex. The negative staining techniques were also used in some works to analyze the structure of the purified proteins and the proteins integrated in the plasma membrane. This method convined with scanning electron microscopy was used also to analyze the function of a protein during the in vitro formation of collagen fibers in collaboration with Dr. Tashima[6] et al in [6]Medical Proteomics Laboratory (ref. Tashima[6] et al).

c. Conventional scanning electron microscopy

Conventional scanning electron microscopy is a technique used to examine the surface structure of the cells, tissues or other non-biological materials. The collaborative work using scanning electron microscopy convined with negative staining method was performed with Dr. Tashima[6] as mentioned above (ref. Tashima[6] et al). Scanning electron microscopy was also used to analyze the morphological changes of cultured macrophages and non-biological materials as a collaborative work with Dr. Cheng[7] in [7]Olympus Co.

Publications

<Group I>

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