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. Integrative analysis of cancer cell signaling networks by high-resolution proteomics and systems biology

Post-translational modifications (PTMs), such as phosphorylation, ubiquitination and acetylation, are known to be widely involved in the regulation of various biological processes through extensive diversification of each protein function at the cellular network level. Previous functional analyses of cancer cell signaling under a variety of experimental conditions revealed many of the key molecules and their associated protein modifications in relation to each type of cancer. In order to systematically discover critical modulators from diversified signaling molecules, we have developed a high-resolution mass spectrometry-based proteomics platform for integrative identification and quantification of multiple post-translational modifications from various types of cancer cells.

1-1. High-resolution proteomic analysis of EGF-regulated ubiquitination dynamics in human cancer cells

Hiroko Kozuka-Hata, Tomoko Hiroki, Aya Kitamura, Aiko Aizawa, Naoaki Miyamura, Kouhei Tsumoto, Jun-ichiro Inoue, and Masaaki Oyama.

Protein ubiquitination is one of the most prevalent post-translational modifications (PTMs) and plays critical roles in regulating protein degradation, signal transduction and DNA repair in cooperation with other PTMs such as phosphorylation and acetylation. Recent mass spectrometry-based proteomics coupled with efficient enrichment technologies for each type of the modified peptides has enabled us to identify precise modification sites and measure their quantitative changes on a global scale. Our previous lysine-modification proteomic analyses of thirteen representative human cancer cell lines led us to identify thousands of ubiquitination (Ub) and acetylation (Ac) sites in total and revealed that their system-wide modification status was mutually different at the cel-

lular network level. In this study, we further applied SILAC (Stable Isotope Labeling by Amino acids in Cell culture) for quantitative description of EGF-dependent lysine-modification site dynamics in HeLa cells in a time-resolved manner. Through integration of large-scale SILAC-encoded data on six time points upon EGF stimulation, we successfully quantified approximately 1,000 kinds of Ub-sites as well as 700 kinds of Ac-sites and found that one-third of these Ub-modified molecules, including several EGF signaling effectors, were subjected to downregulation by proteasomal inhibition.

1-2. Proteome-wide analysis of lysine acetylation and ubiquitination reveals critical signaling regulation in cancer cells

Hiroko Kozuka-Hata, Aya Kitamura, Tomoko Hiroki, Aiko Aizawa, Kouhei Tsumoto, Jun-ichiro Inoue, and Masaaki Oyama.

Post-translational modifications (PTMs), such as phosphorylation, ubiquitination and acetylation, are known to be widely involved in the regulation of various biological processes through extensive diversification of each protein function at the cellular network level. Previous functional analyses of cancer cell signaling under a variety of experimental conditions revealed many of the key molecules and their associated protein modifications in relation to each type of cancer. In order to systematically discover critical modulators from diversified signaling molecules, we have developed a high-resolution mass spectrometry-based proteomics platform for integrative identification and quantification of multiple post-translational modifications from various types of cancer cells. Our large-scale proteomic analysis enabled us to identify more than 5,000 kinds of ubiquitinated sites and 1,600 kinds of acetylated sites from representative human cancer cell lines, leading to identification of approximately 900 novel lysine modification sites in total. Very interestingly, 236 lysine residues derived from 141 proteins were found to be modified with both ubiquitination and acetylation. As a consequence of the subsequent motif extraction analyses, glutamic acid (E) was found to be highly enriched at the position (-1) for the lysine acetylation sites, whereas the same amino acid was relatively dispersed along the neighboring residues of the lysine ubiquitination sites.

1-3. System-wide perturbation of the proteome and phosphoproteome dynamics in glioblastoma stem cells through mTOR signaling inhibition

Hiroko Kozuka-Hata, Tomoko Hiroki, Ryo Koyama-Nasu, Kouhei Tsumoto, Jun-ichiro Inoue, Tetsu Akiyama, 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.

1-4. System-level analysis of CagA-dependent signaling network dynamics by Helicobacter pylori infection

Hiroko Kozuka-Hata, Masato Suzuki, Kotaro Kiga, Shinya Tasaki, Jun-ichiro Inoue, Tadashi Yamamoto, Chihiro Sasakawa, and Masaaki Oyama.

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.

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

Masaaki Oyama, Hiroko Kozuka-Hata, Sumio Sugano, Tadashi Yamamoto, and Jun-ichiro Inoue.

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).

3. In-depth proteomic analysis of drug-responsive signaling pathway elements in human cancer cells

Wei QI, Aya Kitamura, Naoaki Miyamura, Tomoko Hiroki, Aiko Aizawa, Kazuki Mori, Hiroko Kozuka-Hata, and Masaaki Oyama.

Abnormal expression of histone deacetylases (HDACs) in human cancer cells was reported to be associated with angiogenesis, migration, chemotherapy resistance as well as cell differentiation and apoptosis in a wide range of previous studies. Therefore, clinical use of HDAC inhibitors has been discussed as a new therapeutic approach against cancer for a long period. In 2006, suberoylanilide hydroxamic acid (SAHA), a pan-inhibitor targeting HDACs and also known as Vorinostat, was approved by the US Food and Drug Administration (FDA) for the treatment of cutaneous T-cell lymphoma. In addition to the anticancer activity against hematologic cancers, SAHA also shows a significant antitumor effect on solid tumors through inducing apoptosis, arresting cell cycle or elevating radiation sensitization. In order to unveil the underlying complex mechanism, we used human HeLa cells as the model platform for analyzing SAHA-responsive elements on a proteomic scale. According to the experimental pre-evaluation through western blotting for acetylated histone H3 and microscopic observation of cell growth under a variety of drug-perturbed conditions, we determined to treat cultured cells with SAHA for 24 h to perform an in-depth quantitative proteomic analysis of SAHA-responsive elements in human HeLa cells. After SAHA treatment, the cells were lysed, trypsin-digested and analyzed by high-resolution nanoflow liquid chromatography- tandem mass spectrometry. As a result of ultra-deep proteomic analysis by Orbitrap Eclipse Tribrid system coupled with Ultimate3000 RSLCnano liquid chromatography technology, a total of 5,135 proteins was identified using Proteome Discoverer software. Approximately 8 % of the identified proteins were found to be differentially regulated with more than two-fold changes in response to SAHA treatment by Label Free Quantification (LFQ). The subsequent pathway analysis based on Kyoto Encyclopedia of Genes and Genomes (KEGG) indicated that cell cycle and anti-apoptotic pathway elements including p27 and HO-1 were prominently correlated with SAHA-dependent regulation in human HeLa

cells.

4. Real-Time Search-Assisted Multiplexed Quantitative Proteomics Reveals System-Wide Translational Regulation of Non-Canonical Short Open Reading Frames

Hiroko Kozuka-Hata, Tomoko Hiroki, Naoaki Miyamura, Aya Kitamura, Kouhei Tsumoto, Jun-ichiro Inoue, and Masaaki Oyama.

Abnormal expression of histone deacetylases (HDACs) is reported to be associated with angiogenesis, metastasis and chemotherapy resistance regarding cancer in a wide range of previous studies. Suberoylanilide hydroxamic acid (SAHA) is well known to function as a pan-inhibitor for HDACs and recognized as one of the therapeutic drug candidates to epigenetically coordinate cancer cell fate regulation on a genomic scale. Here, we established a Real-Time Search-assisted mass spectrometric platform for system-wide quantification of translated products encoded by non-canonical short open reading frames (ORFs) as well as already annotated protein coding sequences (CDSs) on the human transcriptome and applied this methodology to quantitative proteomic analyses of SAHA-treated human HeLa cells to evaluate proteome-wide regulation in response to drug perturbation. Very intriguingly, our RTS-based in-depth proteomic analysis enabled us to identify approximately 5000 novel peptides from the ribosome profiling-based short ORFs encoded in the diversified regions on presumed 'non-coding' nucleotide sequences of mRNAs as well as lncRNAs and nonsense mediated decay (NMD) transcripts. Furthermore, TMT-based multiplex large-scale quantification of the whole proteome changes upon differential SAHA treatment unveiled dose-dependent selective translational regulation of a limited fraction of the non-canonical short ORFs in addition to key cell cycle/proliferation-related molecules such as UBE2C, CENPF and PRC1. Our study provided the first system-wide landscape of drug-perturbed translational modulation on both canonical and non-canonical proteome dynamics in human cancer cells.

5. Ultra-deep single-cell proteomic analysis of patient-derived glioblastoma cells

Hiroko Kozuka-Hata, Tomoko Hiroki, Aya Kitamura, Naoaki Miyamura, Tetsu Akiyama, Jun-ichiro Inoue, Kouhei Tsumoto, and Masaaki Oyama.

Single-cell analysis is an essential technique for understanding cellular diversity by analyzing mutually unique data from individual cells. The heterogeneity of cancer cells is known to be deeply involved in drug resistance and poor prognosis, thus there is a strong demand for the development of effective cancer therapies based on single-cell analysis. While single-cell transcriptome analysis has rapidly become widespread with the evolution of next-generation sequencers, single-cell proteome analysis is still an emerging approach due to technical limitations in detection sensitivity. However, since proteins more directly reflect cellular functions compared to RNA molecules, it is highly anticipated that in-depth single-cell proteome analysis, based on recent advancements in mass spectrometry technology, will provide more detailed molecular network information on individual cell regulation. Previously, we conducted a high-precision quantitative phosphoproteome analysis of cancer stem cells (GB2) derived from glioblastoma patients to elucidate the signaling mechanisms leading to very high malignant properties with a five-year survival rate of less than 10%. Our analysis revealed that stimulation by epidermal growth factor (EGF), which controls stemness maintenance of these cells, activated mTORC1 and highly phosphorylated the downstream ribosomal protein S6 (Kozuka-Hata et al., PLoS One, 2012). Since mTOR inhibitors exert antitumor effects by inhibiting the functions of various factors necessary for cancer cell proliferation and angiogenesis, we applied Torin 1, a representative mTOR inhibitor, for EGF signaling perturbation of GB2 cells and newly established an experimental system to evaluate EGF-dependent protein expression changes at the single-cell level. Our integrative single-cell proteomic measurement using advanced Orbitrap MS instruments unveiled Torin 1-dependent global dynamics of more than 6,000 proteins including novel peptides encoded by non-canonical translation at single-cell resolution.

<Group II>

Biomolecular recognition is based on collective and specific non-covalent interactions between discrete biological molecules. Our laboratory studies various protein systems, for instance, antibody-antigen and protein-ligand complexes, to understand quantitatively how these coordinated non-covalent interactions contribute to their specific recognition in biological and artificial systems. We seek to elucidate the molecular mechanisms by which biological molecules obtain high-specificity and affinity from multiple angles using advanced instrumentation. To produce functional molecules with higher performance and better properties, we aim to build a solid foundation from which to develop drugs that modulate specific interactions between biomolecules and ultimately to understand the principles of molecular interactions in our lives.

1. Experimental modification in thermal stability of oligomers by alanine substitution and site saturation mutagenesis of interfacial residues

Hoya M, Matsunaga R, Nagatoishi S, and Tsumoto K.

For certain industrial applications, the stability of protein oligomers is important. In this study, we demonstrated an efficient method to improve the thermal stability of oligomers using the trimeric protein chloramphenicol acetyltransferase (CAT) as the model. We substituted all interfacial residues of CAT with alanine to detect residues critical for oligomer stability. Mutation of six of the forty-nine interfacial residues enhanced oligomer thermal stability. Site saturation mutagenesis was performed on these six residues to optimize the side chains. About 15% of mutations enhanced thermal stability by more than 0.5 °C and most did not disrupt activity of CAT. Certain combinations of mutations further improved thermal stability and resistance against heat treatment. The quadruple mutant, H17V/N34S/F134A/D157C, retained the same activity as the wild-type after heat treatment at 9 °C higher temperature than the wild-type CAT. Furthermore, combinations with only alanine substitutions also improved thermal stability, suggesting the method we developed can be used for rapid modification of industrially important proteins.

2. IL-6 Reduces Spheroid Sizes of Osteophytic Cells Derived from Osteoarthritis Knee Joint via Induction of Apoptosis

Negishi Y, Adili Arepati, Susana de Vega, Momoeda M, Kaneko H, Mehmet Zeynel Cilek, Yoshinaga C, Takafuji K, Otsuka Y, Shimoda M, Negishi-Koga T, Ishijima M, and Okada Y

Osteophytes in osteoarthritis (OA) joints contribute to restriction of joint movement, joint pain, and OA progression, but little is known about osteophyte regulators. Examination of gene expression related to cartilage extracellular matrix, endochondral ossification, and growth factor signaling in articular cartilage and osteophytes obtained from OA knee joints showed that several genes such as COL1A1, VCAN, BGLAP, BMP8B, RUNX2, and SOST were overexpressed in osteophytes compared with articular cartilage. Ratios of mesenchymal stem/progenitor cells, which were characterized by co-expression of CD105 and CD166, were significantly higher in osteophytic cells than articular cells. A three-dimensional culture method for cartilage and osteophyte cells was developed by modification of cultures of self-assembled spheroid cell organoids (spheroids). These spheroids cultured in the media for mesenchymal stem cells containing transforming growth factor-β3 showed characteristic morphologies and gene expression profiles of articular cartilage and osteophytes, respectively. The effects of IL-1β, tumor necrosis factor-α, and IL-6 on the spheroids of articular and osteophytic cells were studied. To the best of our knowledge, they provide the first evidence that IL-6 suppresses the spheroid size of osteophytic cells by inducing apoptosis and reducing extracellular matrix molecules. These data show that IL-6 is the suppressor of osteophyte growth and suggest that IL-6 expression and/or activity are implicated in the regulation of osteophyte formation in pathologic joints.

3. Generation of antibodies to an extracellular region of the transporters Glut1/Glut4 by immunization with a designed antigen

Sumikawa T, Nakakido M, Matsunaga R, Kuroda D, Nagatoishi S, and Tsumoto K.

Monoclonal antibodies are one of the fastest growing class of drugs. Nevertheless, relatively few biologics target multispanning membrane proteins because of technical challenges. To target relatively small extracellular regions of multiple membrane-spanning proteins, synthetic peptides, which are composed of amino acids corresponding to an extracellular region of a membrane protein, are often utilized in antibody discovery. However, antibodies to these peptides often do not recognize parental membrane proteins. In this study, we designed fusion proteins in which an extracellular helix of the membrane protein glucose transporter 1 (Glut1) was grafted onto the scaffold protein Adhiron. In the initial design, the grafted fragment did not form a helical conformation. Molecular dynamics simulations of full-length Glut1 suggested the importance of intramolecular interactions formed by surrounding residues in the formation of the helical conformation. A fusion protein designed to maintain such intramolecular interactions did form the desired helical conformation in the grafted region. We then immunized an alpaca with the designed fusion protein and obtained VHH (variable region of heavy-chain antibodies) using the phage display method. The binding of these VHH antibodies to the recombinant Glut1 protein was evaluated by surface plasmon resonance, and their binding to Glut1 on the cell membrane was further validated by flow cytometry. Furthermore, we also succeeded in the generation of a VHH against another integral membrane protein, glucose transporter 4 (Glut4) with the same strategy. These illustrates that our combined biochemical and computational approach can be applied to designing other novel fusion proteins for generating site-specific antibodies.

4. Unveiling the affinity-stability relationship in anti-measles virus antibodies: a computational approach for hotspots prediction

Paul R, Kasahara K, Sasaki J, Pérez JF, Matsunaga R, Hashiguchi T, Kuroda D, and Tsumoto K.

Recent years have seen an uptick in the use of computational applications in antibody engineering. These tools have enhanced our ability to predict interactions with antigens and immunogenicity, facilitate humanization, and serve other critical functions. However, several studies highlight the concern of potential trade-offs between antibody affinity and stability in antibody engineering. In this study, we analyzed anti-measles virus antibodies as a case study, to examine the relationship between binding affinity and stability, upon identifying the binding hotspots. We leverage in silico tools like Rosetta and FoldX, along with molecular dynamics (MD) simulations, offering a cost-effective alternative to traditional in vitro mutagenesis. We introduced a pattern in identifying key residues in pairs, shedding light on hotspots identification. Experimental physicochemical analysis validated the predicted key residues by confirming significant decrease in binding affinity for the high-affinity antibodies to measles virus hemagglutinin. Through the nature of the identified pairs, which represented the relative hydropathy of amino acid side chain, a connection was proposed between affinity and stability. The findings of the study enhance our understanding of the interactions between antibody and measles virus hemagglutinin. Moreover, the implications of the observed correlation between binding affinity and stability extend beyond the field of anti-measles virus antibodies, thereby opening doors for advancements in antibody research.

5. THOUSAND-GRAIN WEIGHT 6, which is an IAA-glucose hydrolase, preferentially recognizes the structure of the indole ring

Akabane T, Suzuki N, Ikeda K, Yonezawa T, Nagatoishi S, Matsumura H, Yoshizawa T, Tsuchiya W, Kamino S, Tsumoto K, Ishimaru K, Katoh E, and Hirotsu N.

An indole-3-acetic acid (IAA)-glucose hydrolase, THOUSAND-GRAIN WEIGHT 6 (TGW6), negatively regulates the grain weight in rice. TGW6 has been used as a target for breeding increased rice yield. Moreover, the activity of TGW6 has been thought to involve auxin homeostasis, yet the details of this putative TGW6 activity remain unclear. Here, we show the three-dimensional structure and substrate preference of TGW6 using X-ray crystallography, thermal shift assays and fluorine nuclear magnetic resonance (19F NMR). The crystal structure of TGW6 was determined at 2.6 Å resolution and exhibited a six-bladed β-propeller structure. Thermal shift assays revealed that TGW6 preferably interacted with indole compounds among the tested substrates, enzyme products and their analogs. Further analysis using 19F NMR with 1,134 fluorinated fragments emphasized the importance of indole fragments in recognition by TGW6. Finally, docking simulation analyses of the substrate and related fragments in the presence of TGW6 supported the interaction specificity for indole compounds. Herein, we describe the structure and substrate preference of TGW6 for interacting with indole fragments during substrate recognition. Uncovering the molecular details of TGW6 activity will stimulate the use of this enzyme for increasing crop yields and contributes to functional studies of IAA glycoconjugate hydrolases in auxin homeostasis.

6. Structural basis for the recognition of human hemoglobin by the heme-acquisition protein Shr from Streptococcus pyogenes

Senoo A, Hoshino M, Shiomi T, Nakakido M, Nagatoishi S, Kuroda D, Nakagawa I, Tame JRH, Caaveiro JMM, and Tsumoto K.

In Gram-positive bacteria, sophisticated machineries to acquire the heme group of hemoglobin (Hb) have evolved to extract the precious iron atom contained in it. In the human pathogen Streptococcus pyogenes, the Shr protein is a key component of this machinery. Herein we present the crystal structure of hemoglobin-interacting domain 2 (HID2) of Shr bound to Hb. HID2 interacts with both, the protein and heme portions of Hb, explaining the specificity of HID2 for the heme-bound form of Hb, but not its heme-depleted form. Further mutational analysis shows little tolerance of HID2 to interfacial mutations, suggesting that its interaction surface with Hb could be a suitable candidate to develop efficient inhibitors abrogating the binding of Shr to Hb.

7. A high-resolution structural characterization and physicochemical study of how a peptoid binds to an oncoprotein MDM2

Yokomine M, Morimoto J, Fukuda Y, Ueda T, Takeuchi K, Umezawa K, Ago H, Matsuura H, Ueno G, Senoo A, Nagatoishi S, Tsumoto K, and Sando S.

Peptoids are a promising drug modality targeting disease-related proteins, but how a peptoid engages in protein binding is poorly understood. This is primarily due to a lack of high-resolution peptoid-protein complex structures and systematic physicochemical studies. Here, we present the first crystal structure of a peptoid bound to a protein, providing high-resolution structural information about how a peptoid binds to a protein. We previously reported a rigid peptoid, oligo(N-substituted alanine) (oligo-NSA), and developed an oligo-NSA-type peptoid that binds to MDM2. X-ray crystallographic analysis of the peptoid bound to MDM2 showed that the peptoid recognizes the MDM2 surface predominantly through the interaction of the N-substituents, while the main chain acts as a scaffold. Additionally, conformational, thermodynamic, and kinetic analysis of the peptoid

and its derivatives with a less rigid main chain revealed that rigidification of the peptoid main chain contributes to improving the protein binding affinity. This improvement is thermodynamically attributed to an increased magnitude of the binding enthalpy change, and kinetically to an increased association rate and decreased dissociation rate. This study provides invaluable insights into the design of protein-targeting peptoids.

8. Thermodynamic and molecular dynamic insights into how fusion influences peptide-tag recognition of an antibody

Miyanabe K, Yamashita T, and Tsumoto K.

To understand the effect of protein fusion on the recognition of a peptide-tag by an antibody, we fused a CCR5-derived peptide-tag (pep1) to GFP and investigated its recognition by an anti-pep1 antibody, 4B08. First, to characterize the thermodynamic properties associated with the pep1-4B08 binding, isothermal titration calorimetry experiments were conducted. It was found that pep1 fused to the C-terminus of GFP (GFP-CT) enhanced the enthalpic gain by 2.1 kcal mol-1 and the entropic loss only by 0.9 kcal mol-1, resulting in an 8-fold increase in the binding affinity compared to the unfused pep1. On the other hand, pep1 fused to the N-terminus of GFP (GFP-NT) enhanced the enthalpic gain by 3.0 kcal mol-1 and the entropic loss by 3.2 kcal mol-1, leading to no significant enhancement of the binding affinity. To gain deeper insights, molecular dynamics simulations of GFP-NT, GFP-CT, and pep1 were performed. The results showed that the location of the fusion point sensitively affects the interaction energy, the solvent accessible surface area, and the fluctuation of pep1 in the unbound state, which explains the difference in the experimental thermodynamic properties.

9. Functional insights of Tyr37 in framework region 2 directly contributing to the binding affinities and dissociation kinetics in single-domain VHH antibodies

Yamamoto K, Nagatoishi S, Nakakido M, Kuroda D, and Tsumoto K.

Single-domain VHH antibody is regarded as one of the promising antibody classes for therapeutic and diagnostic applications. VHH antibodies have amino acids in framework region 2 that are distinct from those in conventional antibodies, such as the Val-37Phe/Tyr (V37F/Y) substitution. Correlations between the residue type at position 37 and the conformation of the CDR3 in VHH antigen recognition have been previously reported. However, few studies focused on the meaning of harboring two residue types in position 37 of VHH antibodies, and the concrete roles of Y37 have been little to be elucidated. Here, we investigated the functional states of position 37 in co-crystal structures and performed analyses of three model antibodies with either F or Y at position 37. Our analysis indicates that Y at position 37 enhances the dissociation rate, which is highly correlated with drug efficacy. Our findings help to explain the molecular mechanisms that distinguish VHH antibodies from conventional antibodies.

10. Cryo-EM structures elucidate the multiligand receptor nature of megalin

Goto S, Tsutsumi A, Lee Y, Hosojima M, Kabasawa H, Komochi K, Nagatoishi S, Takemoto K, Tsumoto K, Nishizawa T, Kikkawa M, and Saito A.

Megalin (low-density lipoprotein receptor-related protein 2) is a giant glycoprotein of about 600 kDa, mediating the endocytosis of more than 60 ligands, including those of proteins, peptides, and drug compounds [S. Goto, M. Hosojima, H. Kabasawa, A. Saito, Int. J. Biochem. Cell Biol. 157, 106393 (2023)]. It is expressed predominantly in renal proximal tubule epithelial cells, as well as in the brain, lungs, eyes, inner ear, thyroid gland, and placenta. Megalin is also known to mediate the endocytosis of toxic compounds, particularly those that cause renal and hearing disorders [Y. Hori et al., J. Am. Soc. Nephrol. 28, 1783-1791 (2017)]. Genetic megalin deficiency causes Donnai-Barrow syndrome/facio-oculo-acoustico-renal syndrome in humans. However, it is not known how megalin interacts with such a wide variety of ligands and plays pathological roles in various organs. In this study, we elucidated the dimeric architecture of megalin, purified from rat kidneys, using cryoelectron microscopy. The maps revealed the densities of endogenous ligands bound to various regions throughout the dimer, elucidating the multiligand receptor nature of megalin. We also determined the structure of megalin in complex with receptor-associated protein, a molecular chaperone for megalin. The results will facilitate further studies on the pathophysiology of megalin-dependent multiligand endocytic pathways in multiple organs and will also be useful for the development of megalin-targeted drugs for renal and hearing disorders, Alzheimer's disease [B. V. Zlokovic et al., Proc. Natl. Acad. Sci. U.S.A. 93, 4229-4234 (1996)], and other illnesses.

11. Next-Generation Anti-TNFα Agents: The Example of Ozoralizumab

Tsumoto K and Takeuchi T.

Biologic therapy involving anti-tumor necrosis factor-α (anti-TNFα) agents has fundamentally changed the management of patients with immune-mediated inflammatory diseases, including

rheumatoid arthritis, thus benefiting many patients. Nevertheless, the inability of some patients to achieve low disease activity or clinical remission remains a major concern. To address such concerns, next-generation anti-TNFα agents that differ from the immunoglobulin G-format anti-TNFα agents that have been used to date are being developed using antibody-engineering technology. Their unique design employing novel molecular characteristics affords several advantages, such as early improvement of clinical symptoms, optimization of drug bioavailability, enhancement of tissue penetration, and a reduction in side effects. This holds promise for a new paradigm shift in biologic therapy via the use of next-generation anti-TNFα agents. Ozoralizumab, a next-generation anti-TNFα agent that was recently approved in Japan, comprises a variable region heavy-chain format. It has a completely different structure from conventional therapeutic antibodies, such as a small molecular size, an albumin-binding module, and a unique format that produces an avidity effect. Ozoralizumab exhibited rapid biodistribution into joints, provided attenuation of Fcγ receptor-mediated inflammatory responses, and had a high binding affinity to TNFα in non-clinical studies. In clinical trials, ozoralizumab yielded an early improvement in clinical symptoms, a sustained efficacy for up to 52 weeks, and an acceptable tolerability in patients with rheumatoid arthritis. This review focuses on the results of pre-clinical and clinical trials for ozoralizumab and outlines the progress in next-generation antibody development.

12. Characterization of a novel format scFv×VHH single-chain biparatopic antibody against metal binding protein MtsA

Asano R, Takeuchi M, Nakakido M, Ito S, Aikawa C, Yokoyama T, Senoo A, Ueno G, Nagatoishi S, Tanaka Y, Nakagawa I, and Tsumoto K.

Biparatopic antibodies (bpAbs) are engineered antibodies that bind to multiple different epitopes within the same antigens. bpAbs comprise diverse formats, including fragment-based formats, and choosing the appropriate molecular format for a desired function against a target molecule is a challenging task. Moreover, optimizing the design of constructs requires selecting appropriate antibody modalities and adjusting linker length for individual bpAbs. Therefore, it is crucial to understand the characteristics of bpAbs at the molecular level. In this study, we first obtained single-chain variable fragments and camelid heavy-chain variable domains targeting distinct epitopes of the metal binding protein MtsA and then developed a novel format single-chain bpAb connecting these fragment antibodies with various linkers. The physicochemical properties, binding activities, complex formation states with antigen, and functions of the bpAb were analyzed using multiple approaches. Notably, we found that the assembly state of the complexes was controlled by a linker and that longer linkers tended to form more compact complexes. These observations provide detailed molecular information that should be considered in the design of bpAbs.

13. Crystal structures of human CD40 in complex with monoclonal antibodies dacetuzumab and bleselumab

Asano R, Nakakido M, Pérez JF, Ise T, Caaveiro JMM, Nagata S, and Tsumoto K.

CD40 is a member of the tumor necrosis factor receptor superfamily, and it is widely expressed on immune and non-immune cell types. The interaction between CD40 and the CD40 ligand (CD40L) plays an essential function in signaling, and the CD40/CD40L complex works as an immune checkpoint molecule. CD40 has become a therapeutic target, and a variety of agonistic/antagonistic anti-CD40 monoclonal antibodies (mAbs) have been developed. To better understand the mode of action of anti-CD40 mAbs, we determined the X-ray crystal structures of dacetuzumab (agonist) and bleselumab (antagonist) in complex with the extracellular domain of human CD40, respectively. The structure reveals that dacetuzumab binds to CD40 on the top of cysteine-rich domain 1 (CRD1), which is the domain most distant from the cell surface, and it does not compete with CD40L binding. The binding interface of bleselumab spread between CRD2 and CRD1, overlapping with the binding surface of the ligand. Our results offer important insights for future structural and functional studies of CD40 and provide clues to understanding the mechanism of biological response. These data can be applied to developing new strategies for designing antibodies with more therapeutic efficacy.

14. High-throughput system for the thermostability analysis of proteins

Ito S, Matsunaga R, Nakakido M, Komura D, Katoh H, Ishikawa S, and Tsumoto K.

Thermal stability of proteins is a primary metric for evaluating their physical properties. Although researchers attempted to predict it using machine learning frameworks, their performance has been dependent on the quality and quantity of published data. This is due to the technical limitation that thermodynamic characterization of protein denaturation by fluorescence or calorimetry in a high-throughput manner has been challenging. Obtaining a melting curve that derives solely from the target protein requires laborious purification, making it far from practical to prepare a hundred or more samples in a single workflow. Here, we aimed to overcome this throughput

limitation by leveraging the high protein secretion efficacy of Brevibacillus and consecutive treatment with plate-scale purification methodologies. By handling the entire process of expression, purification, and analysis on a per-plate basis, we enabled the direct observation of protein denaturation in 384 samples within 4 days. To demonstrate a practical application of the system, we conducted a comprehensive analysis of 186 single mutants of a single-chain variable fragment of nivolumab, harvesting the melting temperature (Tm) ranging from -9.3 up to +10.8°C compared to the wild-type sequence. Our findings will allow for data-driven stabilization in protein design and streamlining the rational approaches.

15. Impact of single-residue mutations on protein thermal stability: The case of threonine 83 of BC2L-CN lectin

Hoya M, Matsunaga R, Nagatoishi S, Ide T, Kuroda D, and Tsumoto K.

The thermal stability of trimeric lectin BC2L-CN was investigated and found to be considerably altered when mutating residue 83, originally a threonine, located at the fucose-binding loop. Mutants were analyzed using differential scanning calorimetry and isothermal microcalorimetry. Although most mutations decreased the affinity of the protein for oligosaccharide H type 1, six mutations increased the melting temperature (Tm) by >5 °C; one mutation, T83P, increased the Tm value by 18.2 °C(T83P, Tm = 96.3 °C). In molecular dynamic simulations, the investigated thermostable mutants, T83P, T83A, and T83S, had decreased fluctuations in the loop containing residue 83. In the T83S mutation, the side-chain hydroxyl group of serine formed a hydrogen bond with a nearby residue, suggesting that the restricted movement of the side-chain resulted in fewer fluctuations and enhanced thermal stability. Residue 83 is located at the interface and near the upstream end of the equivalent loop in a different protomer; therefore, fluctuations by this residue likely propagate throughout the loop. Our study of the dramatic change in thermal stability by a single amino acid mutation provides useful insights into the rational design of protein structures, especially the structures of oligomeric proteins.

16. PDZD8-FKBP8 tethering complex at ER-mitochondria contact sites regulates mitochondrial complexity

Nakamura K, Aoyama-Ishiwatari S, Nagao T, Paaran M, Obara CJ, Sakurai-Saito Y, Johnston J, Du Y, Suga S, Tsuboi M, Nakakido M, Tsumoto K, Kishi Y, Gotoh Y, Kwak C, Rhee HW, Seo JK, Kosako H, Potter C, Carragher B, Lippincott-Schwartz J, Polleux F, and Hirabayashi Y.

Mitochondria-ER membrane contact sites (MERCS) represent a fundamental ultrastructural feature underlying unique biochemistry and physiology in eukaryotic cells. The ER protein PDZD8 is required for the formation of MERCS in many cell types, however, its tethering partner on the outer mitochondrial membrane (OMM) is currently unknown. Here we identified the OMM protein FKBP8 as the tethering partner of PDZD8 using a combination of unbiased proximity proteomics, CRISPR-Cas9 endogenous protein tagging, Cryo-Electron Microscopy (Cryo-EM) tomography, and correlative light-EM (CLEM). Single molecule tracking revealed highly dynamic diffusion properties of PDZD8 along the ER membrane with significant pauses and capture at MERCS. Overexpression of FKBP8 was sufficient to narrow the ER-OMM distance, whereas independent versus combined deletions of these two proteins demonstrated their interdependence for MERCS formation. Furthermore, PDZD8 enhances mitochondrial complexity in a FKBP8-dependent manner. Our results identify a novel ER-mitochondria tethering complex that regulates mitochondrial morphology in mammalian cells.

17. Development of novel humanized VHH synthetic libraries based on physicochemical analyses

Nakakido M, Kinoshita S, and Tsumoto K.

Due to the high affinity and specificity of antibodies toward antigens, various antibody-based applications have been developed. Recently, variable antigen-binding domains of heavy-chain antibodies (VHH) have become an attractive alternative to conventional fragment antibodies due to their unique molecular characteristics. As an antibody-generating strategy, synthetic VHH libraries (including humanized VHH libraries) have been developed using distinct strategies to constrain the diversity of amino acid sequences. In this study, we designed and constructed several novel synthetic humanized VHH libraries based on biophysical analyses conducted using the complementarity determining region-grafting method and comprehensive sequence analyses of VHHs deposited in the protein data bank. We obtained VHHs from the libraries, and hit clones exhibited considerable thermal stability. We also found that VHHs from distinct libraries tended to have different epitopes. Based on our results, we propose a strategy for generating humanized VHHs with distinct epitopes toward various antigens by utilizing our library combinations.

18. Systematic Preparation of a 66-IgG Library with Symmetric and Asymmetric Homogeneous Glycans and Their Functional Evaluation

Manabe S, Iwamoto S, Nagatoishi S, Hoshinoo A, Mitani A, Sumiyoshi W, Kinoshita T, Yamaguchi Y, and Tsumoto K.

Immunoglobulin G (IgG) antibodies possess a conserved N-glycosylation site in the Fc domain. In FcγRIIIa affinity column chromatography, unglycosylated, hemiglycosylated, and fully glycosylated IgG retention times differ considerably. Using retention-time differences, 66 different trastuzumab antibodies with symmetric and asymmetric homogeneous glycans were prepared systematically, substantially expanding the scope of IgGs with homogeneous glycans. Using the prepared trastuzumab with homogeneous glycans, thermal stability and antibody-dependent cellular cytotoxicity were investigated. In some glycan series, a directly proportional relationship was observed between the thermal unfolding temperature (Tm) and the calorimetric unfolding heat (ΔHcal). Antibody function could be deduced from the combination of a pair of glycans in an intact form. Controlling glycan structure through the combination of a pair of glycans permits the precise tuning of stability and effector functions of IgG. Overall, our technology can be used to investigate the effects of glycans on antibody functions.

19. Triphenylphosphonium-modified catiomers enhance in vivo mRNA delivery through stabilized polyion complexation

Norimatsu J, Mizuno HL, Watanabe T, Obara T, Nakakido M, Tsumoto K, Cabral H, Kuroda D, and Anraku Y.

Nanocarriers based on cationic materials play a central role in the success of mRNA-based therapies. Traditionally, amine-bearing lipids and polymers have been successfully employed for creating mRNA-loaded nanocarriers, though they still present challenges, such as physical and biological instability, limiting both delivery efficiency and therapeutic potential. Non-amine cations could be a promising avenue in addressing these limitations. However, such alternatives remain notably underexplored. Herein, we introduced triphenylphosphonium (TPP) as an alternative cationic moiety for mRNA delivery, leveraging its advantageous properties for nucleic acid complexation. Through the modification of amine-bearing catiomers, we replaced traditional amine-based counterparts with TPP to create innovative polymeric micelles as mRNA nanocarriers. A comprehensive analysis, encompassing physicochemical, thermodynamic, and computational approaches, revealed that the TPP substitution significantly influenced polymer self-assembly, mRNA binding, and the overall stability of mRNA-loaded polymeric micelles. Upon intravenous injection, TPP-bearing micelles demonstrated a remarkable increase in mRNA bioavailability, facilitating efficient protein production in solid tumors. These findings provide a compelling rationale for substituting amines with TPP, emphasizing their potential for advancing mRNA therapeutics.

20. Unveiling the structural mechanisms behind high affinity and selectivity in phosphorylated epitope-specific rabbit antibodies

Kasahara K, Kawade R, Nakakido M, Matsunaga R, Akiba H, Entzminger KC, Maruyama T, Okumura SCJ, Caaveiro JMM, Kuroda D, and Tsumoto K.

Protein phosphorylation is a crucial process in various cellular functions, and its irregularities have been implicated in several diseases, including cancer. Antibodies are commonly employed to detect protein phosphorylation in research. However, unlike the extensive studies on recognition mechanisms of the phosphate group by proteins such as kinases and phosphatases, only a few studies have explored antibody mechanisms. In this study, we produced and characterized two rabbit monoclonal antibodies that recognize a monophosphorylated Akt peptide. Through crystallography, thermodynamic mutational analyses, and molecular dynamics simulations, we investigated the unique recognition mechanism that enables higher binding affinity and selectivity of the antibodies compared to other generic proteins with lower binding affinity to phosphorylated epitopes. Our results demonstrate that molecular dynamics simulations provide novel insights into the dynamic aspects of molecular recognition of posttranslational modifications by proteins beyond static crystal structures, highlighting how specific atomic level interactions drive the exceptional affinity and selectivity of antibodies.

21. Structural basis for the ligand promiscuity of the hydroxamate siderophore binding protein FtsB from Streptococcus pyogenes

Fernandez-Perez J, Senoo A, Caaveiro JMM, Nakakido M, de Vega S, Nakagawa I, and Tsumoto K.

Pathogenic bacteria must secure the uptake of nutritional metals such as iron for their growth, making their import systems attractive targets for the development of new antimicrobial modalities. In the pathogenic bacterium Streptococcus pyogenes, the iron uptake system FtsABCD transports iron encapsulated by siderophores of the hydroxamate class. However, the inability of S. pyogenes to produce these metabolites makes the biological and clinical relevance of this

route unresolved. Herein, we demonstrated that the periplasmic binding protein FtsB recognizes not only the hydroxamate siderophore ferrichrome, as previously documented, but also ferrioxamine E (FOE), ferrioxamine B (FOB), and bisucaberin (BIS), each of them with high affinity (nM level). Up to seven aromatic residues in the binding pocket accommodate the variable backbones of the different siderophores through CH-π interactions, explaining ligand promiscuity. Collectively, our observations revealed how S. pyogenes exploits the diverse xenosiderophores produced by other microorganisms as iron sources to secure this precious nutrient.

22. Malaria parasites require a divergent heme oxygenase for apicoplast gene expression and biogenesis

Blackwell AM, Jami-Alahmadi Y, Nasamu AS, Kudo S, Senoo A, Slam C, Tsumoto K, Wohlschlegel JA, Manuel Martinez Caaveiro J, Goldberg DE, and Sigala PA.

Malaria parasites have evolved unusual metabolic adaptations that specialize them for growth within heme-rich human erythrocytes. During blood-stage infection, Plasmodium falciparum parasites internalize and digest abundant host hemoglobin within the digestive vacuole. This massive catabolic process generates copious free heme, most of which is biomineralized into inert hemozoin. Parasites also express a divergent heme oxygenase (HO)-like protein (PfHO) that lacks key active-site residues and has lost canonical HO activity. The cellular role of this unusual protein that underpins its retention by parasites has been unknown. To unravel PfHO function, we first determined a 2.8 Å-resolution X-ray structure that revealed a highly α-helical fold indicative of distant HO homology. Localization studies unveiled PfHO targeting to the apicoplast organelle, where it is imported and undergoes N-terminal processing but retains most of the electropositive transit peptide. We observed that conditional knockdown of PfHO was lethal to parasites, which died from defective apicoplast biogenesis and impaired isoprenoid-precursor synthesis. Complementation and molecular-interaction studies revealed an essential role for the electropositive N-terminus of PfHO, which selectively associates with the apicoplast genome and enzymes involved in nucleic acid metabolism and gene expression. PfHO knockdown resulted in a specific deficiency in levels of apicoplast-encoded RNA but not DNA. These studies reveal an essential function for PfHO in apicoplast maintenance and suggest that Plasmodium repurposed the conserved HO scaffold from its canonical heme-degrading function in the ancestral chloroplast to fulfill a critical adaptive role in organelle gene expression.

23. Specific recognition mechanism of an antibody to sulfated tyrosine and its potential use in biological research

Ujiie K, Nakakido M, Kinoshita S, Jose Caaveiro M M, Entzminger, C J Okumura, Maruyama, Miyauchi k, Matano T, and Tsumoto K

Post-translational modification of proteins is a crucial biological reaction that regulates protein functions by altering molecular properties. The specific detection of such modifications in proteins has made significant contributions to molecular biology research and holds potential for future drug development applications. In HIV research, for example, tyrosine sulfation at the N-terminus of C-C chemokine receptor type 5 (CCR5) is considered to significantly enhance HIV infection efficiency. However, antibodies specific to sulfated CCR5 still need to be developed. In this study, we successfully generated an antibody that specifically recognized the sulfated N-terminal peptide of CCR5 through rabbit immunization and panning via phage display using a CCR5 N-terminal peptide containing sulfate modification. We used various physicochemical methods in combination with molecular dynamics simulation to screen for residues that could be involved in recognition of the sulfated peptide by this antibody. We also confirmed that this antibody recognized the sulfated full-length CCR5 on the cell surface, which suggested it should be useful as a research tool that could lead to the development of novel therapeutics. Although the antibody binding did not inhibit HIV infection, it could be also described as sulfation site-specific binding, beyond sulfation-specific binding.

24. The pericellular function of Fibulin-7 in the adhesion of oligodendrocyte lineage cells to neuronal axons during CNS myelination

Yamada M, Sasaki B,Yamada N, Hayashi C, Tsumoto K, de Vega S, and Suzuki N

Myelin is an electrical insulator that enables saltatory nerve conduction and is essential for proper functioning of the central nervous system (CNS). It is formed by oligodendrocytes (OLs) in the CNS, and during OL development various molecules, including extracellular matrix (ECM) proteins, regulate OL differentiation and myelination; however, the role of ECM proteins in these processes is not well understood. Our present work is centered on the analyses of the expression and function of fibulin-7 (Fbln7), an ECM protein of the fibulin family, in OL differentiation. In the expression analysis of Fbln7 in the CNS, we found that it was expressed at early postnatal stage and localized in the processes of OL precursor cells (OPCs), in the inner region of myelin, and in axons. The functional analysis using recombinant Fbln7

protein (rFbln7) revealed that rFbln7 promoted OPC attachment activity via β1 integrin and heparan sulfate receptors. Further, rFbln7 induced the adhesion to neurites and the differentiation of OLs. Altogether, our results show that Fbln7 promotes the adhesion between OLs and axons and OL differentiation.

<Group III>

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

Neural circuits in the central nervous system are the basis of various higher-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 retinal neural cells have been examined mainly by the electrophysiological methods and models of cell connectivity have been proposed. Morphological studies of the actual neural connection, which constitute the physiological properties of retinal neurons, have been desired. Until recently the only method to reveal the three-dimensional (3D) connectivity of actual neural cells morphologically was to collect ultrathin serial sections and observe them in transmission electron microscope (TEM). But the technical difficulties discouraged us from such a troublesome procedure. Recent progress in scanning electron microscope (SEM) equipment allowed us to develop a new method to observe ultrathin TEM sections in SEM (thin section scanning electron microscopy: TSSEM). To collect huge number of serial sections stably and efficiently, we have been developing new equipment and techniques. By using these techniques, it became possible to collect more than 1000 serial sections of less than 30 nm thickness much easier. 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 terminals. In fish retina, four kinds of cone photoreceptor are arranged in a very regular pattern, forming cone mosaics. Therefore, the first step of color recognition circuit is expected to be morphologically analyzed by using TSSEM method.

TSSEM method developed here was expanded to be used in analyzing mitochondrial 3D structure as a collaborative work. Aside from getting 3D information, TSSEM method can provide us precise information of much wider areas of thin sections more effectively and more easily than transmission electron microscopy. Such studies are also in progress as a collaborative work.

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 individual technique or combination of some of these, we can offer direct visual evidence that cannot be acquired by other methods. This year, 15 projects in 11 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 of about 70 nm 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 sequential reaction with appropriate primary antibodies and colloidal gold labeled secondary antibodies. This year, thin section electron microscopy and those 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[1]'s laboratory: [1]Division of Molecular Virology, Department of Microbiology and Immunology, regarding the infection/replication processes of herpes simplex virus (HSV). Thin section electron microscopy has been used to analyze the function of viral proteins in trans-nuclear membrane processes of the newly formed viruses. By analyzing the virus forming processes in some mutant host cells, we could analyze viral proteins as well as candidate host molecules those may be involved in the trans-nuclear process of the HSV. TSSEM method was also used to observe a specific virus infected single cell.

a-2. Analysis of calcium-binding protein 7 functions on mouse neuromuscular junctions

We have been performing several studies also with research groups in Dr. Yamanashi[2]'s laboratory: [2] Division of Genetics. This year, we analyzed the functions of calcium-binding protein 7 in mouse muscular cell on the morphology of neuromuscular junctions. In this project, fixed pieces of diaphragm were stained with fluorescently labeled alpha bungarotoxin to reveal the acetylcholine receptor rich postsynaptic membrane, observed on fluorescent microscope and then prepared for electron microscopic samples. Flu-

orescently labeled areas of the diaphragm samples were cut into sections and observed in a transmission microscope. Morphological data combined with biochemical experiments showed that the MuSK-mediated signaling induces muscle expression of Cabp7, which suppresses age-related NMJ degeneration likely by attenuating p25 expression, providing insights into prophylactic/therapeutic intervention against age- related motor dysfunction. (ref. Eguchi *et al*)

Some other collaborative research works using thin section electron microscopy and/or immuno-electron microscopy were performed with Dr. Coban[3], in [3]Division of Malaria Immunology, about the function of Paneth cells on Malaria infection, Dr.Takekawa[4] in [4]Division of Cell Signaling and Molecular Medicine, about the changes in nuclear pore size, Dr. Nakahara[5] in [5]Department of Life Science Dentistry, The Nippon Dental University, Dr. Katayama[6], in [6]Laboratory of Viral Infection, Ohmura Satoshi Memorial Institute, Kitasato University and so on.

b. Negative staining techniques

Negative staining techniques are simple and quick method to observe the morphology of the macro-molecules. This year, negative staining techniques were used to analyze exosomes in collaboration with Dr. Hayashi[7] in [7]Division of Vaccine Science, Laboratory of Adjuvant Innovation.

c. 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. Scanning electron microscopy combined with thin section transmission microscopy were used in collaborative work with Dr. Ishikawa[8], [8]Laboratory of Reproductive Systems Biology, about the structure of young and aged mouse oocyte zona pellucida and about the morphology of the sperm and spermatocyte. (ref. Ishikawa-Yamauchi *et al*) Scanning electron microscopy was also used in the work with Dr. Yamagami[9], [9]Division of Ophthalmology, Department of Visual Sciences, Nihon University School of Medicine.

Thin section scanning electron microscope (TS-SEM) methods are also used as a collaborative work with Dr. Kobayashi[10], [10]Division of Protein Metabolism, to analyze the 3D organization of mitochondria in neural stem cells.

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