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. 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) are known to be deeply involved in the regulation of various cellular systems through extensive diversification of protein function at the network level. As ingenious cross-regulation among different modes of PTMs contributes to stringent cell fate control, it is essential to acquire comprehensive information on multiple PTMs for systematic description of cellular information processing.

In this research, we performed global lysine-modification proteome analyses focused on ubiquitination (Ub) and acetylation (Ac), which have not been extensively studied in comparison with phosphorylation. Our results showed that more than 5,000 kinds of Ub-K sites and 1,600 kinds of Ac-K sites were successfully detected, leading to identification of 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.

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: [1]Laboratory of Molecular and Genetic Information, Institute for Quantitative Biosciences, The University of Tokyo

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 Combined with Quantitative Phosphoproteomics Reveals Transforming Growth Factor-beta Receptor type-2 (TGFBR2) as a Novel Regulator of Glioblastoma Stem Cell Properties

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 cancer stem cell properties during cultivation in serum-containing medium, little is known about the molecular mechanisms regulating signaling alteration in relation to reduction of stem cell-like characteristics. To elucidate the global phosphorylation-related signaling events, we performed a SILAC-based quantitative phosphoproteome analysis of serum-induced dynamics in glioblastoma stem cells established from the tumor tissues of the patient. Among a total of 2876 phosphorylation sites on 1584 proteins identified in our analysis, 732 phosphorylation sites on 419 proteins were regulated through the alteration of stem cell-like characteristics. 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-β receptor type-2 (TGFBR2) as a prominent upstream regulator involved in the serum-induced phosphoproteome regulation. The functional association of transforming growth factor-β receptor type-2 with stem cell-like properties was experimentally validated through signaling perturbation using the corresponding inhibitors, which indicated that transforming growth factor-β receptor type-2 could play an important role as a novel cell fate determinant in glioblastoma stem cell regulation.

4. Quantitative phosphoproteomics-based molecular network description for high-resolution kinase-substrate interactome analysis

Yuta Narushima, Hiroko Kozuka-Hata, Kouhei Tsumoto, Jun-ichiro Inoue and Masaaki Oyama.

Phosphorylation-dependent cellular signaling is known to play a diverse role in regulating multiple cellular processes such as proliferation, differentiation and apoptosis. Recent technological advances in mass spectrometry-based phosphoproteomics have enabled us to measure network-wide signaling dynamics in a comprehensive and quantitative manner. As conventional protein-protein interaction (PPI) information-based network analysis is insufficient to systematically analyze phosphorylation site-dependent complex interaction dynamics, here we develop and evaluate a platform to provide a high-resolution molecular network description for kinase-substrate interactome analysis. In this study, we developed a Cytoscape-based bioinformatical platform named "Post Translational Modification mapper (PTMapper)" to integrate PPI data with publicly available kinase-substrate relations at the resolution of phosphorylated amino acid residues. The previous phosphoproteome data on EGF-induced cellular signaling in glioblastoma stem cells was applied to evaluate our platform, leading to discovery of phosphorylation-dependent crucial signaling modulation in the p70S6K1-related pathway. Our study revealed that high-resolution cellular network description of phosphorylation-site dependent kinase-substrate signaling regulation should accelerate phosphoproteomics-based exploration of novel drug targets in the context of each disease-related signaling.

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

Hiroko Kozuka-Hata, Masato Suzuki[2], Kotaro

Kiga[2], Shinya Tasaki, Jun-ichiro Inoue, Tadashi Yamamoto[3], Chihiro Sasakawa[2] and Masaaki Oyama: [2]Division of Bacterial Infection, Department of Microbiology and Immunology, IMSUT, [3]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[4], Tadashi Yamamoto[3] and Jun-ichiro Inoue: [4]Department of Computational Biology and Medical 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).

7. Shotgun proteomics deciphered age/division of labor-related functional specification of three honeybee (*Apis mellifera* L.) exocrine glands

Toshiyuki Fujita[5], Hiroko Kozuka-Hata, Yutaro Hori[5], Jun Takeuchi[5], Takeo Kubo[5], and Masaaki Oyama: [5]Department of Biological Sciences, Graduate School of Science, The University of Tokyo

The honeybee (*Apis mellifera* L.) uses various chemical signals produced by the worker exocrine glands to maintain the functioning of its colony. The roles of worker postcerebral glands (PcGs), thoracic glands (TGs), and mandibular glands (MGs) and the functional changes they undergo according to the division of labor from nursing to foraging are not as well studied. To comprehensively characterize the molecular roles of these glands in workers and their changes according to the division of labor of workers, we analyzed the proteomes of PcGs, TGs, and MGs from nurse bees and foragers using shotgun proteomics technology. We identi-

fied approximately 2000 proteins from each of the nurse bee or forager glands and highlighted the features of these glands at the molecular level by semiquantitative enrichment analyses of frequently detected, gland-selective, and labor-selective proteins. First, we found the high potential to produce lipids in PcGs and MGs, suggesting their relation to pheromone production. Second, we also found the proton pumps abundant in TGs and propose some transporters possibly related to the saliva production. Finally, our data unveiled candidate enzymes involved in labor-dependent acid production in MGs.

<Group II>

Biomolecular recognition is based on collective and specific non-covalent interactions between discrete biological molecules. Our laboratory studies a variety of 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. We aim to produce functional molecules with higher performance and better properties, 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. Thermodynamic and computational analyses reveal the functional roles of the galloyl group of tea catechins in molecular recognition.

Takahashi T, Nagatoishi S, Kuroda D and Tsumoto K.

Catechins, biologically active polyphenols in green tea, exhibit various biological activities, such as anticancer and antiviral activities, arising from interactions with functional proteins. However, the molecular details of these interactions remain unclear. In this study, we investigated the interactions between human serum albumin (HSA) and various catechins, including some with a galloyl group, by means of isothermal titration calorimetry (ITC), differential scanning calorimetry (DSC), and docking simulations. Our results indicate that the galloyl group was important for recognition by HSA and was responsible for enthalpic gains derived from a larger buried surface area and more van der Waals contacts. Thus, our thermodynamic and computational analyses suggest that the galloyl group plays important functional roles in the specific binding of catechins to proteins, implying that the biological activities of these compounds may be due in part to the physicochemical characteristics of the galloyl group.

2. Molecular basis for governing the morphology of type-I collagen fibrils by Osteomodulin.

Tashima T, Nagatoishi S, Caaveiro JMM, Nakakido M, Sagara H, Kusano-Arai O, Iwanari H, Mimuro H, Hamakubo T, Ohnuma SI and Tsumoto K.

Small leucine-rich repeat proteoglycan (SLRP) proteins have an important role in the organization of the extracellular matrix, especially in the formation of collagen fibrils. However, the mechanism governing the shape of collagen fibrils is poorly understood. Here, we report that the protein Osteomodulin (OMD) of the SLRP family is a monomeric protein in solution that interacts with type-I collagen. This interaction is dominated by weak electrostatic forces employing negatively charged residues of OMD, in particular Glu284 and Glu303, and controlled by entropic factors. The protein OMD establishes a fast-binding equilibrium with collagen, where OMD may engage not only with individual collagen molecules, but also with the growing fibrils. This weak electrostatic interaction is carefully balanced so it modulates the shape of the fibrils without compromising their viability.

3. Antibody Affinity Maturation by Computational Design.

Kuroda D and Tsumoto K.

The immune systems protect our bodies from foreign molecules or antigens, where antibodies play important roles. Antibodies evolve over time upon antigen encounter by somatically mutating their genome sequences. The end result is a series of antibodies that display higher affinities and specificities to specific antigens. This process is called affinity maturation. Recent improvements in computer hardware and modeling algorithms now enable the rational design of protein structures and functions, and several works on computer-aided antibody design have been published. In this chapter, we briefly describe computational methods for antibody affinity maturation, focusing on methods for sampling antibody conformations and for scoring designed antibody variants. We also discuss lessons learned from the successful computer-aided design of antibodies.

4. Structural behavior of keratin-associated protein 8.1 in human hair as revealed by a monoclonal antibody.

Akiba H, Ikeuchi E, Ganbat J, Fujikawa H, Arai-Kusano O, Iwanari H, Nakakido M, Hamakubo T, Shimomura Y and Tsumoto K.

Keratin-associated protein 8.1 (KAP8.1) is a hair protein whose structure, biochemical roles, and protein distribution patterns have not been well characterized. In this study, we generated a monoclonal antibody against human KAP8.1 to analyze the protein's roles and distribution in the human hair shaft. Using this antibody, we revealed that KAP8.1 was predominantly expressed in discrete regions of the keratinizing zone of the hair shaft cortex. The protein expression patterns paralleled the distribution of KAP8.1 mRNA and suggested that KAP8.1 plays a role associated with cells to control hair curvature. Cross-reactivity among species and epitope analysis indicated that the monoclonal antibody recognized a linear epitope shared among human, mouse, and sheep KAP8.1. The antibody failed to interact with sheep KAP8.1 in native conformation, suggesting that structural features of KAP8.1 vary among species.

5. Fukunaga A, Maeta S, Reema B, Nakakido M and Tsumoto K.

Improvement of antibody affinity by introduction of basic amino acid residues into the framework region.

Antibodies are widely used not only as therapeutic agents but also as research tools and diagnostic agents, and extensive efforts have been made to generate antibodies that have higher affinity. It was recently reported that introduction of charged residues into the framework region of an antibody improved its affinity; however, the underlying molecular mechanism has not been elucidated. In this study, we used kinetic and thermodynamic analyses of the antibody-antigen interaction to investigate the molecular mechanism by which an antibody with introduced charged residues recognizes its antigen with higher affinity. The introduction of basic amino acid residues resulted in improvement of the affinity whereas the introduction of acidic residues weakened the interaction. For two mutant antigen-binding fragments (Fabs) with improved affinity (named K5- and R5-mutants), the balance between the association rate constant kon and the dissociation rate constant koff was distinct despite each mutant having the same number of charged residues. Moreover, thermodynamic analysis of the interactions in the transition state revealed a difference between the K5- and R5-mutants in terms of enthalpic energy change following formation of the encounter complex with the antigen. These results suggest that the affinity of the K5- and R5-mutants is improved by distinct mechanisms. Although the mutations destabilize the Fab and necessitate further studies, our strategy is expected to become a versatile and simple means to improve the affinity of antibodies to their antigens.

6. Biophysical Analysis of the Protein-Small Molecule Interactions to Develop Small Molecule Drug Discovery.

Nagatoishi S, Caaveiro JMM and Tsumoto K.

In small molecule drug discovery, researchers must find specific binders that interact with a target protein and inhibit its function in connection with human diseases. It is of critical importance to know the binding mode of compounds interacting with a target protein to assure hit validation and optimization. Biophysical analysis is a powerful quantitative approach to evaluate the binding modes of such candidates. Since the level of sensitivity of biophysical analysis is suitable to quantitatively detect the binding of fragment compounds, and because of the remarkable success of compound libraries of small molecules, the development and adaptation of biophysical analysis for these applications is in great demand. Herein, we describe the technical developments of biophysical methods, especially thermodynamic and kinetic analysis, for the purpose of screenings which employ small molecules. In addition, we discuss the interaction mechanisms of small molecules to find hit compounds based on these biophysical analyses.

7. Discovery and Optimization of Inhibitors of the Parkinson's Disease Associated Protein DJ-1.

Tashiro S, Caaveiro JMM, Nakakido M, Tanabe A, Nagatoishi S, Tamura Y, Matsuda N, Liu D, Hoang QQ and Tsumoto K.

DJ-1 is a Parkinson's disease associated protein endowed with enzymatic, redox sensing, regulatory, chaperoning, and neuroprotective activities. Although DJ-1 has been vigorously studied for the past decade and a half, its exact role in the progression of the disease remains uncertain. In addition, little is known about the spatiotemporal regulation of DJ-1, or the biochemical basis explaining its numerous biological functions. Progress has been hampered by the lack of inhibitors with precisely known mechanisms of action. Herein, we have employed biophysical methodologies and X-ray crystallography to identify and to optimize a family of compounds inactivating the critical Cys106 residue

of human DJ-1. We demonstrate these compounds are potent inhibitors of various activities of DJ-1 in vitro and in cell-based assays. This study reports a new family of DJ-1 inhibitors with a defined mechanism of action and contributes toward the understanding of the biological function of DJ-1.

8. Long-Term Stability and Reversible Thermal Unfolding of Antibody Structure at Low pH: Case Study.

Fukada H, Tsumoto K, Arakawa T and Ejima D.

We have here observed that the differential scanning calorimetry profiles and melting temperatures of a humanized antibody were unchanged over a 10-year span when stored at 4°C and at different pH values, even at pH 2.7. This is somewhat surprising, as this particular antibody undergoes conformational changes below pH 4.0. Differential scanning calorimetry analysis showed that melting of the antibody at pH 2.7 was highly reversible, suggesting a possibility that the observed reversibility is at least in part responsible for a 10-year stability at low pH. Conversely, it showed thermal unfolding followed by aggregation at higher pH.

9. Tyrosine Sulfation Restricts the Conformational Ensemble of a Flexible Peptide, Strengthening the Binding Affinity for an Antibody.

Miyanabe K, Yamashita T, Abe Y, Akiba H, Takamatsu Y, Nakakido M, Hamakubo T, Ueda T, Caaveiro JMM and Tsumoto K.

Protein tyrosine sulfation (PTS) is a post-translational modification regulating numerous biological events. PTS generally occurs at flexible regions of proteins, enhancing intermolecular interactions between proteins. Because of the high flexibility associated with the regions where PTS is generally encountered, an atomic-level understanding has been difficult to achieve by X-ray crystallography or nuclear magnetic resonance techniques. In this study, we focused on the conformational behavior of a flexible sulfated peptide and its interaction with an antibody. Molecular dynamics simulations and thermodynamic analysis indicated that PTS reduced the main-chain fluctuations upon the appearance of sulfate-mediated intramolecular H-bonds. Collectively, our data suggested that one of the mechanisms by which PTS may enhance protein-protein interactions consists of the limitation of conformational dynamics in the unbound state, thus reducing the loss of entropy upon binding and boosting the affinity for its partner.

10. Intramolecular H-bonds govern the recognition of a flexible peptide by an antibody.

Miyanabe K, Akiba H, Kuroda D, Nakakido M, Kusano-Arai O, Iwanari H, Hamakubo T, Caaveiro JMM and Tsumoto K.

Molecular recognition is a fundamental event at the core of essentially every biological process. In particular, intermolecular H-bonds have been recognized as key stabilizing forces in antibody-antigen interactions resulting in exquisite specificity and high affinity. Although equally abundant, the role of intramolecular H-bonds is far less clear and not universally acknowledged. Herein, we have carried out a molecular-level study to dissect the contribution of intramolecular H-bonds in a flexible peptide for the recognition by an antibody. We show that intramolecular H-bonds may have a profound, multifaceted and favorable effect on the binding affinity by up to 2 kcal mol-1 of free energy. Collectively, our results suggest that antibodies are fine tuned to recognize transiently stabilized structures of flexible peptides in solution, for which intramolecular H-bonds play a key role.

11. Roles of the disulfide bond between the variable and the constant domains of rabbit immunoglobulin kappa chains in thermal stability and affinity.

Kawade R, Akiba H, Entzminger K, Maruyama T, Okumura CJ and Tsumoto K.

Rabbit antibodies show unique structural characteristics in that kappa chains have an inter-domain disulfide bond between the variable and constant domains. Here we characterized this disulfide bond from physicochemical viewpoints both in stability and affinity. It was revealed that the disulfide bond contributed to the thermal stability of the antibody, but the affinity and mechanism of antigen recognition was not altered by the mutation. The present result expands the understanding of how rabbit antibodies with kappa light chains gain affinity under characteristic mechanism to gain thermal stability, and would give suggestions for the methods to artificially stabilize antibody molecules.

12. Inhibition of homophilic dimerization and disruption of cell adhesion by P-cadherin-specific small molecules from SPR-based assays.

Senoo A, Nagatoishi S, Moberg A, Babol LN, Mitani T, Tashima T, Kudo S and Tsumoto K.

The inhibitor for the homophilic dimerization of P-cadherin was discovered by SPR-based screening

using fragment compounds. Our SPR assays identified a specific P-cadherin binder, which was able to inhibit the cell adhesion of living CHO cells that expressed P-cadherin.

13. Repertoire Analysis of Antibody CDR-H3 Loops Suggests Affinity Maturation Does Not Typically Result in Rigidification.

Jeliazkov JR, Sljoka A, Kuroda D, Tsuchimura N, Katoh N, Tsumoto K and Gray JJ.

Antibodies can rapidly evolve in specific response to antigens. Affinity maturation drives this evolution through cycles of mutation and selection leading to enhanced antibody specificity and affinity. Elucidating the biophysical mechanisms that underlie affinity maturation is fundamental to understanding B-cell immunity. An emergent hypothesis is that affinity maturation reduces the conformational flexibility of the antibody's antigen-binding paratope to minimize entropic losses incurred upon binding. In recent years, computational and experimental approaches have tested this hypothesis on a small number of antibodies, often observing a decrease in the flexibility of the complementarity determining region (CDR) loops that typically comprise the paratope and in particular the CDR-H3 loop, which contributes a plurality of antigen contacts. However, there were a few exceptions and previous studies were limited to a small handful of cases. Here, we determined the structural flexibility of the CDR-H3 loop for thousands of recent homology models of the human peripheral blood cell antibody repertoire using rigidity theory. We found no clear delineation in the flexibility of naïve and antigen-experienced antibodies. To account for possible sources of error, we additionally analyzed hundreds of human and mouse antibodies in the Protein Data Bank through both rigidity theory and B-factor analysis. By both metrics, we observed only a slight decrease in the CDR-H3 loop flexibility when comparing affinity matured antibodies to naïve antibodies, and the decrease was not as drastic as previously reported. Further analysis, incorporating molecular dynamics simulations, revealed a spectrum of changes in flexibility. Our results suggest that rigidification may be just one of many biophysical mechanisms for increasing affinity.

14. A combination of 19F NMR and surface plasmon resonance for site-specific hit selection and validation of fragment molecules that bind to the ATP-binding site of a kinase.

Nagatoishi S, Yamaguchi S, Katoh E, Kajita K, Yokotagawa T, Kanai S, Furuya T and Tsumoto K.

19F NMR has recently emerged as an efficient, sensitive tool for analyzing protein binding to small molecules, and surface plasmon resonance (SPR) is also a popular tool for this purpose. Herein a combination of 19F NMR and SPR was used to find novel binders to the ATP-binding pocket of MAP kinase extracellular regulated kinase 2 (ERK2) by fragment screening with an original fluorinated-fragment library. The 19F NMR screening yielded a high primary hit rate of binders to the ERK2 ATP-binding pocket compared with the rate for the SPR screening. Hit compounds were evaluated and categorized according to their ability to bind to different binding sites in the ATP-binding pocket. The binding manner was characterized by using isothermal titration calorimetry and docking simulation. Combining 19F NMR with other biophysical methods allows the identification of multiple types of hit compounds, thereby increasing opportunities for drug design using preferred fragments.

15. Assessing the Heterogeneity of the Fc-Glycan of a Therapeutic Antibody Using an engineered FcγReceptor IIIa-Immobilized Column.

Kiyoshi M, Caaveiro JMM, Tada M, Tamura H, Tanaka T, Terao Y, Morante K, Harazono A, Hashii N, Shibata H, Kuroda D, Nagatoishi S, Oe S, Ide T, Tsumoto K and Ishii-Watabe A.

The N-glycan moiety of IgG-Fc has a significant impact on multifaceted properties of antibodies such as in their effector function, structure, and stability. Numerous studies have been devoted to understanding its biological effect since the exact composition of the Fc N-glycan modulates the magnitude of effector functions such as the antibody-dependent cell mediated cytotoxicity (ADCC), and the complement-dependent cytotoxicity (CDC). To date, systematic analyses of the properties and influence of glycan variants have been of great interest. Understanding the principles on how N-glycosylation modulates those properties is important for the molecular design, manufacturing, process optimization, and quality control of therapeutic antibodies. In this study, we have separated a model therapeutic antibody into three fractions according to the composition of the N-glycan by using a novel FcγRIIIa chromatography column. Notably, Fc galactosylation was a major factor influencing the affinity of IgG-Fc to the FcγRIIIa immobilized on the column. Each antibody fraction was employed for structural, biological, and physicochemical analysis, illustrating the mechanism by which galactose modulates the affinity to FcγRIIIa. In addition, we discuss the benefits of the FcγRIIIa chromatography column to assess the heterogeneity of the N-glycan.

16. Synergistic Cytotoxic Effect on Gastric Cancer Cells of an Immunotoxin Cocktail in Which Antibodies Recognize Different Epitopes on CDH17.

Kusano-Arai O, Iwanari H, Kudo S, Kikuchi C, Yui A, Akiba H, Matsusaka K, Kaneda A, Fukayama M, Tsumoto K and Hamakubo T.

Cadherin-17 (CDH17) is highly expressed in gastric cancer and is thus considered to be a good target for antibody therapy. CDH17 is classified as a nonclassical cadherin, in that it is composed of seven extracellular cadherin domains. We generated anti-CDH17 monoclonal antibodies (mAbs) which recognize the extracellular domain of CDH17. Competitive assay using AGS, a gastric cancer cell line, cells revealed that five selected anti-CDH17 mAbs recognize different epitopes on CDH17. As AGS cells were shown to exhibit broad expression pattern of CDH17 by flow cytometry, we separated three clones with a low (10,000/cell), medium (50,000/cell), and high (200,000/cell) expression level, designating them as AGSlow, AGSmed, and AGShigh, respectively. The mAbs, coupled with saporin, exhibited effective cytotoxicity to AGShigh, but poor cytotoxicity to AGSlow. By contrast, the immunotoxin cocktail using the three clones D2101, D2005, and D2008, which recognize different epitopes, exhibited efficient cytotoxicity, even to the AGSlow group. The effect of the immunotoxin cocktail is synergistic, as the combination index was demonstrated to be below 1.0, as calculated by the method of Chou and Talalay using CalcuSyn software. These results suggest that the immunotoxin cocktail targeted to multiple epitopes has synergistic effects on low expression level cells, which expand the applicable range of immunotoxin therapy for cancer.

17. Structures of the prefusion form of measles virus fusion protein in complex with inhibitors.

Hashiguchi T, Fukuda Y, Matsuoka R, Kuroda D, Kubota M, Shirogane Y, Watanabe S, Tsumoto K, Kohda D, Plemper RK and Yanagi Y.

easles virus (MeV), a major cause of childhood morbidity and mortality, is highly immunotropic and one of the most contagious pathogens. MeV may establish, albeit rarely, persistent infection in the central nervous system, causing fatal and intractable neurodegenerative diseases such as subacute sclerosing panencephalitis and measles inclusion body encephalitis. Recent studies have suggested that particular substitutions in the MeV fusion (F) protein are involved in the pathogenesis by destabilizing the F protein and endowing it with hyperfusogenicity. Here we show the crystal structures of the prefusion MeV-F alone and in complex with the small compound AS-48 or a fusion inhibitor peptide. Notably, these independently developed inhibitors bind the same hydrophobic pocket located at the region connecting the head and stalk of MeV-F, where a number of substitutions in MeV isolates from neurodegenerative diseases are also localized. Since these inhibitors could suppress membrane fusion mediated by most of the hyperfusogenic MeV-F mutants, the development of more effective inhibitors based on the structures may be warranted to treat MeV-induced neurodegenerative diseases.

18. Development of drug discovery screening system by molecular interaction kinetics-mass spectrometry.

Obi N, Fukuda T, Nakayama N, Ervin J, Bando Y, Nishimura T, Nagatoishi S, Tsumoto K and Kawamura T.

Six small-molecule binders of CAII were analyzed quantitatively using nPOI and MIK-MS, and the results were compared to published surface plasmon resonance (SPR) results. The nPOI and SPR results show good agreement, confirming the reliability of the analysis. Time-dependent binding results may be obtained by our MS sensorgram approach. Drugs that meet medical needs in a short period are required; this nPOI-LC-MS system is considered an important tool for rapid drug discovery.

19. A secondary RET mutation in the activation loop conferring resistance to vandetanib.

Nakaoku T, Kohno T, Araki M, Niho S, Chauhan R, Knowles PP, Tsuchihara K, Matsumoto S, Shimada Y, Mimaki S, Ishii G, Ichikawa H, Nagatoishi S, Tsumoto K, Okuno Y, Yoh K, McDonald NQ and Goto K.

Resistance to vandetanib, a type I RET kinase inhibitor, developed in a patient with metastatic lung adenocarcinoma harboring a CCDC6-RET fusion that initially exhibited a response to treatment. The resistant tumor acquired a secondary mutation resulting in a serine-to-phenylalanine substitution at codon 904 in the activation loop of the RET kinase domain. The S904F mutation confers resistance to vandetanib by increasing the ATP affinity and autophosphorylation activity of RET kinase. A reduced interaction with the drug is also observed in vitro for the S904F mutant by thermal shift assay. A crystal structure of the S904F mutant reveals a small hydrophobic core around F904 likely to enhance basal kinase activity by stabilizing an active con-

former. Our findings indicate that missense mutations in the activation loop of the kinase domain are able to increase kinase activity and confer drug resistance through allosteric effects.

20. Characterization of glycoengineered anti-HER2 monoclonal antibodies produced by using a silkworm-baculovirus expression system.

Egashira Y, Nagatoishi S, Kiyoshi M, Ishii-Watabe A and Tsumoto K.

Silkworm-baculovirus expression systems are efficient means for the production of recombinant proteins that provide high expression levels and post-translational modifications. Here, we characterized the stability, glycosylation pattern and antibody-dependent cell-mediated cytotoxicity activity of anti-HER2 monoclonal antibodies containing native or glycoengineered mammalian-like N-glycans that were produced by using a silkworm-baculovirus expression system. Compared with a monoclonal antibody produced by using a Chinese hamster ovary cell expression system, the glycoengineered monoclonal antibody had comparable thermal stability and a higher antibody-dependent cell-mediated cytotoxicity activity. These results suggest that silkworm-baculovirus expression systems are next-generation expression systems potentially useful for the cost-effective production of therapeutic antibodies.

21. Enzymatically cleavable traceless biotin tags for protein PEGylation and purification.

Wawro AM, Aoki Y, Muraoka T, Tsumoto K and Kinbara K.

Here we report an example of a protein-PEG conjugate with a biotin tag cleavable by lipase-catalyzed hydrolysis. Very mild cleavage conditions, heterogeneous, easily separable catalysts, and traceless design make this method attractive for the preparation and purification of PEGylated proteins.

22. Production and characterization of a novel site-specific-modifiable anti-OX40-receptor single-chain variable fragment for targeted drug delivery.

Tanabe A, Nakano K, Nakakido M, Nagatoishi S, Tanaka Y, Tsumoto K, Uchimaru K and Watanabe T.

OX40 receptor (tumor necrosis factor receptor superfamily, member 4; CD134) is a T-cell co-stimulatory molecule that plays an important role in T-cell activation and survival. OX40 receptor is activated by its ligand, OX40L; and modulation of the OX40-OX40L interaction is a promising target for the treatment of autoimmune diseases and cancers. Here, we generated a high-affinity anti-OX40 single-chain variable fragment carrying a C-terminal cysteine residue (scFvC). Physicochemical and functional analyses revealed that the scFvC bound to OX40-expressing cells and was internalized via OX 40-mediated endocytosis without inducing phosphorylation of IκBα (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha), an important complex in the classical NFκB (nuclear factor kappa-light-chain-enhancer of activated B cells) signaling pathway. In addition, mutation of the 36th cysteine residue in variable region of light chain enabled site-specific chemical modification to carboxy terminal cysteine and improved the thermal stability of the scFvC. These results suggest that this novel high-affinity anti-OX40 scFvC may be useful as a transporter for targeted delivery of small compounds, proteins, peptides, liposomes, and nanoparticles, into OX40-expressing cells for the treatment of autoimmune diseases and cancers.

23. PRDM14 directly interacts with heat shock proteins HSP90α and glucose-regulated protein 78.

Moriya C, Taniguchi H, Nagatoishi S, Igarashi H, Tsumoto K and Imai K.

PRDM14 is overexpressed in various cancers and can regulate cancer phenotype under certain conditions. Inhibiting PRDM14 expression in breast and pancreatic cancers has been reported to reduce cancer stem-like phenotypes, which are associated with aggressive tumor properties. Therefore, PRDM14 is considered a promising target for cancer therapy. To develop a pharmaceutical treatment, the mechanism and interacting partners of PRDM14 need to be clarified. Here, we identified the proteins interacting with PRDM14 in triple-negative breast cancer (TNBC) cells, which do not express the three most common types of receptor (estrogen receptors, progesterone receptors, and HER2). We obtained 13 candidates that were pulled down with PRDM14 in TNBC HCC1937 cells and identified them by mass spectrometry. Two candidates-glucose-regulated protein 78 (GRP78) and heat shock protein 90-α (HSP90α)-were confirmed in immunoprecipitation assay in two TNBC cell lines (HCC1937 and MDA-MB231). Surface plasmon resonance analysis using GST-PRDM14 showed that these two proteins directly interacted with PRDM14 and that the interactions required the C-terminal region of PRDM14, which includes zinc finger motifs. We also confirmed the interactions in living cells by NanoLuc luciferase-based bioluminescence resonance energy

transfer (NanoBRET) assay. Moreover, HSP90 inhibitors (17DMAG and HSP990) significantly decreased breast cancer stem-like CD24- CD44+ and side population (SP) cells in HCC1937 cells, but not in PRDM14 knockdown HCC1937 cells. The combination of the GRP78 inhibitor HA15 and PRDM14 knockdown significantly decreased cell proliferation and SP cell number in HCC1937 cells. These results suggest that HSP90α and GRP78 interact with PRDM14 and participate in cancer regulation.

<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 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 retinal neural cell functions 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 retinal neurons, have been desired. Until recently the only method to reveal the connectivity of actual neural cells morphologically was to collect ultrathin serial sections and observe in transmission electron microscope (TEM). But the technical difficulties discouraged us from such troublesome procedures. Recent progress in scanning electron microscope (SEM) equipment allowed us to develop a new method to observe ultrathin TEM sections in SEM. To observe thin TEM sections, we have developed new sample staining methods to enhance electron contrast. To collect huge number of serial sections stably and efficiently, we have been developing new equipment and techniques. By using this equipment, it became possible to cut more than 1000 serial sections of less than 30nm 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.

Aside from getting 3D information, it became possible to analyze much wider areas by using SEM to observe thin TEM sections than by using TEM. These methods are also applicable for analyzing other cells and tissues and such studies are currently in progress.

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, 13 projects in 10 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 was 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.

a-2. Roles of Fezf2 in functional maturation of cone ON bipolar cells in retina.

We have been performing several studies also with research groups in Dr. Watanabe[2]'s laboratory: [2]Project Division of Molecular and Developmental Biology. This year, we analyzed the role of Fezf2 transcriptional repressor in retinal development. With the analysis of the retina by electron microscopy, we revealed that invaginations of ON bipolar cells into cone pedicles only are significantly shorter in *Fezf2*-KO mouse retinas than wild type, suggesting that the structural changes are cone-spe-

cific (ref. Suzuki-Kerr H[2] *et al*).

Some other collaborative research works using thin section electron microscopy and/or immuno-electron microscopy were performed with Dr. Noda[3], [3]Laboratory of Ultrastructural Virology, Department of Virus Research, Institute of Frontier Life and Medical Sciences, Kyoto University (ref. Noda T. *et al*, Nakatsu S. *et al*), Dr. Kamioka[4], [4]Division of Mucosal Immunology, concerning function of intestinal Paneth cells in mucosal immunity, Dr. Eguchi[5], in [5]Division of Genetics, Dr. Takekawa[6] in [6]Division of Cell Signaling and Molecular Medicine 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 combined with scanning electron microscopy were used to analyze the function of a protein during in vitro formation of collagen fibers in collaboration with Dr. Tashima[7] *et al* in [7]Medical Proteomics Laboratory.

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 works using scanning electron microscopy were done with Dr. Mimuro's group[8], in [8]Division of Bacteriology, Department of Infectious Disease Control, International Research Center for Infectious Diseases, about the morphological conversions of Helicobacter pylori under anaerobic conditions (ref. Hirukawa S. *et al*).

Scanning electron microscopy was also used to analyze the morphological changes of collagen fibers as a collaborative work with Tashima[7] *et al* (ref. Tashima T. *et al*).

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