Department of Cancer Biology

Division of Oncology
癌細胞シグナル研究分野

Associate Professor　Miho Ohsugi, Ph.D.

准教授　理学博士　　大　杉　美　穂

Protein kinases are important for the various cellular events including regulation of growth and function of normal cells and development of malignant tumors. Our current interest is to characterize cell signaling downstream of protein kinases that are relevant respectively to cancer development. We are also interested in protein kinase signaling and chromosome dynamics that are involved in the regulation of cell cycle progression and cell.

1. Function and regulation of mitotic motor proteins

Shou Soeda, Noriko Tokai-Nishizumi, Tetsuhiro Shimodaira, Kaori Yamada and Miho Ohsugi

Mitosis is a process whereby a complete copy of the genetic information is distributed to each daughter cell. This process is critically important, with even small errors leading to aneuploidy or cell death. The chromosomal and/or centrosomal abnormalities are often observed in tumor cells and those abnormalities may often be the first events in the development of a cancer. It is well known that microtubule-based motor proteins are involved in spindle formation and chromosome movements in mitosis. In addition, orchestrated regulation by mitotic kinases is important for the progression of each step of mitosis.

The human chromokinesin Kid/Kinesin-10 is a member of the chromosome-associated kinesin family. Kid has been implicated in multiple process throughout the cell division process; generation of the polar ejection force that pushes the chromosome arms away from the spindle poles toward the spindle equator, the maintenance of spindle length during prometaphase and metaphase, and the tight clustering of anaphase chromosomes (anaphase chromsome compaction). We are interested in the mechanisms by which multiple functions of Kid during M-phase are regulated. We previously found that Kid is phosphorylated on Thr463 by Cdc2/cyclin B kinase. We further showed that the Thr463 dephosphorylation triggered the activation of the microtubule-binding domain of Kid, which is essential to switch the functions of Kid. We further addressed the mechanism underlying Kid-mediated anaphase chromosome clustering. For this purpose, we are currently analyzing a series of Kid mutants for their ability to cluster telophase II chromosomes in activated oocytes.

Plk1 (polo-like kinase 1) is a highly conserved serine/threonine kinase that plays multiple pivotal roles in mitosis, meiosis, and also in oncogenesis. Through the solid-phase phosphorylation screening, we previously identified several Plk1 substrates including Kif18A/kinesin-8. Kif18A, a member of the kinesin-8 motor proteins, is an essential regulator of chromosome alignment, possesses microtubule plus-end directed motility and microtubule plus-end attenuating activity. Kif18A remarkably accumulates at the kinetochore- microtubule (kMT) plus-end in metaphase but not in prometaphase. However, the underlying mechanism and importance of Kif18A localization are unknown. We have been addressing this issue and found the importance of C-terminal tail region of Kif18A in its unique localization and function in chromosome

alignment.

2. Regulation of mitotic events

Shou Soeda, Kenji Iemura, Tetsuhiro Shimodaira, Akinori Takahashi[1], Chisato Kikuguchi[1], Tetsuhiro Shimodaira, Masahiro Morita, Noriko Tokai-Nishizumi, Kazumasa Yokoyama, Toru Suzuki[1], Tadashi Yamamoto[1] and Miho Ohsugi: [1]Cell Signal Unit, Okinawa Institute of Science and Technology

The CCR4-NOT complex is a large protein complex consisting of at least nine subunits, CNOT1-CNOT3, CNOT6, CNOT6L, and CNOT7-CNOT10. Among the subunits, CNOT6, CNOT6L, CNOT7, and CNOT8 are shown to have the mRNA deadeny- lase activity, thus regulating the amount of cytoplasmic mRNA. We found that CNOT3 depletion from HeLa cells increases the expression of MAD1 mRNA and its protein product that plays a part in the spindle assembly checkpoint, leading to the increase of population of mitotic cells. Indeed, the MAD1 mRNA was more stable in CNOT3-depleted cells than in the control cells. Our results suggest that the CNOT3 subunit controls the deadenylase activity of the CCR4-NOT complex toward the MAD1 mRNA and is involvedin the regulation of the spindle assembly checkpoint.

We are currently investigating other newly identified substrates of Plk1, including Tropomyosin family protein and regulatory subunit of AMP-activated protein kinase. which will uncover the molecular mechanisms underlying the Plk1-mediated control of cell divisions and oncogenesis.

3. Structure, regulation and function of centrosomes

Tsubasa Ohashi, Toshiyuki Oikawa, Kenji Iemura, Michiko Koizumi and Miho Ohsugi

The centrosome is the primary microtubule-organizing center (MTOC) in many animal cells. γ-tubulin localizes to the pericentriolar material (PCM) as one of the subunits of the γ-tubulin ring complex, and functions as MTOC. We previously identified Kizuna (Kiz) as a centrosomal Plk1 substrate. In HeLa cells, Kiz is critical for establishing a robust mitotic centrosome architecture that can endure the forces that converge on the centrosomes during spindle formation and centrosomal Plk1 maintains spindle pole integrity through Kiz Thr 379 phosphorylation. We further identified Kiz-interacting protein Cep72. Cep72 is essential for localization of CG-NAP, a large coiled-coil protein forming the structural framework of the PCM, as well as Kiz. Cep72 is also involved in γ-tubulin ring complexes recruitment to the centrosome and CG-NAP confers the microtubule nucleation activity on the γTuRCs. During mitosis, the Cep72-mediated centrosomal MTOC activity helps connect spindle microtubules to the centrosome so that forces generated by chromosome movement along microtubules converge on the PCM. The involvement of Kiz and Cep72 in acentrosomal spindle formation in meiotic oocyte is a question we are addressing.

In mammalian cells, twoγ-tubulin genes TUBG1 and TUBG2 encode closely related isotypes. The protein encoded by TUBG1 and TUBG2 share more than 95% amino acid identity and the functional difference between twoγ-tubulins remains unnkown. TUBG1 is ubiquitously expressed in all cell types, whereas TUBG2 has been found mainly in the brain and early embryo. It has been reported that some cancer cells ectopically express genes whose expression is normally limited to particular cell type or tissue. We recently developed a method to separate γ-tubulin1 and γ-tubulin2 proteins on SDS-PAGE. Using this method, we are trying to examine the expression pattern of γ-tubulins in human cancer and normal cell lines.

4. Chromosome dynamics in early mice embryo

Shou Soeda, Tsubasa Ohashi, Toshiyuki Oikawa, Kaori Yamada, Fukashi Inoue, and Miho Ohsugi

In vertebrates, oocytes are arrested at metaphase of the second meiosis, and fertilization triggers the anaphase onset and emission of the second polar body. Then, nuclear envelopes are assembled around maternal and paternal chromosomes, forming separate haploid mail and female pronuclei. In mouse embryo, this topological genome separation appears to be preserved up to the four-cell embryo stage and then gradually disappears. Maternal proteins and transcripts stored in oocytes control embryonic development, until zygotic gene activation (ZGA) begins. In mice, minor gene activation begins at the 1-cell stage that is followed by a major gene activation at the 2-cell stage. Therefore, completion of oocyte meiosis II and first couple of mitosis after fertilization are almost exclusively under maternal control. We are interested in how the structure and behavior of maternal and paternal chromosomes change around the transition point from maternal to embryonic control. We are addressing these issues by several approaches including time-lapse imaging of chromosomes dynamics in early mouse embryos and establishment of in vitro cell-free system derived from mouse oocytes. We also conduct comperehensive transcriptome analyses of unfertilized mouse oocytes and 1- to 8-cell embryos.

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Department of Cancer Biology

Division of Cancer Cell Research
腫瘍細胞社会学分野

Professor	Motoharu Seiki, D.M.Sc	教　授　医学博士	清　木　元　治
Associate Professor	Naohiko Koshikawa, Ph.D.	准教授　理学博士	越　川　直　彦
Assistant Professor	Takeharu Sakamoto, Ph.D.	助　教　医学博士	坂　本　毅　治
Assistant Professor	Daisuke Hoshino, Ph.D.	助　教　医学博士	星　野　大　輔

Membrane proteins and their ligands including signaling molecules and extracellular matrix proteins mediate complex arrays of cell signaling. Fate and activities of these proteins are often regulated by proteases in the extracellular space. We are currently focused on studying biological roles of membrane-anchored type of matrix metalloproteinases (MT-MMPs) which are potent modulators of pericellular microenvironment and eventually regulate varieties of cellular functions such as proliferation, migration, apoptosis, and differentiation. Since uncontrolled expression of MT-MMPs in tumors contributes to their malignant characteristics, they are potential targets for cancer therapy.

1. Network analysis of the focal adhesion to invadopodia transition identifies a PI3K-PKCα invasive signaling axis.

In cancer, deregulated signaling can produce an invasive cellular phenotype. We modeled the invasive transition as a theoretical switch between two cytoskeletal structures: focal adhesions and extracellular matrix-degrading invadopodia. We constructed molecular interaction networks of each structure and identified upstream regulatory hubs through computational analyses. We compared these regulatory hubs to the status of signaling components from head and neck carcinomas, which led us to analyze phosphatidylinositol 3-kinase (PI3K) and protein kinase C α (PKCα). Consistent with previous studies, PI3K activity promoted both the formation and the activity of invadopodia. We found that PI3K induction of invadopodia was increased by overexpression of SH2 (Src homology 2) domain-containing inositol 5'-phosphatase 2 (SHIP2), which converts the phosphatidylinositol 3,4,5-trisphosphate [PI(3,4,5)P(3)] that is produced by PI3K activity to phosphatidylinositol 3,4-bisphosphate [PI(3,4)P(2)], which is believed to promote invadopodia formation. Knockdown of PKCα had divergent effects on invadopodia formation, depending on the status of PI3K. Loss of PKCα inhibited invadopodia formation in cells with wild-type PI3K pathway status. Conversely, in cells with constitutively active PI3K (through activating PI3K mutants or lacking the endogenous opposing enzyme PTEN), PKCα knockdown increased invadopodia formation. Mechanistic studies revealed a negative feedback loop from PKCα that dampened PI3K activity and invasive behavior in cells with genetic hyperactivation of the PI3K pathway. These studies demonstrated the potential of network modeling as a discovery tool and identified PI3K and PKCα as interacting regulators of invasive behavior.

2. Detection of the heterogeneous O-glycosylation profile of MT1-MMP expressed in cancer cells by a simple MALDI-MS method.

Glycosylation is an important and universal post-translational modification for many proteins, and regulates protein functions. However, simple and

rapid methods to analyze glycans on individual proteins have not been available until recently. A new technique to analyze glycopeptides in a highly sensitive manner by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) using the liquid matrix 3AQ/CHCA was developed recently and we optimized this technique to analyze a small amount of transmembrane protein separated by SDS-PAGE. We used the MALDI-MS method to evaluate glycosylation status of membrane-type 1 matrix metalloproteinase (MT1-MMP). O-glycosylation of MT1-MMP is reported to modulate its protease activity and thereby to affect cancer cell invasion. MT1-MMP expressed in human fibrosarcoma HT1080 cells was immunoprecipitated and resolved by SDS-PAGE. After in-gel tryptic digestion of the protein, a single droplet of the digest was applied directly to the liquid matrix on a MALDI target plate. Concentration of hydrophilic glycopeptides within the central area occurred due to gradual evaporation of the sample solution, whereas nonglycosylated hydrophobic peptides remained at the periphery. This specific separation and concentration of the glycopeptides enabled comprehensive analysis of the MT1-MMP O-glycosylation. We demonstrate, for the first time, heterogeneous O-glycosylation profile of a protein by a whole protein analysis using MALDI-MS. Since cancer cells are reported to have altered glycosylation of proteins, this easy-to-use method for glycopeptide analysis opens up the possibility to identify specific glycosylation patterns of proteins that can be used as new biomarkers for malignant tumors.

3. Genetic screening of new genes responsible for cellular adaptation to hypoxia using a genome-wide shRNA library.

Oxygen is a vital requirement for multi-cellular organisms to generate energy and cells have developed multiple compensatory mechanisms to adapt to stressful hypoxic conditions. Such adaptive mechanisms are intricately interconnected with other signaling pathways that regulate cellular functions such as cell growth. However, our understanding of the overall system governing the cellular response to the availability of oxygen remains limited. To identify new genes involved in the response to hypoxic stress, we have performed a genome-wide gene knockdown analysis in human lung carcinoma PC8 cells using an shRNA library carried by a lentiviral vector. The knockdown analysis was performed under both normoxic and hypoxic conditions to identify shRNA sequences enriched or lost in the resulting selected cell populations. Consequently, we identified 56 candidate genes that might contribute to the cellular response to hypoxia. Subsequent individual knockdown of each gene demonstrated that 13 of these have a significant effect upon oxygen-sensitive cell growth. The identification of BCL2L1, which encodes a Bcl-2 family protein that plays a role in cell survival by preventing apoptosis, validates the successful design of our screen. The other selected genes have not previously been directly implicated in the cellular response to hypoxia. Interestingly, hypoxia did not directly enhance the expression of any of the identified genes, suggesting that we have identified a new class of genes that have been missed by conventional gene expression analyses to identify hypoxia response genes. Thus, our genetic screening method using a genome-wide shRNA library and the newly-identified genes represent useful tools to analyze the cellular systems that respond to hypoxic stress.

4. Establishment and validation of computational model for MT1-MMP dependent ECM degradation and intervention strategies.

MT1-MMP is a potent invasion-promoting membrane protease employed by aggressive cancer cells. MT1-MMP localizes preferentially at membrane protrusions called invadopodia where it plays a central role in degradation of the surrounding extracellular matrix (ECM). Previous reports suggested a role for a continuous supply of MT1-MMP in ECM degradation. However, the turnover rate of MT1-MMP and the extent to which the turnover contributes to the ECM degradation at invadopodia have not been clarified. To approach this problem, we first performed FRAP (Fluorescence Recovery after Photobleaching) experiments with fluorescence-tagged MT1-MMP focusing on a single invadopodium and found very rapid recovery in FRAP signals, approximated by double-exponential plots with time constants of 26s and 259s. The recovery depended primarily on vesicle transport, but negligibly on lateral diffusion. Next we constructed a computational model employing the observed kinetics of the FRAP experiments. The simulations successfully reproduced our FRAP experiments. Next we inhibited the vesicle transport both experimentally, and in simulation. Addition of drugs inhibiting vesicle transport blocked ECM degradation experimentally, and the simulation showed no appreciable ECM degradation under conditions inhibiting vesicle transport. In addition, the degree of the reduction in ECM degradation depended on the degree of the reduction in the MT1-MMP turnover. Thus, our experiments and simulations have established the role of the rapid turnover of MT1-MMP in ECM degradation at invadopodia. Furthermore, our simulations suggested synergetic contributions of proteolytic activity and the MT1-MMP turnover to ECM degradation because there was a nonlinear and marked reduction in ECM degradation if both factors were reduced simultaneously. Thus our

computational model provides a new in silico tool to design and evaluate intervention strategies in cancer cell invasion.

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Department of Cancer Biology

Division of Molecular Pathology
人癌病因遺伝子分野

Professor	Yoshinori Murakami, M.D., Ph.D.	教	授	医学博士	村 上 善 則
Senior Assistant Professor	Daisuke Matsubara, M.D., Ph.D.	講	師	医学博士	松 原 大 祐
Assistant Professor	Mika Sakurai-Yageta, Ph.D.	助	教	医学博士	櫻 井 美 佳

Human cancers develop and progress toward malignancy through accumulation of multiple genetic and epigenetic alterations. Elucidation of these alterations is essential to provide molecular targets for prevention, diagnosis, and treatment of cancer. Our current interest is to understand the role of cell adhesion in cancer invasion and metastasis. Genetic and epigenetic abnormalities involved in human tumors, including cholangiocarcinoma, adult T-cell leukemia, lung, breast and urological cancers, are also being investigated.

1. The biological functions of CADM1/TSLC1 protein in cell adhesion

Mika Sakurai-Yageta, Megumi Ishimura, Yumi Tsuboi, Masayoshi Nagata, Taketo Kawai, Yuka Takahashi, Hideki Kuwano, Takeshi Ito, Yuki Ikeda, Suejen Shiu, Hiroki Nakaoka, Yuki Kumagai, Hiroyuki Kogai, Siew-Pei Gan, Tomoko Masuda, Hiromi Ichihara and Yoshinori Murakami:

Disruption of cell adhesion is a critical step to invasion and metastasis of human cancer. CADM1/TSLC1 is an immunoglobulin superfamily cell adhesion molecule and primarily involved in epithelial cell adhesion, whereas its expression is often lost in various epithelial cancers in their advanced stages. In order to understand the molecular mechanisms underlying inactivation of CADM1 other than the promoter methylation, we investigated the possible involvement of microRNA (miRNA) in the down-regulation of CADM1. Using computational algorithms, miR-214 and miR-375 were identified as candidate miRNAs targeting CADM1. A luciferase reporter assay demonstrated that miR-214 and miR-375 repressed the promoter activity through 3'-UTR of *CADM1*. Quantitative RT-PCR analysis demonstrated that miR-214 and miR-375 were highly expressed in 21 (62%) and 17 (50%) cases of 34 primary NSCLCs. Notably, increased expression of miR-214 was preferentially observed in tumors with advanced pathological stages and in those lacking CADM1 expression but were not associated with the promoter methylation, suggesting that miR-214-mediated silencing would be another mechanism to suppress CADM1 expression. On the other hand, introduction of miR-214 or miR-375 into NSCLC cells decreased CADM1 protein expression. Furthermore, overexpression of miR-214 enhanced anchorage-independent growth of NSCLC cells, A549, whereas transfection of miRNA inhibitors of miR-214 or miR-375 significantly suppressed the *in vitro* wound healing activity of HCC827 cells. These findings suggest that overexpression of miR-214 and miR-375 could participate in the malignant features of NSCLC through down-regulating CADM1 and would provide a potential target for the treatment of a subset of NSCLC. We also reported that CADM1 and its binding protein 4.1B provide a tumor suppressor cascade of human breast cancers, whereas CADM4, a homologue of CADM1, and 4.1B play a critical role in suppression of renal clear cell carcinoma. Furthermore, possible cross-talk of CADM1 cascade with other known signal transduction pathways, in-

cluding those driven by tyrosine kinases, are being elucidated. Dynamic regulation of CADM1 protein on the cell membrane is also being analyzed using photo-bleaching assay.

2. Analysis of oncogenic function of CADM1 in adult T-cell leukemia (ATL) and small cell lung cancer (SCLC)

Mika Sakurai-Yageta, Shigefumi Murakami, Shinji Kikuchi[1], Miwako Iwai, Tomoko Masuda, Yoshinori Murakami: [1]Department of Surgery, Institute of Clinical Medicine, University of Tsukuba.

In contrast to a tumor suppressor function of CADM1 in epithelial cancers, CADM1 is ectopically expressed in adult T-cell leukemia (ATL), conferring an invasive phenotype characteristic to ATL. We investigate the roles of CADM1 in small cell lung cancer (SCLC). Immunohistochemistry demonstrates that 10 of 35 (29%) primary SCLC tumors express CADM1 protein. Western blotting and RT-PCR analyses have revealed that CADM1 is significantly expressed in 11 of 14 SCLC cells growing in suspension cultures but in neither of 2 SCLC cells showing attached growth to plastic dishes, suggesting that CADM1 is involved in anchorage-independent growth in SCLC. Then, we demonstrate that SCLC expresses a unique splicing variant of CADM1 (variant 8/9) containing additional extracellular fragments corresponding to exon 9 in addition to variant 8, a common isoform in epithelia. Variant 8/9 of CADM1 is almost exclusively observed in SCLC and testis, although this variant protein localizes along the membrane and shows similar cell aggregation activity to variant 8. Interestingly, both variant 8/9 and variant 8 of CADM1 show enhanced tumorigenicity in nude mice when transfected into SBC5, a SCLC cell lacking CADM1. Inversely, suppression of CADM1 expression by shRNA reduced spheroid-like cell aggregation of NCI-H69, a SCLC cell expressing a high amount of CADM1. These findings suggest that CADM1 enhances the malignant features of SCLC, as is observed in ATL, and could provide a molecular marker specific to SCLC. To establish CADM1 as a diagnostic and therapeutic target of ATL or SCLC, we analyzed N- and O-glycosylation structure of CADM1 from each cancer cell in collaboration with Shimadzu Co. Ltd. Furthermore, we investigated downstream cascade of CADM1 characteristic to ATL or SCLC and identified several candidate pathways by cell-based screening of chemical reagents that inhibit CADM1-mediated cell spreading in combination with molecular biological analysis.

3. Molecular pathological analysis of cholangiocarcinoma from Thailand and Japan

Takeshi Ito, Akiteru Goto[2], Mika Sakurai-Yageta and Yoshinori Murakami: [2]Department of Pathology, Akita University Graduate School of Medicine.

Cholangiocarcinoma (CCA) is one of the cancers refractory to any therapeutic approach, and its incidence is known to be highest in the northeastern part of Thailand, including Khon Kaen province. Chronic inflammation caused by the liver fluke (*Opisthorchis viverrini*) infection is identified as the major etiological factor of CCA development in northeastern Thailand. The incidence of CCA is also increasing worldwide, including Japan, possibly due to chronic inflammation of the liver caused by HCV infection. Further epidemiological and etiological studies of CCA in Thailand as well as in Japan are expected to provide novel approaches to prediction, early diagnosis and treatment of this nearly always fatal malignancy. We started active international collaboration between Thailand and Japan in 2010 towards the control of CCA associated with liver fluke infection as an International Collaboration Project supported by the Japan Science and Technology Agency (JST). This is a multidisciplinary collaboration by investigators in epidemiology, parasitology, surgical and molecular pathology, biochemistry, internal medicine and surgery in both Thailand and Japan and several promising findings are being obtained. We examined the features of CCA in Thailand and Japan by molecular pathological approaches. Microarray analysis of CCA cells from patients in Thailand infected with Ov and from patients in other countries, including Japan, has demonstrated that CCA associated with Ov infections shows a distinct profile in gene expression, suggesting that such CCA develops and progresses through distinct molecular pathways. These distinct features arise through chronic inflammation caused by Ov. A tumor suppressor, CADM1, is identified as one of the genes downregulated in CCA by Ov. CADM1 expression is known to be frequently lost in various advanced stage cancers, including hepatocellular carcinoma. We have found by immunohistochemical studies that CADM1 expression is already lost in 10 of 40 (25%) bile duct epithelia in the Ov-infected liver tissues from which the CCAs arose, but not in any bile duct epithelia from Japanese CCA patients, suggesting that bile duct epithelia in patients infected with Ov shows pre-cancerous features.

4. Analyses of genetic and epigenetic alterations in human tumors

Daisuke Matsubara, Toshiro Niki[3], Takahiro Mimae, Akihiko Ito[4], Anri Saito, Osman Wael, Ibrahim Reem, Fumi Taira, Ken Akashi, Atsushi Kanetani, Takeshi Ito and Yoshinori Murakami:

[3]Department of Integrative Pathology, Jichi Medical University; [4]Department of Pathology, Kinki University School of Medicine

To understand the molecular features of multistage carcinogenesis, aberrations in additional key molecules in human tumorigenesis were examined in various cancers. Microarray analysis in combination with laser capture microscopy identified Nocth2 and Six1 as candidate oncogenes upregulated in early-stage lung adenocarcinoma in collaboration with Kinki University School of Medicine. Microarray analysis of 40 lung adenocarcinoma cells have identified a group of tumors that shows neither oncogene activation nor tumor-suppressor inactivation but presents mesenchymal features. We have further demonstrated that the expression of chromosome remodeling factors, BRG1 and BRM, are frequently lost in the group of tumors with mesenchymal phenotype. The same microarray-based analysis also identified that a human lung adenocarcinoma cell line, LC-2/ad, expressed CDC6-RET fusion protein in collaboration with Jichi Medical University. This is the first report of cell line carrying the *RET* fusion gene, providing a useful tool to examine the molecular mechanisms of RET-related carcinogenesis. In addition, as a novel driving force of tumor progression, copy number alterations (CNA) in human solid tumors are being analyzed in various cancers using DNA chip-based method.

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Department of Cancer Biology

Division of Cellular and Molecular Biology
分子発癌分野

Professor	Jun-ichiro Inoue, Ph.D.		教　授	薬学博士	井　上　純一郎
Associate Professor	Taishin Akiyama, Ph.D.		准教授	薬学博士	秋　山　泰　身
Assistant Professor	Yuu Taguchi, Ph.D.		助　教	医学博士	田　口　　祐
Project Assistant Professor	Yuri Shibata, Ph.D.		特任助教	生命科学博士	柴　田　佑　里

Gene expression is largely regulated by signal transduction triggered by various stimulations. Several lines of evidence indicate that genetic defects of molecules involved in the signal transduction or the gene expression lead to abnormal cell differentiation or tumor formation. Our goal is to understand the molecular mechanisms of disease pathogenesis and oncogenesis by elucidating normal regulation of intracellular signal transduction and gene expression involved in cell proliferation and differentiation. We have identified and been interested in Tumor necrosis factor receptor-associated factor 6 (TRAF6), which transduces signal emanating from the TNFR superfamily and the TLR/IL-1R family leading to activation of transcription factor NF-κB and AP-1. By generating TRAF6-deficient mice, we found that TRAF6 is essential for osteoclastogenesis, self-tolerance, lymph node organogenesis and formation of skin appendices. We are currently focusing on molecular mechanisms underlying TRAF6-mediated activation of signal transduction pathways and how TRAF6 is involved in osteoclastogenesis and self-tolerance. In addition, NF-κB is constitutively activated in various cancer cells and this activation could be involved in the malignancy of tumors. Thus, we are also investigating the molecular mechanisms of the constitutive activation of NF-κB.

1. Molecular mechanism of the regulation of Rel/NF-κB transcription factor

Michiyasu Ito, Aya Kojima, Yuko Hata[1], Masaaki Oyama[1], Jin Gohda[2], and Jun-ichiro Inoue: [1]Medical Proteomics Laboratory, and [2]Center for Asian Infectious Diseases, IMSUT.

Transcription factor Rel/NF-κB binds specifically to a decameric motif of nucleotide, κB site, and activates transcription. The activation of Rel/NF-κB has been demonstrated to be carried out post-translationally upon extracellular stimuli through membrane receptors such as members of the TLR/IL-1R family and of TNFR superfamily. Rel/NF-κB forms a complex with regulatory protein, IκB, and is sequestered in the cytoplasm prior to stimulation. Upon stimulation, IκB is rapidly phosphorylated on two specific serine residues by IκB kinase (IKK) complex followed by lysine 48 (K48)-linked ubiquitination and proteasome-dependent degradation of IκB. Rel/NF-κB subsequently translocates to the nucleus to activate transcription of target genes. This project is to identify molecules that regulate signal from membrane receptors to Rel/NF-κB/IκB complex. We have previously identified upstream activators of Rel/NF-κB, tumor necrosis factor receptor-associated factor (TRAF) 6. TRAF6 contains RING domain in the N-terminus and acts as an E3 ubiquitin-ligase to catalyze the lysine 63 (K63)-linked polyubiquitination of several signaling molecules and TRAF6 itself. We have reported that K63-

linked polyubiquitination of TAK1 at Lys-209 by TRAF6 and Ubc13, an E2 ubiquitin-conjugating enzyme, is required for the IL-1-mediated formation of TRAF6/MEKK3/TAK1 complex, which is essential for the activation of TAK1 and subsequent activation of NF-κB. However, it is not understood how the polyubiquitin chain conjugated to TAK1 mediates formation of this signal complex. We hypothesized that some unidentified component of the complex could bind the polyubiquitin chain of TAK1, thereby linking TAK1 to the complex. Therefore, we are currently looking for such proteins. We already have a candidate, whose knock down resulted in inhibition of NF-κB activation induced by IL-1.

2. p47 negatively regulates IKK activation by inducing the lysosomal degradation of polyubiquitinated NEMO.

Yuri Shibata, Masaaki Oyama[1], Hiroko Kozuka-Hata[1], Xiao Han, Jin Gohda[2] and Jun-ichiro Inoue

We have identified p47 (also known as NSFL1C) as a novel IKK-interacting protein. p47 is originally identified as a major cofactor of the cytosolic ATPase associated with various cellular activities p97, and p47 is required for p97-madiated reassembly of Golgi stacks. Co-immunoprecipitation assay revealed that p47 bound to the IKK complex thorough NEMO upon TNF-α and IL-1 stimulation. Silencing of p47 enhanced IKK activation, NF-κB nuclear translocation, and its transcriptional activity. These results indicate that p47 is a negative regulator in IKK/NF-κB activation. To elucidate the molecular mechanisms of p47-mediated IKK inhibition, we sought to determine the structural requirements for the inhibitory effect of p47 on IKK. Wild-type and mutant p47 lacking p97-binding domain blocked NF-κB activation equally well. In contrast, p47 deletion mutant lacking ubiquitin-binding domain had a significantly reduced ability to inhibit NF-κB activation, indicating that the ubiquitin-binding activity, but not the p97-binding activity, of p47 is crucial for IKK inhibition. Consistent with this result, *in vitro* binding assay revealed that p47 interacted with NEMO only when NEMO was conjugated to polyubiquitin chains, and p47 preferentially bound to Lys63-linked and linear polyubiquitin chains than Lys48-linked polyubiquitin chains. The enhanced accumulation of polyubiquitinated NEMO was observed in p47-knockdown cells and in the lysosome inhibitor-treated cells, but not in the proteasome inhibitor-treated cells. Moreover, confocal microscopy analysis revealed that NEMO colocalized with LAMP-1, a late endosomal-lysosomal marker, upon TNF-α stimulation. Collectively, these results strongly suggest that p47 binds to polyubiquitinated NEMO and target them for lysosomal degradation, thereby negatively regulating NF-κB activation. Furthermore, the expression of p47 was reduced in adult T-cell leukemia (ATL) cells, in which the constitutive activation of NF-κB is crucial in leukemogenesis and survival of tumor cells. The reduced expression of p47 may result in constitutive NF-κB activation, which may trigger development of various cancers.

3. Molecular mechanism of HTLV-1 Tax-induced IKK activation

Yuri Shibata, Masaaki Oyama[1], Hiroko Kozuka-Hata[1], Jin Gohda[2] and Jun-ichiro Inoue

Activation of NF-κB by human T-cell leukemia virus type 1 (HTLV-1) Tax is thought to be crucial in T-cell transformation and the onset of adult T-cell leukemia (ATL). Although it is well known that Tax interacts with NEMO and induces IKK activation, the molecular mechanism of Tax-induced IKK activation remains largely unknown. To elucidate this mechanism, we established a cell-free assay system, in which the IKK complex can be activated by adding recombinant Tax to cytosolic extracts. Whereas recombinant Tax induced activation of IKK complex in cytosolic extracts, it failed to activate the purified IKK complex. Moreover, Tax-induced IKK activation was blocked by addition of lysine-free, methylated or K63R ubiquitin. These results suggest that Lys63-linked polyubiquitination is critical for Tax-induced IKK activation. Polyubiquitin chains might be required to recruit IKK kinase (IKKK) or induce *trans*-autophosphorylation of the IKK complex, which leads to IKK activation.

4. Molecular mechanism of RANK signaling in osteoclastogenesis

Yuu Taguchi, Kazuaki Tsumura, Fukutoshi Shirai, Mami Yamamoto, Masaaki Oyama[1], Hiroko Kozuka-Hata[1], Jin Gohda[2], and Jun-ichiro Inoue

Osteoclasts are bone-resorbing cells derived from hematopoietic progenitor cells. Osteoclasts play a crucial role in bone homeostasis in concert with osteoblasts, which mediate bone-formation. Excess formation or activation of osteoclasts results in pathological bone resorption, such as postmenopausal osteoporosis. Therefore, precise elucidation of the regulatory mechanisms of osteoclastogenesis is essential for understanding skeletal diseases and for developing drugs to treat such diseases.

Osteoclastogenesis depends on receptor activator of nuclear factor-κB (RANK) signaling in progenitor cells. The RANK signaling activates NF-κB and AP-1 through the adaptor molecule TRAF6, and induces PLCγ2-mediated Ca^{2+} oscillation, which is required for induction of NFATc1, a master transcrip-

tional factor in osteoclastogenesis. We have previously identified a novel domain in cytoplasmic region of RANK, named Highly Conserved domain in RANK (HCR), which did not share any significant homology with other proteins. HCR functions as a platform for formation of signal complex including TRAF6, PLCγ2, and Gab2, and emanates sustained RANK signaling, which is essential for osteoclastogenesis.

In this year, we reported that ectopic expression of the HCR-peptide composed of 62 amino acids inhibited RANK-mediated osteoclastogenesis. Moreover, we also reported that although expression of either the amino- or carboxyl-terminal half of the HCR peptide (N- or C-peptide) independently inhibited RANK signaling prior to cell-cell fusion, expression of the GY-peptide, which is a part of the C-peptide, inhibited cell-cell fusion to prevent formation of multinucleated osteoclasts. Since Gab2 bound the C-peptide but not N- and GY-peptide, and Vav3, which mediates signaling related to cell-cell fusion, bound only GY-peptide, we thought that HCR region in RANK can mediate both signaling related to induction of NFATc1 and cell-cell fusion. To further elucidate the mechanisms of the HCR-mediated signaling, we tried to identify the novel binding protein to the HCR-fragment by using proteomics approaches, and already have some candidate proteins.

On the other hand, we also tried to identify the novel protein, which is involved in osteoclastogenesis. Because regulatory mechanisms, especially negative regulatory system in osteoclastogenesis, were not fully understood, microarray analyses were performed to identify such genes. We already have some candidate genes whose expression are regulated by RANK signaling, and are now confirming whether such genes are involved in osteoclastogenesis. We think that these genes are suitable targets for developing drugs to treat bone disease.

5. Analysis of the role of ubiquitin E3 ligases cIAP1/2 in RANKL-induced osteoclastogenesis

Noritaka Yamaguchi[3], Yuu Taguchi, Jin Gohda[2], and Jun-ichiro Inoue: [3]Graduate School of Pharmaceutical Sciences, Chiba University

Osteoclast is known to be formed by the fusion of hematopoietic cells of the monocyte-macrophage lineage. One of the essential signaling molecules for osteoclast differentiation is RANK, a member of the TNF receptor superfamily expressing on the surface of the precursor cells. Binding of RANK to its ligand activates NF-κB and MAP kinase pathways and finally induces expression of NFATc1, a master transcription factor of osteoclastgenesis. Recently, emerging evidence indicates that cellular inhibitor of apoptosis protein 1 and 2 (cIAP1/2) are involved in the signaling of the TNF receptor superfamily. cIAP1/2 are ubiquitin E3 ligases that induce K63-linked polyubiquitination of RIP1 to activate NF-κB in the TNF signaling. In the CD40 signaling, cIAP1/2 induce K48-linked polyubiquitination and proteasomal degradation of TRAF3, and its degradation leads to activation of MAP kinases. Although RANK is a member of the TNF receptor superfamily, the role of cIAP1/2 in this signaling is not clear. In this study, we reveal the involvement of cIAP1/2 in RANKL-induced osteoclastogenesis. The overexpression of cIAP1 or cIAP2 in the mouse monocytic cell line Raw264.7 resulted in the significant suppression of RANKL-induced NFATc1 mRNA expression and osteoclastogenesis, whereas the activation of the NF-κB and MAPK pathways was barely changed by these overexpressions. The depletion of endogenous cIAP1/2 by their specific inhibitor MV1 or their siRNA-mediated knockdown resulted in enhanced RANKL-induced NFATc1 expression and osteoclastogenesis without affecting the activation of the NF-κB and MAPK pathways. In combination, these results indicate that cIAP1/2 negatively regulate osteoclastogenesis by inhibiting NFATc1 mRNA expression in a manner that is distinct from the previously identified functions of cIAP1/2.

6. The role of NF-κB activity in regulating population of cancer stem cell of breast cancers

Mizuki Yamamoto, Eerdunfu, Takeshi Yokoyama Noritaka Yamaguchi[3], Kentaro Semba[4] and Junichiro Inoue: [4]Department of Life Science and Medical Bio-Science, Waseda University

Recent studies indicate that breast cancer arise from rare self-renewing cancer stem cells (CSCs), which exhibit CD24-low, CD44-high, and EpCAM-positive phenotype. Because CSCs have particular profiles such as resistibility against variant stress as well as normal stem cells, it is thought that CSCs are also involved in recurrence and metastasis. However, it remains unclear which signaling pathways are crucial for the maintenance and functions of CSCs. Recently, we have found that NF-κB is constitutively and highly active in basal-like subtype breast cancer cells, which have high rates of early relapse when compared with patients with other breast cancer subtypes. These results led us to hypothesise that NF-κB may regulate its CSC population. In this project, to analyze significance of NF-κB in breast cancer CSCs, we modulated NF-κB activity in basal-like subtype cells using retrovirus vector encoding IKKβ or IκBα super-repressor and examined population of CSCs by FACS analysis, sphere culture assay, and xenograft model of NOD/SCID mice. We then found that the population of

CSCs was increased or decreased depending on the level of the NF-κB activity, suggesting that NF-κB is involved in the maintenance of CSCs. We are currently analyzing the molecular mechanism of the NF-κB-mediated maintenance of breast cancer CSCs.

7. Regulatory mechanisms for development and functions of medullary thymic epithelial cells

Nobuko Akiyama, Miho Shinzawa, Nobukazu Takizawa, Maki Miyauchi, Jun-ichiro Inoue, and Taishin Akiyama.

Clonal deletion is one of the main mechanisms to maintain T-cell tolerance. T-cell clones that have high avidity for self-antigens are eliminated during their development in the thymus. Self-antigens are predominantly expressed and presented by epithelial cells (TECs) and dendritic cells. Medullary thymic epithelial cells (mTECs) have a unique property; mTECs promiscuously express a wide variety of self-antigens that are normally expressed in tissue specific manner (TSAs). Therefore, it has been proposed that developing T-cells encounter TSAs in the thymic medullar for clonal deletion. Even though an increasing body of evidence indicates crucial roles of mTECs on preventing autoimmunity by establishing self-tolerance in thymus, signaling pathways underlying the differentiation and proliferation of mTECs expressing Aire and TSAs are poorly understood. We previously found that TNF receptor family members RANK and CD40 cooperatively regulate the development of mTECs expressing Aire and TSAs. In this context, RANK and CD40 regulate the mTEC development by activating signal transducers TNF receptor-associated factor 6 (TRAF6) and NF-kB inducing kinase (NIK). However, the downstream target genes of these signaling remain to be determined. We obtained several candidates up-regulated by treatment of *in vitro* organ culture of fetal thymic stroma with recombinant RANK ligand and are currently investigating roles of these factors in the development and functions of mTECs *in vivo*.

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井上純一郎，武川睦寛，徳永文稔，今井浩三　編集　シグナル伝達研究最前線2012−翻訳後修飾，解析技術，疾患との連関から創薬応用まで−実験医学　増刊号

Department of Cancer Biology

Division of Genetics
腫瘍抑制分野

Professor	Yuji Yamanashi, Ph.D.	教　授	理学博士	山　梨　裕　司
Assistant Professor	Ryuichi Mashima, Ph.D.	助　教	工学博士	真　嶋　隆　一
Assistant Professor	Tohru Tezuka, Ph.D.	助　教	理学博士	手　塚　　徹
Assistant Professor	Ryo Ueta, Ph.D.	助　教	博士(生命科学)	植　田　　亮

The major interest of this division is in molecular signals that regulate a variety of cellular activities. Our aim is to address how deregulated cellular signals give rise to neoplastic, immune, neural, metabolic, or developmental disorders. Our goal is to understand the molecular bases of tumorigenesis and the development of other intractable diseases as a path toward uncovering therapeutic targets. Currently, we are investigating regulatory mechanisms in protein-tyrosine kinase (PTK)-mediated signaling pathways and their pathophysiological roles in tumorigenesis, metastasis, inflammation, and myasthenia.

1. Activation of the receptor tyrosine kinase MuSK by the cytoplasmic adaptor protein Dok-7.

Ueta, R., Nakatani, N., Ikegami, T., Miyoshi, S., Tezuka, T., and Yamanashi, Y.:

Protein-tyrosine kinases (PTKs) play crucial roles in a variety of signaling pathways that regulate proliferation, differentiation, motility, and other activities of cells. Therefore, deregulated PTK signals give rise to a wide range of diseases such as neoplastic disorders. To understand the molecular bases of PTK-mediated signaling pathways, we identified Dok-1 as a common substrate of many PTKs in 1997. Since then, the Dok-family has been expanded to seven members, Dok-1 to Dok-7, which share structural similarities characterized by NH_2-terminal pleckstrin homology (PH) and phosphotyrosine binding (PTB) domains, followed by Src homology 2 (SH2) target motifs in the COOH-terminal moiety, suggesting an adaptor function. Indeed, as described below, Dok-1 and Dok-2 recruit p120 rasGAP upon tyrosine phosphorylation to suppress Ras-Erk signaling. However, we found that Dok-7 acts as an essential cytoplasmic activator of the muscle-specific receptor tyrosine kinase (RTK) MuSK in the formation of the neuromuscular junction (NMJ), providing a new insight into RTK-mediated signaling. It now seems possible that local levels of cytoplasmic activators, like Dok-7, control the activity of RTKs in concert with their extracellular ligands.

The NMJ is a synapse between a motor neuron and skeletal muscle, where the motor nerve terminal is apposed to the endplate. The contraction of skeletal muscle is controlled by the neurotransmitter acetylcholine (ACh), which is released from the presynaptic motor nerve terminal. To achieve efficient neuromuscular transmission, acetylcholine receptors (AChRs) must be densely clustered on the postsynaptic muscle membrane of the NMJ. Failure of AChR clustering is associated with disorders of neuromuscular transmission such as congenital myasthenic syndromes and myasthenia gravis, which are characterized by fatigable muscle weakness. The formation of NMJs is orchestrated by MuSK and by neural Agrin, an extracellular activator of MuSK. However, experimentally when motor nerves are ablated, AChRs form clusters in the correct, central

region of muscle during embryogenesis in a MuSK-dependent process known as prepatterning of the receptors. In addition, in vivo overexpression of MuSK causes neuromuscular synapse formation in the absence of Agrin, suggesting that muscle-intrinsic, cell-autonomous activation of MuSK may be adequate to trigger presynaptic and postsynaptic differentiation in vivo. However, the mechanisms by which MuSK is activated independently of nerve and Agrin had long been unclear.

Because both MuSK and the adaptor-like cytoplasmic protein Dok-7 are localized to the postsynaptic region of NMJs, we previously examined their interaction and found that Dok-7 is an essential cytoplasmic activator of MuSK. In addition, we found that Dok-7 directly interacts with the cytoplasmic portion of MuSK and activates the RTK, and that neural Agrin requires Dok-7 in order to activate MuSK. Indeed, in vivo overexpression of Dok-7 increased MuSK activation and promoted NMJ formation. Conversely, mice lacking Dok-7 formed neither NMJs nor AChR clusters.

We are currently investigating the signaling mechanisms involving Dok-7, Agrin, MuSK, and Lrp4. The last forms a complex with MuSK and acts as an Agrin-binding module in the complex. Interestingly, Dok-7 does not require its PTB domain and C-terminal moiety for activation of MuSK in myoblasts, but does in myotubes, at least in cultured cells. We are particularly interested in the molecular mechanisms underlying these cell-type specific requirements. In addition, it was recently reported the N-terminal part of Lrp4 is essential for presynaptic differentiation of the motor nerve terminal at NMJs (*Nature* 489: 438-442, 2012). Therefore, we are also interested in the effects of forced expression of Dok-7 in skeletal muscle on NMJ formation in the absence of Lrp4 or Agrin.

2. Pathophysiological mechanisms underlying *DOK7* myasthenia.

Tezuka, T., Arimura, S., Miyoshi, S., Hoshi, T., Spearman, H.[1], Beeson, D.[1], and Yamanashi, Y.: [1]Weatherall Institute of Molecular Medicine, University of Oxford.

As mentioned above, impaired clustering of AChRs could underlie NMJ disorders, be they autoimmune (MuSK antibody-positive myasthenia gravis) or genetic (congenital myasthenic syndromes (CMS)) in origin. Therefore, our findings that Dok-7 activates MuSK to cluster AChRs and to form NMJs suggested *DOK7* as a candidate gene for mutations associated with CMS. Indeed, we previously demonstrated that biallelic mutations in *DOK7* underlie a major subgroup of CMS with predominantly proximal muscle weakness that did not show tubular aggregates on muscle biopsy but were found to have normal AChR function despite abnormally small and simplified NMJs. We further demonstrated that several mutations, including one associated with the majority of patients with the disease, impaired Dok-7's ability to activate MuSK. This new disease entity is termed "*DOK7* myasthenia."

To investigate pathophysiological mechanisms underlying *DOK7* myasthenia, we have established mice ectopically expressing Dok-7 proteins with mutations in the COOH-terminal moiety. Also, we established knock-in mice that have a mutation associated with the majority of patients with *DOK7* myasthenia. Some of these mice showed NMJ disorders and the effects of these mutations in vivo are under comprehensive investigation.

3. Preparation of an Adeno-associated virus vector for Dok-7 expression.

Arimura, S., Okada, T.[1], Chiyo, T.[1], Tezuka, T., Takeda, S.[1], and Yamanashi, Y.: [1]Department of Molecular Therapy, National Institute of Neuroscience.

As mentioned above, *DOK7* myasthenia is associated with impaired NMJ formation due to decreased ability of Dok-7 to activate MuSK in myotubes at least in part. Interestingly, in vivo overexpression of Dok-7 increased MuSK activation and promoted NMJ formation in the correct, central region of the skeletal muscle. Because these genetically manipulated mice did not show any sign of disease, overexpression of Dok-7 in the skeletal muscle of patients with *DOK7* myasthenia might ameliorate NMJ formation and muscle weakness. As an initial step toward a potential therapy, we generated an Adeno-associated virus-based vector, which strongly expressed Dok-7 in myotubes and induced AChR cluster formation. Mice with mutations in the *dok-7* gene have been infected with the vector. The effects of these treatments are under investigation.

4. Lrp4 antibodies in patients with myasthenia gravis.

Tezuka, T., Motomura, M.[1], Hoshi, T., and Yamanashi, Y.: [1]First Department of Internal Medicine, Graduate School of Biomedical Sciences, Nagasaki University.

Myasthenia gravis (MG) is an autoimmune disease of the NMJ. About 80% of patients with generalized MG have AChR antibodies, the presence of which is a causative factor for the disease, and a variable proportion of the remaining patients (0-50% throughout the world) have MuSK antibodies. However, diagnosis and clinical management remain complicated for patients who are negative for

MuSK and AChR antibodies. Given the essential roles and postsynaptic localization of Lrp4 in the NMJ, we hypothesized that Lrp4 autoantibodies might be a pathogenic factor in MG. To test this hypothesis, we developed a luminescence-based method to efficiently detect serum autoantibodies to Lrp4 in patients, and found that 9 patients were positive for antibodies to the extracellular portion of Lrp4 from a cohort of 300 patients with AChR antibody-negative MG. 6 of these 9 patients with Lrp4 antibody-positive MG were also negative for MuSK antibodies, and generalized MG was diagnosed in all 9 patients, who showed severe limb muscle weakness or progressive bulbar palsy or both. Thymoma was not observed in any of these patients, unlike the situation in patients with AChR antibody-positive MG. Furthermore, we confirmed that serum antibodies to Lrp4 recognize its native form and inhibit binding of Agrin to Lrp4, which is crucial for NMJs. Also, we found that Lrp4 autoantibodies were predominantly comprised of IgG1, a complement activator, suggesting the potential for these antibodies to cause complement-mediated impairment of NMJs. Together, our findings indicate the involvement of Lrp4 antibodies in the pathogenesis of AChR antibody-negative MG. Following this study, two groups in Germany and USA reported respectively that about 50% and 10% of MG patients, who were negative for both MuSK and AChR antibodies, were positive for antibodies to Lrp4 (*J. Neurol.*, 259: 427-435, 2012; *Arch. Neurol.*, 69: 445-451, 2012). Consistent with our observation, these groups reported that some Lrp4 antibodies from patients suppressed Agrin-induced AChR cluster formation in cultured myotubes. We are further investigating the etiology and pathology of Lrp4 antibody-positive MG.

5. Anti-inflammatory roles of Dok-family proteins.

Mashima, R., Arimura, S., Waseda, M., Kajikawa, S., Oda, H.[1], Nakae, S.[2], and Yamanashi, Y.: [1]Department of Pathology, Tokyo Women's Medical University, [2]Laboratory of Systems Biology, IMSUT.

Dok-family proteins can be classified into three subgroups based on their structural similarities and expression patterns; namely, 1) Dok-1, -2, and -3, which are preferentially expressed in hematopoietic cells, 2) Dok-4, -5, and -6, which are preferentially expressed in non-hematopoietic cells, and 3) Dok-7, which is preferentially expressed in muscle cells. As mentioned above, Dok-1 and its closest paralog, Dok-2, recruit p120 rasGAP upon tyrosine phosphorylation to suppress Ras-Erk signaling. Although Dok-3 does not bind with p120 rasGAP, it also inhibits Ras-Erk signaling. Consistently, we previously demonstrated that Dok-1, Dok-2 and Dok-3 are key negative regulators of hematopoietic growth and survival signaling. For example, Dok-1, Dok-2, and Dok-3 cooperatively inhibit macrophage proliferation and *Dok-1*$^{-/-}$*Dok-2*$^{-/-}$*Dok-3*$^{-/-}$ (TKO) mice develop histiocytic sarcoma, an aggressive malignancy of macrophages. In addition, we found that these Dok proteins negatively regulate antigen receptor-mediated adaptive immune signaling as well as TLR4-mediated innate immune signaling. Therefore, we investigated roles of the Dok proteins in inflammatory responses, which are involved in both adaptive and innate immune systems.

We first examined the lung histopathology and bronchoalveolar lavage fluid (BALF) cellularity of TKO mice, because we previously found abnormal accumulation of macrophages, a sign of pulmonary inflammation. These tests demonstrated that spontaneous pulmonary inflammation in the mutant mice, but not in *Dok-1*$^{-/-}$*Dok-2*$^{-/-}$ or *Dok-3*$^{-/-}$ mice, with hallmarks of asthma, including eosinophilia, goblet cell hyperplasia, and subepithelial fibrosis. Consistently, TKO mice, but not the other mutants, showed increased airway hyperresponsiveness to methacholine inhalation, another hallmark of asthma. In addition, Th2-type cytokine concentrations in BALF were increased in TKO mice, suggesting asthma-like pulmonary inflammation. Together, our findings provide strong evidence that Dok-1, Dok-2, and Dok-3 cooperatively play critical anti-inflammatory roles in lung homeostasis. We are currently investigating involvement of the Dok proteins in other types of inflammation.

6. Proteomic analyses.

Tezuka, T., Hoshi, T., Arimura, S., Iemura, S.[1], Natsume, T.[1], Oyama M.[2], and Yamanashi, Y.: [1]National Institute of Advanced Science and Technology, Biological Information Research Center. [2]Medical Proteomics Laboratory, IMSUT

To gain insights into signaling mechanisms underlying a variety of physiological and pathophysiological events, including NMJ formation, tumorigenesis, and tumor metastasis, we have performed mass spectrometry-based proteomic analyses. We are investigating the roles of candidate proteins that appear to be involved in each of these biological events.

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Department of Cancer Biology

Division of Mathematical Oncology
腫瘍数理分野

Project Professor	Kazuhisa Ichikawa	
Project Researcher	Daisuke Oshima	
JSPS Research Fellowships for Young Scientists (PD)	Ayako Watanabe	
特任教授	理学博士	市 川 一 寿
特任研究員	博士(生命科学)	大 島 大 輔
特別研究員	博士(農学)	渡 部 綾 子

Intracellular space is crazy, because it is crowded with spatio-temporally organized organelles, and several ten thousands of proteins are interacting with each other. Events arising from these complicated and complex intracellular spaces are the basis for the cellular functions. Our goal is to elucidate underlying mechanisms for cellular functions by the methods of computational cell biology. Currently, there are two main topics. First one is the initial step of cancer cell invasion. Our computational simulation predicted the requirement of repetitive activation of MT1-MMP for the degradation of extracellular matrix (ECM). This prediction was combined with the experimental observations leading to the new model for the ECM degradation by MT1-MMP showing the importance of rapid turnover of MT1-MMP. Second one is the regulation of transcription factor NF-κB. We have found that the spatial parameters play a critical role for the pattern of NF-κB oscillation.

1. Spatio-temporal dynamics of MT1-MMP at invadopodia

Ayako Watanabe, Daisuke Hoshino[1], Naohiko Koshikawa[1], Takashi Suzuki[2], Motoharu Seiki[1] and Kazuhisa Ichikawa: [1]Division of Cancer Cell Research, Institute of Medical Science, University of Tokyo, [2]Division of Mathematical Science, Graduate School of Engineering Science, Osaka University

Focal degradation of extracellular matrix (ECM) is the first step in the invasion of cancer cells. MT1-MMP is a potent membrane proteinase employed by aggressive cancer cells. In our previous study, we reported that MT1-MMP was preferentially localized at membrane protrusions called invadopodia, where MT1-MMP underwent quick turnover. Our computer simulation and experiments showed that this quick turnover was essential for the degradation of ECM at invadopodia. We characterized the behavior of the model on the ECM-degrading activity of MT1-MMP. First, in our computational model, we found a very narrow transient peak in the activity of MT1-MMP followed by steady state activity. This transient activity was due to the inhibition by TIMP-2. The steady state activity of MT1-MMP decreased dramatically at higher TIMP-2 concentrations. Second, we evaluated the role of the narrow transient activity in the ECM degradation. When the transient activity was forcibly suppressed in computer simulations, the ECM degradation was heavily suppressed indicating the essential role of this transient peak in the ECM degradation. Third, we compared continuous and pulsatile turnover of MT1-MMP in the ECM degradation at invadopodia. The pulsatile insertion model showed basically consistent results with the continuous insertion model in the ECM degradation, and the ECM degrading efficacy depended heavily on the transient activity of MT1-MMP in both models. Unexpectedly, however, low-frequency/high-concentration insertions of MT1-MMP was more effective in ECM degradation than high-frequency/low-concentration insertions

even if the time-averaged amount of inserted MT1-MMP was the same. The present analysis and characterization of ECM degradation by MT1-MMP together with our previous report indicate a dynamic nature of MT1-MMP at invadopodia and the importance of its transient peak in the degradation of the ECM.

2. Roles of Spatial Parameters on the Oscillation of Nuclear NF-κB: Computer Simulations of a 3D Spherical Cell

Daisuke Oshima, Jun-ichiro Inoue[4] and Kazuhisa Ichikawa: [4]Division of Cellular and Molecular Biology, Institute of Medical Science, University of Tokyo

Transcription factor NF-κB is activated by various stimuli, and shuttles between cytoplasm and nucleus. Interestingly, nuclear NF-κB oscillates. This oscillation is implicated to be important for gene expressions, because it was shown that different oscillation pattern led to different expression of genes. Nearly forty reports have been published to date on the computational and mathematical models of the oscillation of NF-κB. These reports showed fairly good agreement with experimentally observed oscillations of nuclear NF-κB.

These computational models, however, are temporal or two-dimensional, and the discussion on spatial parameters, such as diffusion coefficient, cytoplasmic location of translation, and the volume ratio of nuclear to cytoplasmic space (N/C ratio) have not been involved or limited. Since these spatial parameters will be varied in different cell types or environment that a cell is facing, it is important to find relationships between spatial parameters and the oscillation patterns in three-dimensional (3D) cell. We constructed a 3D spherical computational cell-model for the nuclear NF-κB oscillation using A-Cell software. First, we found that the biochemical kinetic constants used in the temporal model cannot replicate the experimentally observed oscillation in 3D model. Thus, the parameters should be changed in the 3D model. Second, all spatial parameters, diffusion constants, the location of protein synthesis, and N/C ratio, affected to the oscillation pattern. Among them, N/C ratio largely altered the oscillation pattern showing larger N/C ratio and larger nuclear membrane area resulted in prolonged oscillation of nuclear NF-κB. Since N/C ratio is increased by the increase in the malignancy of cancer in many types of cancers, our results suggested that the increased N/C ratio in cancer cell is one of the possible alteration mechanisms for gene expression.

3. Parallelization of simulation software generated by A-Cell

Kazuhisa Ichikawa

A-Cell software is free software for simulations of cellular temporal and spatio-temporal models. We implemented the parallelization of A-Cell simulation program, which is automatically generated by A-Cell, for running on the super computer "K". Since automatically generated A-Cell simulation software depends on the model that A-Cell user constructed, the parallelization of simulation software should be robust for the change in the model. For example, shape of the 2D or 3D model is divided into many small compartments, but the shape is different in different models. We developed a method for automatic segmentation of compartments into groups balancing a load for each processing element (PE) with minimum communication between PEs. The parallelization depends on the number of compartments. By running simulations by changing number of compartments, we estimated the parallelization of more than 99.99% up to 32,768 PEs, when the number of compartments is 70,000,000.

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