Department of Cancer Biology

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

Associate Professor Naohiko Koshikawa, Ph.D.

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Membrane-type 1 matrix metalloproteinase 1 (MT1-MMP) is a potent modulator of the pericellular microenvironment and plays crucial roles in physiological and pathological settings in mammals. MT1-MMP mediates its biological roles through the cleavage of specific substrates on cell surface. However our knowledge of MT 1-MMP substrates remains very limited. Our goal is to understand the molecular mechanism of tumor malignant progression regulated by the pericellular proteolysis of MT1-MMP, and to develop novel diagnostic and therapeutic methods for malignant tumors.

1. Membrane type-1 matrix metalloproteinase promotes malignant tumor growth signals by pericellular proteolysis of cancer cells

Naohiko KOSHIKAWA, and Motoharu SEIKI

MT1-MMP is a potent proinvasive and growth promoting membrane protease in cancer. The roles of MT1-MMP are mediated by its pericellular proteolysis activity. Its major substrates are extracellular matrices. It also cleaves membrane proteins and modulates their biological activities in cancer progression. However, little is known about its cell surface substrates and identification of the substrates should enable a better understanding of its multiple biological functions. To identify its new substrates, we purified MT1-MMP complex in cancer cells and analyzed them by mass analysis. As a result, we found two membrane proteins. One was an ErbB ligand HB-EGF, and the other was receptor tyrosine kinase tentatively named NK-2. Processing of HB-EGF by MT1-MMP increased its mitogenic activity, whereas NK-2 processing caused a loss of its growth inhibitory activity, and consequently promoted invasive cancer growth *in vivo*. Co-expression of MT1-MMP and both proteins, and their cleaved fragments was frequently shown in invasive cancer tissues. Taken together, we demonstrated for the first time that MT1-MMP directly promotes invasive cancer growth by the pericellular proteolysis.

2. The phosphoinositide-binding protein ZF21 regulates ECM degradation by invadopodia

Daisuke Hoshino, Makoto Nagano, AnriSaitoh, Naohiko Koshikawa, Takashi Suzuki, Motoharu Seiki.

During the process of tumor invasion, cells require footholds on extracellular matrices (ECM) that are created by forming focal adhesions (FAs) using integrins. On the other hand, cells must degrade the ECM barrier using extracellular proteases including MMPs in the direction of cell movement. Degradation occurs at the leading edges or invadopodia of cells, which are enriched in proteases and adhesion molecules. Recently, we showed that the phosphoinositide-binding protein ZF21 regulates FA disassembly. ZF21 increased cell migration by promoting the turnover of FAs. In addition, ZF21 promotes experimental tumor metastasis to lung in mice and its depletion suppresses it. However, it is not known whether ZF21 regulates cancer cell invasion in addition to its activity on FAs. In this study, we demonstrate that ZF21 also regulates invasion

of tumor cells, whereas it does not affect the overall production of MMP-2, MMP-9, and MT1-MMP by the cells. Also, we observe that the ECM-degrading activity specifically at the invadopodia is severely abrogated. In the ZF21 depleted cells MT1-MMP cannot accumulate to the invadopodia and thereby cannot contribute to the ECM degradation. Thus, this study demonstrates that ZF21 is a key player regulating multiple aspects of cancer cell migration and invasion. Possible mechanisms regulating ECM degradation at the invadopodia are discussed.

Publications

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Department of Cancer Biology

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

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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 roles of cell adhesion in cancer invasion and metastasis. Genetic and epigenetic abnormalities involved in human tumors, including cholangiocarcinoma, adult T-cell leukemia, lung, breast, head and neck and urological cancers, are also being investigated.

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

Mika Sakurai-Yageta, Shigefumi Murakami, Takahiro Mimae, Akihiko Ito[1], Yumi Tsuboi, Anri Saito, Takeshi Ito, Yuki Hanaoka-Ikeda, Shuejen Shiu, Osman Wael, Yuki Kumagai, Hiroyuki Kogai, Takehiro Tsuchiya, Tomoko Maruyama, Hiromi Ichihara and Yoshinori Murakami: [1]Department of Pathology, Kinki University School of Medicine

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 acting as a tumor suppressor. In order to understand the intracellular signaling pathways activated by CADM1-mediated cell adhesion, we establisheda cell-based spreading assay to analyze the signaling pathway specifically activated by the *trans*-homophilic interaction of CADM1. In the assay, MDCK cells expressing exogenous CADM1 were incubated on the glass coated with a recombinant extracellular fragment of CADM1, and the degree of cell spreading was quantified by measuring their surface area. Assay screening of 104 chemical inhibitors with known functions revealed that LY 294002, an inhibitor of phosphoinositide 3-kinase (PI3K), efficiently suppressed cell spreading in a dose-dependent manner. Inhibitors of Akt and Rac 1, downstream effectors of PI3K, also partially suppressed cell spreading, while the addition of both inhibitors blocked cell spreading to the same extent as did LY294002. Furthermore, MPP3 and Dlg, membrane-associated guanylate kinase homolog (MAGuK) proteins, connect CADM1 with p85 of PI3K by forming a multi-protein complex at the periphery of cells. These results suggest that *trans*-homophilic interaction mediated by CADM1 activates the PI3K pathway to reorganize the actin cytoskeleton and form epithelial cell structure. We also examined dynamic regulation of CADM1, 4.1B and MPP3 proteins on the cell membrane using photobleaching assay in combination with a novel mathematical approach. Furthermore, possible cross-talk of CADM1 cascade with other known signal transduction pathways, including those driven by tyrosine kinases, is being elucidated. Possible roles of CADM1 in pulmonary emphysema, hormonal re-

lease in pancreatic islands, infectious bowel diseases, gut immune system and apoptosis induction triggered by disruption of cell adhesion are also investigated partly in collaboration with others.

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

Mika Sakurai-Yageta, Takeshi Ito, Hiroyuki Kogai, Siew-Pei Gan, Kanae Makiya, Masakane Muroi, Misaki Noguchi, Yui Okada, Tomoko Maruyama, Hiromi Ichihara, Akiteru Goto[2] and Yoshinori Murakami. [2]Department of Pathology, Akita University Graduate School of Medicine.

In contrast to a tumor suppressor function of CADM1 in epithelial cancers, CADM1 is overexpressed in adult T-cell leukemia (ATL), conferring an invasive phenotype that is characteristic to ATL. We also reported expression of a splicing variant of CADM1, v8/9, specific to small cell lung cancer (SCLC). A common epithelial variant, v8, and a SCLC-specific variant, v8/9, share 3 immunoglobulin(Ig)-like loops with 6 predicted *N*-glycosylation sites in their extracellular domains. By contrast, v8 and v8/9 contains 17 and 21 possible *O*-glycosylation sites of threonines, respectively, in each unique region between the third Ig-like loop and the transmembrane domain. To establish CADM1 as a diagnostic and therapeutic target of ATL or SCLC, we analyzed the biological significance of *N*- and *O*-glycosylation structure of CADM1 from ATL and SCLC using a cell-based assay for adhesion mediated by *trans*-homophilic interaction of CADM1, as well as a cell scattering assay triggered by HGF. Examination of 2 CADM1 variants and their mutants at one or multiple N-glycosylation sites from glutamine to alanine revealed that *N*-glycosylation at the 1st Ig-like loop of CADM1 played important roles in cell adhesion and also in cell scattering of MDCK. Mass spectrometric analyses of the *N*- and *O*-glycan structures of CADM1 are being investigated in collaboration with Shimadzu Co. Ltd. Specific antibody against the unique fragment of v8/9 is being generated, whereas lentiviral transfer system of shRNA against CADM1 is being prepared to establish CADM1 as a candidate molecular target for the diagnosis and treatment of SCLC and ATL. 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. Analyses of genetic and epigenetic alterations and genetic susceptibility in human tumors

Takeshi Ito, Mika Sakurai-Yageta, Akiteru Goto[2], Fumi Taira, Ken Akashi, Atsushi Kanatani, Hasaya Dokdang, Tomoko Maruyama, Hiromi Ichihara and Yoshinori Murakami

To unveil the molecular mechanisms of multistage carcinogenesis, genetic and epigenetic aberrations in key molecules in human tumorigenesis were examined in various cancers. The genetic susceptibility to individual cancers is also being investigated by genome-wide analysis of structural alterations. In the analysis of cholangiocarcinoma (CCA) that was developed on the basis of chronic infection of liver fluke (*Opisthorchis viverrini: Ov*) in Thailand, microarray analysis demonstrated that CCA with Ov showed enhanced expression of the genes involved in xenobiotic metabolism and chronic inflammatory responses, including cytokine signaling, whereas CCA without Ov showed enhanced expression of the growth factor signaling, such as HER2. These distinct features are already detected in the pre-cancerous bile duct epithelia. Moreover, Ov-associated CCA shows frequent mutation of the *KRAS*, *TP53*, *SMAD4*, and *CDKN2A* genes and preferential nucleotide substitutions from C:G to T:A, suggesting that mutation characteristics are similar to those of pancreatic ductal carcinoma but distinct from those of hepatocellular carcinoma. In the analysis of structural alterations of human genome in cancer, we focused on the copy number alteration (CNA), a new category of somatic alterations at the highly polymorphic sites of more than 1 kb, called copy number variation (CNV). We examined CNAs in breast, bile duct, bladder and head and neck cancers and found a number of CNAs on all chromosomes. Genetic and epigenetic alterations of various genes in human cancer and pre-cancerous lesion, such as pulmonary emphysema, are being investigated.

4. Molecular pathological analyses of human lung cancer

Daisuke Matsubara, Ibrahim Reem, Kana Makiya, Yui Okada, Toshiro Niki[3] and Yoshinori Murakami: [3]Department of Integrative Pathology, Jichi Medical University.

Lung cancer is a leading cause of cancer death in Japan. Understanding the molecular pathological characteristics of human lung cancer is prerequisite to overcome this malignant cancer. We have been interested in the epithelial-mesenchymal transition (EMT) observed in malignant cancer cells with invasive and metastatic ability. On the basis of our previous studies, protein arginine methyltransferase 5 (PRMT5) was identified as a candidate histone methyltransferase gene whose expression was correlated with epithelial mesenchymal transition by

microarray data analysis of 40 non-small cell lung carcinoma cell lines. Immunocytochemical analysis indicated that PRMT5 expression was frequently localized to the cytoplasms of E-cadherin-low and vimentin-high cancer cells, while it was predominant in the nuclei but was faint in the cytoplasms of E-cadherin-high and vimentin-low cancer cells. Immunohistochemical analysis of 130 lung adenocarcinoma cases showed cytoplasmic high expression of PRMT5 in 47 (36%), and nuclear high expression of PRMT5 in 34 (26%). High cytoplasmic PRMT5 expression was frequently observed in high grade subtypes such as solid adenocarcinoma component with low thyroid transcription factor-1 (TTF-1, master regulator of lung) and low cytokeratin 7 and E-cadherin (two markers for bronchial epithelial differentiation), while high nuclear PRMT5 expression was frequent in lepidic growth component, a low-grade subtype. Moreover, cytoplasmic expression of PRMT5 was significantly correlated with vessel invasion and poor prognosis. We have demonstrated the importance of PRMT5 expression, especially in the cytoplasmic expression, in the process of epithelial mesenchymal transition and loss of bronchial epithelial phenotype of lung adenocarcinoma.

5. Pathophysiological analyses of cell growth regulation mediated by enzymatic digestion through the membrane protease, MT1-MMP

Takeharu Sakamoto

A new project on investigating novel roles of hypoxia-inducible factor 1 (HIF-1) in normoxia condition of malignant tumor cells that express the membrane protease MT1-MMP has been started by Dr. Takeharu Sakamoto in collaboration with the members in the Division of Antibody, Vaccine & Experimental Therapy in IMSUT.

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Department of Cancer Biology

Division of Cellular and Molecular Biology
分子発癌分野

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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 acts as an E3 ubiquitin ligase to generate Lys63-linked polyubiquitin chains that are crucial for transducing signals emanating from the TNFR superfamily or 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, immune 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 is likely involved in the malignancy of tumors. Thus, we are also investigating the molecular mechanisms of the constitutive activation of NF-κB and how this activation leads to the malignancy of breast cancers.

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

Aya Kojima, Takao Seki, Mami Yamamoto, Noriyuki Ishibashi, Hironori Ohtsuki, 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. To further understand the molecular mechanisms of TRAF6-mediated NF-κB activation, we try to identify proteins that are ubiquitinated by TRAF6 upon stimulation in addition to TAK1. We took advantage of using the peptide that specifically binds K63-linked polyubiquitin chain to purify such proteins. We have confirmed that the peptide-based affinity column is useful for specific concentration of recombinant K63-linked polyubiquitin chain, suggesting that it also works for purification of the proteins of our interest.

2. Analysis of the physiological role of p47

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

p47 (also known as NSFL1C) is originally identified as a major cofactor of the cytosolic ATPase associated with various cellular activities p97, and the p47/p97 complex is required for the reassembly of Golgi stacks at the end of mitosis. We have previously reported that p47 is a novel negative regulator in IKK/NF-κB activation. Silencing of p47 enhanced IKK activation, NF-κB nuclear translocation, and its transcriptional activity. p47 interacted with NEMO only when NEMO was conjugated to polyubiquitin chains. Moreover, The enhanced accumulation of polyubiquitinated NEMO was observed in p 47-knockdown cells and in the lysosome inhibitor-treated cells, but not in the proteasome inhibitor-treated cells. Collectively, these results strongly suggest that p47 targets polyubiquitinated NEMO for lysosomal degradation, thereby negatively regulating NF-κB activation. To further investigate the physiological significance of p47, we tried to generate p47 knockout mice. Heterozygous knockout mice were viable and fertile, while homozygous knockout mice exhibited embryonic lethality. Then, p47-loxP-flanked mice were crossed with LysM-Cre knock-in mice to disrupt p47 specifically in macrophages. We obtained p47-deficient macrophages and are investigating the effect of p47 deficiency on NF-κB signaling pathway.

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

Yuri Shibata, Masaaki Oyama[1], Hiroko Kozuka-Hata[1] 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 the IKK complex in cytosolic extracts, it failed to activate the purified IKK complex. These results suggest that Tax requires intermediary molecules for IKK activation. To identify such molecules, we purified the Tax-IKK complex and analyzed its interacting proteins by mass spectrometry. We identified a novel Tax-IKK-interacting protein, whose knockdown resulted in decreased binding of Tax to the IKK complex. Correspondingly, knockdown of this protein inhibited the Tax-induced, but not the cytokine-induced IKK activation. Taken together, the novel Tax-IKK-interacting protein functions as an adaptor protein required for full IKK activation induced by Tax.

4. Molecular mechanism of RANK signaling in osteoclastogenesis

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

Osteoclasts are differentiated from hematopoietic stem cells, and play a crucial role in bone homeostasis in concert with osteoblasts. Excess formation or activation of osteoclasts leads to pathological bone resorption as observed in postmenopausal osteoporosis and bone destruction in rheumatoid arthritis. Therefore, it is essential to elucidate precisely the molecular mechanisms of osteoclastogenesis for understanding such bone diseases and developing drugs. Activation of signal transduction pathway emanating from receptor activator of nuclear factor-κB (RANK) is essential for osteoclastogenesis. This RANK signaling activates NF-κB and AP-1 through the E3 ubiquitin ligase TRAF6, and induces PLCγ2-mediated Ca^{2+} oscillation. Co-operation of these signals leads to the induction of NFATc1, a master transcriptional factor in osteoclastogenesis. We have previously identified a unique functional domain in the cytoplasmic region of RANK, named Highly Conserved domain in RANK (HCR), which does not share any significant homology with other proteins. The HCR functions as a platform for formation of signal complex including TRAF6, PLCγ2, and Gab2, and emanates

sustained RANK signaling, which is essential for the NFATc1 induction and osteoclastogenesis. In this year, we found another HCR-dependent event, which is likely to be involved in osteoclastogenic signal. To elucidate the molecular mechanism of the HCR-dependent events, we try to identify the binding protein to HCR by using proteomics approaches. In addition, we also try to identify the novel negative regulator of osteoclastogenesis based on the microarray analyses.

5. Involvement of A20 in the molecular switch that activates the non-canonical NF-κB pathway

Noritaka Yamaguchi[3], Masaaki Oyama[1], Hiroko Kozuka-Hata[1], and Jun-ichiro Inoue: [3]Graduate School of Pharmaceutical Sciences, Chiba University

The non-canonical NF-κB pathway is crucial for the immune system. A critical event in activation of the non-canonical pathway is the attenuation of NF-κB-inducing kinase (NIK) degradation, which is promoted by continuous polyubiquitination of NIK catalyzed by the NIK ubiquitin-ligase complex composed of cellular inhibitor of apoptosis protein 1 and 2 (cIAP1/2), TNF receptor-associated factor 2 (TRAF2), and TRAF3. However, the molecular mechanism of stimulation-dependent NIK stabilization remains poorly understood. Here, we show that A20, a ubiquitin-editing enzyme, promotes efficient activation of the non-canonical pathway independent of its catalytic activity. A20 directly binds to cIAP1 through the seventh zinc finger of A20, resulting in dissociation of the TRAF2/TRAF3 interaction, thereby inactivating the ligase complex to stabilize NIK. Given that A20 negatively regulates the canonical pathway, A20 is likely involved in the molecular switch that promotes the transition from canonical to non-canonical activation for proper control of the immune system.

6. NF-κB non-cell-autonomously regulates cancer stem cell populations in the basal-like breast cancer subtype

Mizuki Yamamoto[5], Sakura Wakinaga, Yuu Taguchi, Noritaka Yamaguchi[3], Taku Ito-Kureha[4], Kentaro Semba[5] and Jun-ichiro Inoue: [4]Cell Signal Unit, Okinawa Institute of Science and Technology, and [5]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. Patients with triple-negative breast cancer display the highest rates of early relapse of all patients with breast cancer. The basal-like subtype, a subgroup of triple-negative breast cancer, exhibits high levels of constitutively active NF-κB signalling. Here we show that NF-κB activation, induced by inflammatory cytokines or by epigenetically dysregulated NIK expression, cell-autonomously upregulates JAG1 expression in non-cancer stem cells. This upregulation stimulates NOTCH signalling in cancer stem cells *in trans*, leading to an expansion of cancer stem cell populations. Among breast cancers, the NF-κB-dependent induction of JAG1 and the NOTCH-dependent expansion of the CSC population occur only in the basal-like subtype. Collectively, our results indicate that NF-κB plays a non-cell-autonomous role in regulating cancer stem cell populations by forming intratumoural microenvironments composed of JAG1-expressing non-cancer-stem cells with a basal-like subtype.

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

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

T-cell clones that have high avidity for self-antigens are eliminated during their development in the thymus. Such mechanism -clonal deletion is one of the main mechanisms to maintain T-cell tolerance to self-antigens. Self-antigens are predominantly expressed and presented by epithelial cells (TECs) and dendritic cells. Among them, 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 shows 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 addition to RANK and CD40, lymphotoxin β receptor (LtβR) is involved in mTEC development. However, the role of LtβR on mTEC development remains elusive. We are currently investigating its role in embryonic development of mTECs.

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Department of Cancer Biology

Division of Genetics
腫瘍抑制分野

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The major interest of this division is in molecular signals that regulate a variety of cellular activities. Our aim is to address how dysregulated 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 protein Dok-7.

Ueta, R., Sasaki Y., Ikegami, T., 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 region of synaptic specialization on the muscle). 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 investigating 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.

Arimura, S., Tezuka, T., Miyoshi, S., Hoshi, T., 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.

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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.

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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. Recently, it was also reported that antibodies to Lrp4 induced MG in model animals (*J. Clin. Invest.*, 123: 5190-5202, 2013). We are further investigating the etiology and pathology of Lrp4 antibody-positive MG.

5. Roles of Dok-1 to Dok-6.

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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*$^{-/-}$ mice develop histiocytic sarcoma, an aggressive malignancy of macrophages. In addition, we found that these Dok proteins cooperatively play critical anti-inflammatory roles in lung homeostasis. We are investigating roles of Dok-1 to Dok-6 in other organs.

6. Role of Mesdc2 in postsynaptic specialization in myotubes.

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To gain insights into signaling mechanisms underlying NMJ formation, we have performed mass spectrometric analysis of Lrp4-binding proteins and found the chaperon Mesdc2 as a candidate. We confirmed their binding in cells, and revealed that Mesdc2 bind selectively to the lower molecular mass form of Lrp4 (lower Lrp4) but not to the upper, more glycosylated form (upper Lrp4). Although the Mesdc2 binds to lower Lrp4, forced expression of Mesdc2 increased upper Lrp4, implying a role for Mesdc2 in the Lrp4 glycosylation, which might facilitate the receptor's cell surface expression. Indeed, we found that down regulation of Mesdc2 expression in cultured myotubes suppressed cell-surface expression of Lrp4, or upper Lrp4 more specifically. Furthermore, down regulation of Mesdc2 also inhibited Agrin-induced postsynaptic specialization in myotubes, which requires binding of Lrp4 to its extracellular ligand, the neural Agrin. Together, these findings demonstrated that Mesdc2 plays a key role in Lrp4-dependent postsynaptic specialization probably by promoting glycosylation and cell-surface expression of Lrp4 in myotubes.

7. Proteomic and genomic analyses.

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To gain insights into signaling mechanisms underlying a variety of physiological and pathophysiological events, including NMJ formation, tumorigenesis, and tumor metastasis, we have performed proteomic and genomic analyses. We are investigat-

ing the roles of candidate proteins and genes that appear to be involved in each of these biological events.

8. Screening of chemical compound libraries.

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In addition to the omics analyses described above, we have performed high throughput screenings of chemical libraries, aiming to intervene in pathogenic signals or to gain insights into signaling mechanisms underlying a variety of biological events. We continue the ongoing screenings to collect appropriate hit compounds.

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Intracellular space is crowded with spatio-temporally organized organelles, and huge number of proteins are interacting in this complex space. Events arising from this complex system are the basis for the cellular functions. Dysregulation of spatio-temporally organized organelles and protein-protein interactions causes various diseases. Our goal is to elucidate mechanisms for cellular functions arising from these spatio-temporal interactions by a method of computer simulation of a cell. We are focusing on the following topics: regulation of transcription factor NF-κB, the initial step of cancer invasion, and development of a new methods for the computation of spatio-temporal dynamics in a realistic intracellular space.

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

Daisuke Ohshima and Kazuhisa Ichikawa

The transcription factor NF-κB shuttles between the cytoplasm and the nucleus, and the nuclear NF-κB is known to oscillate with a cycle of 1.5-2.5 h by the application of external stimuli. Oscillation pattern of NF-κB is implicated to regulate the gene expression profile. Previously, we found that the oscillation pattern of nuclear NF-κB in our computational 3D spherical cell is regulated by spatial parameters such as nuclear to cytoplasmic volume ratio, nuclear transport, locus of protein synthesis, and diffusion coefficient. We have analyzed our 3D model and found an important biological implication for the regulation of oscillation pattern by diffusion coefficient of proteins. Our analyses showed that the "reset" of nuclear NF-κB, which was the returning of nuclear NF-κB to the same or lower than the initial level, was crucial for the oscillation, which was confirmed by the flux analysis. In addition we found that the distant cytoplasmic location from the nucleus acted as a "reservoir" for storing newly synthesized IκB. When diffusion coefficient of proteins was large ($\geq 10^{-11}$ m^2/s), larger amount of IκB was stored in the "reservoir" with a large flux by diffusion. Subsequently, stored IκB diffused back to the nucleus, where nuclear NF-κB was "reset" to the initial state. This initiated the next oscillation cycle. When diffusion coefficient was small ($\leq 10^{-13}$ m^2/s), oscillation of nuclear NF-κB was not observed because of a smaller amount of stored IκB in the "reservoir" and incomplete "reset" of nuclear NF-κB. If the diffusion coefficient for IκB was increased to 10^{-11} m^2/s keeping other proteins in 10^{-13} m^2/s, the oscillation was rescued confirming the "reset" and "reservoir" hypothesis. In addition, we found altered effective value of diffusion coefficient by diffusion obstacles. It is known that mitochondria are crowded around the nucleus upon hypoxia in pulmonary artery endothelial cells. Furthermore, the distribution of mitochondria is also changed by viral infection. Both hypoxia and viral infection activate NF-κB. Our findings strongly suggest that the organelle crowding seen in stressed cells changes the oscillation pattern of NF-κB by controlling effective diffusion coefficient.

2. Dynamics of cell-cell adhesion by CADM1

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CADM1 (Cell adhesion molecules-1) is a cell-cell adhesion molecule and is a tumor suppressor belonging to an immunoglobulin superfamily cell adhesion molecule. CADM1 forms a complex with an actin binding proteins, protein 4.1s, and scaffolding molecules, membrane-associated guanylate kinases (MAGuKs), including MPPs. We investigated dynamic regulation of the CADM1-4.1B-MPP3 complex in the mature cell-cell adhesion of Madin-Darby canine kidney (MDCK) cells. FRAP measurements and mathematical analysis revealed a different time constants for CADM1 and other proteins (4.1B and MPP3) in the complex suggesting that there is different dynamics among CADM1 and other proteins in addition to that as a complex.

Next we constructed a mathematical model for CADM1 dynamics at cell-cell adhesion site. We searched a possible mechanisms and parameter sets accounting for our observation. By simulating several models with varying parameters (rate constants for binding and diffusion coefficients), we have found a model and a parameter set that can account for the observation.

3. Simulations of signal transduction in true intracellular space (TiCS)

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Cell is a three-dimensional (3D) entity with complex and complicated structures of organelles. Spatial parameters, such as effective value of diffusion coefficient, organelles volume, surface area, density, distribution and population, and their shape could alter the signal transduction in a cell. In fact, it is known that the size of the nucleus is larger at malignant cancer cells, and the density and area of nuclear pore are also increased in malignant cancer cells. It is also known that the shape of the nucleus is aberrant in progeria patients. Thus, these spatial parameters will possess some role on the intracellular signal transduction. The next important step toward the elucidation of cellular functions is to reveal roles of spatial parameters. To this purpose, we imaged serial section of a mouse hepatocyte by scanning electron microscopy. Organelles were extracted from 2D images of 500 serial sections. We developed software for the segmentation of nucleus and mitochondria and analyzed images from cancer cells. LLE (largest Lyapunov exponent), which is a measure of chaotic behavior of the system, of images from cancer cells has a positive value suggesting the chaotic nature of the images. In addition, LLE of cancer cells was larger than normal cells. The LLE of images from natural scene (pictures of mountains and cities) was much smaller than those from normal and cancer cells.

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