Department of Cancer Biology

Division of Oncology
癌細胞シグナル研究分野

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Protein-tyrosine kinases are important not only for the development of malignant tumors but also for the regulation of growth and function of normal cells. Our current interest is to characterize cell signaling downstream of protein-tyrosine kinases that are relevant respectively to cancer development and to neuronal function. We are also interested in protein kinase signaling and chromosome dynamics that are involved in the regulation of cell cycle progression and cell

1. The biological role of Tob family proteins and CCR4/Not complex

Toru Suzuki, Shin-ichi Ogawa, Akinori Takahashi, Xue Li, Akihiko Miura, Chisato Kikuguchi and Tadashi Yamamoto

By screening a cDNA expression library with c-ErbB-2 protein, we identified Tob which encodes a 45kDa protein displaying homology with the growth suppressing proteins, Btg1 and Btg2/Pc3. We have also identified and characterized *tob2* and *ana* that are homologous to *tob* gene. These proteins compose a functionally related anti-proliferative protein family, called the Tob/Btg family.

To elucidate the physiological function of the Tob/Btg family proteins, we generated mice lacking *tob* (i), *tob2* (ii), or *ana* (iii). (i) *tob*-deficient mice ($tob^{-/-}$) had a greater bone mass resulting from increased number of osteoblasts in comparison with wild-type mice. We also found that aged $tob^{-/-}$ mice develop a variety of tumors. (ii) In contrast, mice lacking *tob2* had decreased bone mass, and the number of osteoclasts which are differentiated from bone marrow cells was increased. Furthermore, expression of *RANKL* mRNA in stromal cells was increased in the absence of Tob2 and decreased in the presence of Tob2. Tob2 interacted with vitamin D(3) receptor (VDR), suggesting its involvement in vitamin D(3) receptor-mediated regulation of transcription. In conclusion, Tob2 negatively regulates formation of osteoclasts by suppressing RANKL expression through its interaction with VDR. (iii) Unlike the other Tob family proteins, Ana is specifically expressed in type II alveolar epithelial cells. Since lung adenocarcinoma is thought to be mainly derived from type II alveolar epithelial cells, Ana may be involved in development of lung tumor. Indeed, *ana*-deficient mice developed spontaneous tumors including lung adenocarcinoma. We also found that expression of *ana* gene was largely reduced in almost all of the lung cancer cell lines and clinical samples of lung adenocarcinoma examined. These data suggested that downregulation of *ana* gene might be responsible for lung adenocarcinoma progression.

As other biological functions of Tob family

proteins, we obtained several evidences showing that Tob is involved in DNA damage response. UV-induced stress promotes proteasome-dependent degradation of Tob, triggering an apoptotic signal. Whereas expression of the degradation-resistant Tob suppressed UV-induced apoptosis, suppression of Tob by small-interfering RNA resulted in frequent induction of apoptosis irrespective of the presence of functional p53 even at UV doses that do not promote Tob degradation. Thus, proteosomal clearance of Tob provides a novel p53-independent pathway for UV-induced apoptosis. Furthermore, we have identified several molecules which are responsible for UV-induced Tob stabilization. Those might contribute to keep cell viability when DNA damage is not severe. We also found that expression of Tob and Tob2 changes depending on the state of the adipocyte differentiation and suppression of the proteins results in impairment of the differentiation. We start to understand the molecular mechanism by which Tob and Tob2 regulate the adipocyte differentiation.

Other studies to establish biological significance of the *tob* family members are following. (i) We purified Tob-containing complexes from HeLa cells that stably express Flag epitope-tagged Tob by immunoprecipitation with anti-Flag antibody. Mass spectrometric analysis showed that CNot1, CNot2, CNot3, CNot6, CNot7 and CNot9 were included in the Tob complex. All the molecules form a large protein complex which is conserved from yeast to humans, called CNot complex. The yeast CNot complex exists in two different forms, 2.0 MDa and 1.2 MDa, and both forms share the following subunits; Not proteins (Not1p to Not5p), Ccr4p, Caf1p, Caf40p, and Caf130p. Ccr4p and Caf1p are shown to have the mRNA deadenylase activity, thus regulating the amount of cytoplasmic mRNA. The mammalian orthologs of yeast Ccr4p and Caf1p, CNot6 and CNot7 also have the deadenylase activity. We showed that Tob suppresses the deadenylase activity of CNot complex at least *in vitro*. Although the CNot complex possesses mRNA deadenylase activity, thus regulating the stability of mRNAs, biological roles of the complex in mammals are largely unknown. (ii) We identified CNot6L, which is homologous to yeast Ccr4 and mammalian CNot6. CNot6L forms a multi-subunit complex similar to the yeast CNot complex. Suppression of CNot6L by RNA interference (RNAi) results in growth retardation of NIH3T3 cells accompanied by elevation of both $p27^{Kip1}$ mRNA and $p27^{Kip1}$ protein. Reintroduction of wild-type CNot6L but not mutant CNot6L lacking deadenylase activity restores the growth of CNot6L-depleted NIH3T3 cells. The data suggest that CNot6L regulates the cell growth in a manner dependent on its deadenylase activity. Our findings suggest that CNot6L regulates the turnover rate of specific target mRNAs. It should be elucidated how the CNot deadenylase complex recognize the specific mRNAs. (iii) We examine the effect of RNAi-mediated knock down of the other components of the CNot complex on cell proliferation and other biological phenomenon. We found that depletion of CNot1 or CNot2 resulted in extensive cell death. The cell death occurred caspase-dependent manner, indicating apoptosis. In contrast, depletion of CNot3 resulted in impaired M-phase checkpoint response due to the elevated *mad1* mRNA. (iv) To examine the biological functions of CNot complex, we generated gene-engineered mice lacking *cnot 3, cnot6, cnot6L, cnot8*, or *cnot9* gene. We have already obtained the data that most of the components in the complex are involved in embryonic development and energy homeostasis. Namely, $cnot3^{-/-}$ and $cnot9^{-/-}$ mice show an embryonic lethality and $cnot3^{+/-}$ and $cnot6L^{-/-}$ mice show both the impaired energy homeostasis and the resistance to high-fat diet-induced obesity. To understand the mechanism by which each molecule in the complex regulates such biological events, expression change of various mRNAs should be monitored. Several approaches such as expression microarray analysis, Real-time PCR, and Northern blot analysis are ongoing. To support the idea that CNot complex targets specific group of mRNA for deadenylation, a subset of mRNA (not all) are increased in each gene-engineered mice. To determine whether those mRNA are true targets of CNot complex and to elucidate the molecular mechanism by which CNot complex recognizes specific mRNAs are included in our near future works.

2. Roles of protein kinases in the central nervous system

Takanobu Nakazawa, Kazumasa Yokoyama, Takeshi Inoue, Naosuke Hoshina, and Tadashi Yamamoto

The Src-family protein-tyrosine kinases are implicated in various neural functions such as formation of neural network, myelination, and synaptic plasticity. To analyze the roles of Src and Fyn, we have been focusing on various substrates of these kinases, including *N*-methyl-D-aspartate (NMDA) type of ionotropic glutamate receptors. Our own studies have shown that GluN2A and GluN2B subunits of NMDA receptors, which play important roles in learning,

memory formation, and emotional behavior, are the major substrates of Fyn and Src. We identified Tyr-1472 phosphorylation on GluN2B and Tyr-1325 phosphorylation on GluN2A as the major tyrosine phosphorylation site of GluN2B and GluN2A, respectively. Using the knock-in mouse lines expressing mutant GluN2B with a Tyr-1472-Phe (Y1472F) mutation or expressing mutant GluN2A with a Tyr-1325-Phe (Y1325F) mutation, we showed that Tyr-1472 phosphorylation is a key mediator of fear-related learning in the amygdala and that Tyr-1325 regulates depression-related behavior. Furthermore, we found that Tyr-1472 phosphorylation regulates anxiety-like behavior and CRF expression in the amygdala. Recently, we also found that Tyr-1472 phosphorylation is involved in thermal nociception in mice. We are now doing further characterization of the role of these phosphorylation events *in vivo.*

In parallel of these studies, to uncover Src- and Fyn-mediated signaling pathways, we have been trying to identify binding partners and substrates of these kinases in the brain using solid-phase phosphorylation screening, yeast two-hybrid screening, and proteomic approaches. As a result, we have identified a number of putative mediators of Fyn- and Src-mediated signaling, including NYAP, FAK, p250 GAP, TCGAP, Nogo-A, and RhoGEFs. Among these proteins, we demonstrated that NYAP family proteins are the most heavily tyrosine-phosphorylated proteins in the developing neuron. We generated NYAP family knockout mice and demonstrated that the NYAP family plays a pivotal role as the central scaffold in the PI3K signaling pathway. We also generated TCGAP knockout mice and found that TCGAP regulates neural morphogenesis *in vivo.*

In database search for novel protein-tyrosine kinases, we identified a kinase that is highly expressed in the brain and termed it as BREK (Brain-Enriched Kinase). It turned out that BREK is the same as AATYK2/LMTK2 found at the almost same time by others. BREK is a member of a family consisting of AATYK1/LMTK1, AATYK2/BREK/LMTK2, and AATYK3/LMTK3. BREK has also been termed as KPI-2/CPRK. All family members are predominantly expressed in the brain. We showed that BREK plays a role in NGF signaling in PC12 cells, suggesting that BREK is involved in neural development and function in early postnatal development. To further investigate the physiological role of BREK family kinases, we focused on LMTK3 because of its high expression in the brain. We generated LMTK3 knockout mice and found that LMTK3 as well as BREK regulates higher brain functions.

3. Roles of chromokinesin Kid and mitotic kinases in execution of cell division

Miho Ohsugi, Noriko Tokai-Nishizumi, Fukashi Inoue, Kenji Iemura, Tsubasa Ohashi, Tetsuhiro Shimodaira, Shou Soeda, Kaori Yamada and Tadashi Yamamoto

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.

i) The chromokinesin Kid

The human chromokinesin Kid/Kinesin-10 is a member of the chromosome-associated kinesin family identified in our lab in 1996. Kid has been implicated in generating the polar ejection force that pushes the chromosome arms away from the spindle poles toward the spindle equator, as well as in the maintenance of spindle length during prometaphase and metaphase. We previously showed that chromosome localization of Kid during prometaphase and metaphase requires Cdc2/cyclin B-mediated phosphorylation on Thr463. In addition, Importin α directly associates with Kid via nuclear localization signals (NLSs) and Ran-GTP-mediated dissociation of importin α/β from Kid promotes the accumulation of Kid on chromosomes. During anaphase and telophase, Kid is localized in the interstices between adjacent chromosomes and contribute to the tight clustering of anaphase chromosomes (anaphase chromsome compaction). Kid deficiency often leads to micro- or multinuclear formation at oocyte meiosis II and the first couple of mitoses after fertilization, causing embryonic death in mice. Later somatic mitoses are not fatally affected by the absence of Kid, suggesting that cell divisions under significant influence of the maternal factor specifically require Kid to prevent formation of multinucleated cells. 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.

ii) Mitotic kinase Plk1 and its substrates

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 Kizuna (Kiz). We showed that 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 (γTuRCs) 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 addition, we are currently investigating other newly identified substrates of Plk1, which will uncover the molecular mechanisms underlying the Plk1-mediated control of cell divisions and oncogenesis.

iii) Chromosome dynamics in early mice embryo

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 and some nuclear proteins in early mouse embryos. We also conduct comperehensive transcriptome analyses of unfertilized mouse oocytes and 1- to 8-cell embryos.

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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. Regulation of Hypoxia-inducible Factor-1 by MT1-MMP via a non-proteolytic mechanism

Takeharu Sakamoto, Toshiro Hara, Seiko Yoshino, Kohei Mimura, Jane S. Weng, Yuka Takahashi, and Motoharu Seiki

Hypoxia inducible factor-1 (HIF-1) is a key transcription factor required for cellular adaptation to hypoxia, although its physiological roles and activation mechanisms during normoxia have not been studied sufficiently. The Warburg effect, which is a hallmark of malignant tumors that is characterized by increased activity of aerobic glycolysis, accompanies activation of HIF-1 during normoxia. Besides tumor cells that have multiple genetic and epigenetic alterations, normal macrophages also use glycolysis for ATP production by depending upon elevated HIF-1 activity even during normoxia. We recently found that activity of factor inhibiting HIF-1 (FIH-1) is specifically suppressed in macrophages by a nonproteolytic activity of membrane type-1 matrix metalloproteinase (MT1-MMP/MMP-14). Thus, MT1-MMP expressed in macrophages plays a significant role in regulating HIF-1 activity during normoxia. In the light of this finding, we examined here whether MT1-MMP contributes to the Warburg effect of tumor cells. All the tumor cell lines that express MT1-MMP exhibit increased glycolytic activity, and forced expression of MT1-MMP in MT1-MMP-negative tumor cells is sufficient to induce the Warburg effect. The cytoplasmic tail of MT1-MMP mediates the stimulation of aerobic glycolysis by increasing the expression of HIF-1 target genes. Specific intervention of the MT1-MMP-mediated activation of HIF-1 in tumor cells retarded tumor growth in mice. Systemic administration of a membrane-penetrating form of the cytoplasmic tail peptide in mice to inhibit HIF-1 activation competitively also exhibited a therapeutic effect on tumors.

2. A $p27^{kip1}$-binding protein, p27RF-Rho, promotes cancer metastasis via activation of RhoA and RhoC

Daisuke Hoshino, Ayaka Yoshida, Naohiko Koshikawa and Motoharu Seiki

Rho proteins control diverse cellular functions by regulating actin polymerization and gene expression. In particular, the expression of RhoA and RhoC is frequently associated with malignant tumors and plays a key role in invasion and metastasis. However, the identity of the upstream factors that regulate the metastasis-promoting activities of the Rho proteins is not clear. We recently identified a factor that regulates the activation of RhoA, which we termed p27RF-Rho ($p27^{kip1}$ releasing factor from RhoA) (Hoshino et al. JBC. 40: 27315, 2009). The cell cycle regulator $p27^{kip1}$ inhibits RhoA activation when localized to the cytoplasm, and p27RF-Rho antagonizes this activity. In this study, we evaluated whether p27RF-Rho regulates the pro-metastatic activity of RhoA and RhoC in mouse and human tumor cells. We first analyzed the expression of these proteins in metastatic (F10) and non-metastatic (F0) variants of the B16 melanoma cells. F10 cells expressed greater levels of RhoA, RhoC, and p27RF-Rho than the non-metastatic F0 cells. Depletion of p27RF-Rho in F10 cells reduced the activation of both RhoA and RhoC. Consistent with this reduced Rho activity, cell adhesion to the extracellular matrix and pericellular proteolysis mediated by invadopodia were both also decreased. Depletion of any of p27RF-Rho, RhoA, or RhoC in F10 cells markedly suppressed lung metastasis of cells injected into the mouse tail vein. Knockdown of p27RF-Rho expression in mouse and human tumor cells affected an early step of experimental lung metastasis, presumably reflecting inefficient extravasasion and subsequent invasion. Thus, p27RF-Rho is a key upstream regulator of RhoA and RhoC that controls spreading of tumor cells.

3. ZF21 regulates focal adhesion disassembly and cell spreading

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Directional migration of adherent cells on an extracellular matrix requires repeated formation and disassembly of focal adhesions (FAs). We have identified ZF21 as a regulator of disassembly of FAs and cell migration, and increased expression of the gene has been linked to metastatic colon cancer. ZF21 is a member of a protein family characterized by the presence of the FYVE domain, which is conserved among Fab1 p, YOPB, Vps27p, and EEA1 proteins, and has been shown to mediate the binding of such proteins to phosphoinositides in the lipid layers of cell membranes. ZF21 binds multiple factors that promote disassembly of FAs such as FAK, beta-tubulin, m-calpain, and SHP-2. ZF21 does not contain any other known protein motifs other than the FYVE domain, but a region of the protein C-terminal to the FYVE domain is sufficient to mediate binding to beta-tubulin. In this study, we demonstrate that the C-terminal region is important for the ability of ZF21 to induce disassembly of FAs and cell migration, and to promote an early step of experimental metastasis to the lung in mice. In light of the importance of the C-terminal region, we analyzed its ternary structure using NMR spectroscopy. We demonstrate that this region exhibits a structure similar to that of a canonical pleckstrin homology domain, but that it lacks a positively charged interface to bind phosphatidylinositol phosphate. Thus, ZF21 contains a novel non-canonical PH-like domain that is a possible target to develop a therapeutic strategy to treat metastatic cancer.

4. MT1-MMP activates HB-EGF in ovarian carcinomas

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Increased expression of heparin-binding EGF-like growth factor (HB-EGF) and membrane-type matrix metalloproteinase-1 (MT1-MMP) is frequently associated with various types of malignant tumor. HB EGF-like growth factor has been reported to promote the malignant progression of ovarian carcinoma. Based on this finding, inhibition of HB-EGF activity with CRM 197 is now under phase I clinical evaluation. On the other hand, MT1-MMP expressed in ovarian carcinoma cells is thought to promote invasion and growth of tumor cells by degrading the extracellular matrix. However, we recently demonstrated that co-expression of MT1-MMP and HB-EGF in gastric carcinoma cells leads to cleavage of HB-EGF within its N-terminal heparin-binding region, converting it into a potent heparin-independent growth factor. In this study, we evaluated the importance of regula-

tion of HB-EGF by MT1-MMP in clinical samples of ovarian carcinoma. We detected co-expression of HB-EGF and MT1-MMP in clear cell ovarian carcinoma tissues, particularly at the invasion front and in tumor cells that had disseminated into the ascites, whereas HB-EGF alone was expressed in non-invasive borderline ovarian tumor tissue. Furthermore, a soluble HB-EGF fragment that corresponds to that processed by MT1-MMP was detected in malignant ascites obtained from patients with metastatic ovarian carcinoma. Ovarian carcinoma cells that express MT1-MMP and HB-EGF exhibited enhanced cell growth in a 3D-collagen matrix and anchorage-independent growth in suspension. These results indicate that MT1-MMP co-expressed with HB-EGF in ovarian carcinoma cells potentiates the activity of HB-EGF to promote invasive tumor growth and spreading *in vivo*.

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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 role of cell adhesion in cancer invasion and metastasis. To this end, an immunoglobulin superfamily cell adhesion molecule, CADM1/TSLC1, and its cascade were identified and are being characterized. Genetic and epigenetic abnormalities involved in human tumors are also being investigated.

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

Mika Sakurai-Yageta, Yumi Tsuboi, Shigefumi Murakami, Mitsuru Hagiyama, Takeshi Ito, Yuki Ikeda, Suejen Shiu, Hiroki Nakaoka, Yuki Kumagai, Tomoko Masuda, Naoki Ichiyanagi, Hiroyuki Kogai, Azusa Yanagawa, Akiteru Goto, Akihiko Ito[1], Yusuke Nagara[2], Shoichi Ishiura[2], and Yoshinori Murakami: [1]Department of Pathology, Kinki University School of Medicine, [2]Department of Life Sciences, Graduate School of Arts and Sciences, University of Medicine,[2]

CADM1/TSLC1 is an immunoglobulin superfamily cell adhesion molecule and primarily involved in epithelial cell adhesion, whereas its expression is often lost in various cancers in their advanced stages. In order to provide possible molecular tools to replace CADM1 expression in cancer cells, molecular mechanism of transcriptional regulation of the *CADM1* gene is investigated. The expression of the *Cadm1* gene is induced during the neural differentiation of murine embryonal carcinoma P19 cells by treatment with retinoic acid (RA). We demonstrate that suppression of CADM1 expression using RNAi interfered with P19 cell aggregation and reduced cell populations expressing MAP2 after RA treatment. Non-aggregated P19 cells were not differentiated into neurons, suggesting that CADM1 participates in neuronal differentiation of P19 *in vitro*. A luciferase assay localized an RA-responsive element to an around 90-bp fragment upstream of the translational start site. Within this fragment, we identified several putative binding sites for transcription factor Sp1, which showed enhanced transcriptional activity by RA. Moreover, a chromatin immunoprecipitation revealed that RARα was associated with a DNA fragment containing Sp1BS-1, while suppression of RARα expression using siRNA reduced the responsiveness of the CADM1 promoter to RA. These results suggest that Sp1 plays a critical role in RA-induced CADM1 expression through possible interaction with RARα in the neural differentiation of P19. On the other hand, CADM1 undergoes membrane-proximal cleavage called shedding. We have determined the cleavage site by LC/MS/MS and showed that ADAM10 and γ-secretase are re-

sponsible for proteolysis of CADM1 in collaboration with Graduate School of Arts and Sciences, University of Tokyo. To further investigate the physiological function of CADM1 cascade, additional binding proteins to CADM1, including a gap junction protein connexin 36, are identified by molecular biological and proteomic approaches. Dynamic regulation of CADM1 protein on the cell membrane is also being analyzed using photo-bleaching assay. In addition, roles of CADM1 in nerve-mast cell interaction were investigated in collaboration with Kinki University, School of Medicine.

2. Analyses of genetic and epigenetic alterations in human tumors

Taketo Kawai, Takahiro Mimae, Hideki Kuwano, Megumi Ishimura, Masayoshi Nagata, Yuka Takahashi, Yasuhiro Ebihara, Miwako Iwai, Mika Sakurai-Yageta, Akiteru Goto, Akihiko Ito[1] and Yoshinori Murakami

To understand the molecular features of multistage carcinogenesis in human, aberrations of CADM1and the molecules in its cascade, as well as other key molecules in human tumorigenesis, were examined in various cancers. In 67 breast cancers, loss or reduced expression of CADM1 and 4.1B protein was observed in 45 (67%) and 49 (73%) tumors, respectively, and associated with advanced pathological stages. Furthermore, aberrant expression of CADM1 and 4.1B was preferentially observed in invasive lesions in comparison with non-invasive lesions from the same specimen. These findings suggest that aberrant expression of CADM1 and 4.1B is involved in the progression of breast cancer, especially in invasion into the stroma and metastasis. On the other hand, immunohistochemical analysis of the kidney revealed that CADM4, another member of the CADM family protein, as well as 4.1B, was expressed not in the distal tubules but in the proximal tubules, which was the precursor cells of renal clear cell carcinoma (RCCC). Subsequent analysis of 40 renal clear cell carcinomas (RCCC) revealed that loss of CADM4 expression occurred in 28 (70%) tumors and was associated with advanced pathological stages, higher nuclear grade and vascular involvement. Moreover, introduction of CADM4 into RCCC cells, 786-O, suppressed tumor formation in nude mice. These results suggest that CADM4 functions as a tumor suppressor in RCCC. In addition, microarray analysis in combination with laser capture microscopy identified Nocth2 and Six1 as candidate oncogenes upregulated in early-stage lung adenocarcinoma. Genetic and epigenetic alterations of various cancer related genes in additional human tumors, including those from the lung, head and neck, bile duct and bladder, as well as thymomas, mesothelioma and sarcomas, are being investigated.

3. Functional analyses of CADM1 overexpression in highly metastatic tumors, including adult T-cell leukemia-lymphoma (ATL)

Shigefumi Murakami, Mika Sakurai-Yageta, Miwako Iwai, Akiteru Goto and Yoshinori Murakami:

We have previously reported in collaboration with others that CADM1 is overexpressed in adult T-cell leukemia-lymphoma (ATL) cells. Subsequent studies have demonstrated that CADM1 directly interacts with T-lymphoma invasion and metastasis 1 (Tiam1), a Rac-specific guanine nucleotide exchange factor (RacGEF), through its cytoplasic domain. This interaction induces lamellipodia formation through Rac activation in HTLV-I transformed cell lines, as well as ATL cell lines, suggesting that the CADM1-Tiam1 interaction promotes cell motility leading to tissue infiltration of leukemic cells in ATL patients. We have also identified that CADM1 is overexpressed in a subset of tumors with highly metastatic potentials. Down-stream pathways of CADM1 in these tumors are being analyzed. Furthermore, possible cross-talk of CADM1 cascade with other known signal transduction pathways is being examined by screening the inhibitors of CADM1-mediated cell adhesion and motility.

4. Analysis of human papilloma virus in human lung and esophageal cancer

Akiteru Goto and Masashi Fukayama[3]: [3]Department of Pathology, Graduate School of Medicine, University of Tokyo

To elucidate the role of human papilloma virus (HPV) in the development of lung and esophageal cancer, 485 lung and esophageal cancers (176 lung squamous cell carcinoma, 128 lung adenocarcinoma, 181 esophageal carcinoma) in eight institutions in Asia (Tokyo, Kochi, Kagoshima, and Okinawa, Japan; Seoul and Daegu, Korea; Changhua, Republic of China (Taiwan); Singapore, Singapore) were examined for the presence of HPV genome by a combination of polymerase chain reaction and in situ hybridization (ISH). HPV was found in 6.3%, 7%, and 9.4% of patients with lung squamous cell carcinoma, lung adenocarcinoma, and esophageal cancer, respectively. Among the geographic

areas surveyed, Kagoshima exhibited a significantly higher prevalence of HPV infection in cases of esophageal carcinoma (24.1%). There was no geographical difference in the infection rates of HPV in lung carcinomas. Subtype-specific ISH was also performed, which identified the high-risk HPV types 16/18 in the majority (75.7%) of the patients with lung and esophageal cancer positive for HPV.

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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 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 thought to be constitutively activated in cancer cells and this activation could be involved in the malignancy of tumors. Thus, we are also investigating the molecular mechanisms and target genes of the constitutive activation of NF-κB.

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

Yuri Shibata, Michiyasu Ito, Takao Seki, Xiaochuan Chen, Xiao Han, 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 TRAF 6 itself. We have previously 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.

On the other hand, we identified a novel IKK complex-associated protein X. Immunoprecipitation assay showed that the protein X interacted with IKK complex through directly binding to NEMO in a stimulation-dependent manner. Knockdown of protein X by RNAi enhanced kinase activity of IKK, and nuclear-translocation and transcriptional activation of NF-κB, and up-regulated production of TNF-α and IL-6, which are NF-κB target genes. In contrast, overexpression of protein X significantly inhibited NF-κB-driven transcriptional activation. These results indicate that protein X could function as a negative regulator in IKK activation. *In vitro* binding assay showed that protein X bound to polyubiquitinated NEMO but not to un-ubiquitinated NEMO, and protein X more efficiently bound to K63- and linear-linked polyubiquitin chains than K48-linked chains. In addition, overexpression of protein X selectively reduced exogenous NEMO expression. In contrast, knockdown of protein X expression by RNAi accumulated the polyubiquitinated NEMO upon stimulation with TNF-α. Taken together, these data strongly suggest that protein X is a novel binding factor of the IKK complex that negatively regulates NF-κB activation by binding to the K63- and/or linear-polyubiquitin chains of NEMO, which somehow leads to degradation of NEMO.

We are also interested in the signal pathways that are activated upon sensing cytosolic nucleic acids such as RNA and DNA because we have demonstrated that TRAF6 is involved in these pathways. We are trying to identify critical molecules involved in these pathways using proteomics approaches.

2. RANK signaling induces interferon-stimulated genes in the fetal thymic stroma

Daisuke Ohshima, Junwen Qin, Hiroyasu Konno, Akihisa Hirosawa, Takuma Shiraishi, Hiromi Yanai, Yusuke Shimo, Miho Shinzawa, Nobuko Akiyama, Riu Yamashita[3], Kenta Nakai[4], Taishin Akiyama, and Jun-ichiro Inoue: [3]Frontier Research Initiative, and [4]Laboratory for Functional Analysis in Silico, Human Genome Center, IMSUT.

Immunological self-tolerance is established by several mechanisms. One of the crucial mechanisms for this is thymic negative selection-specifically, the elimination of potentially self-reactive T-cell clones bearing T-cell receptors (TCRs) with a high affinity for self-antigens. It is known that thymic epithelial cells localized in the medulla (medullary thymic epithelial cells; mTECs) play an essential role in thymic negative selection. Previous studies revealed that mTEC development depends on the signal transducers TRAF6 and NIK. However, the downstream target genes of signals controlled by these molecules remain to be determined. We carried out a microarray analysis on mRNAs down-regulated by absence of TRAF6 or functional NIK in an *in vitro* organ culture of fetal thymic stroma (2DG-FTOC). An *in silico* analysis of transcription factor binding sites in plausible promoter regions of differentially expressed genes suggests an involvement of STAT1 in TRAF6- and NIK-dependent gene expression. Indeed, the signal of RANK, a TNF receptor family member that activates TRAF6 and NIK, induces the phosphorylation of STAT1 in 2DG-FTOC. Moreover, RANK signaling induces the up-regulation of interferon (IFN)-stimulated gene (ISG) expression, suggesting that the RANKL-dependent activation of STAT1 up-regulates ISG expression. The RANKL-dependent expression levels of ISGs were reduced but not completely abolished in 2DG obtained from mouse embryo deficient in interferon receptor a, which is essential for the type-I interferon signal. Our data suggest that RANK signaling induces ISG expression in both type I interferon-independent and interferon-dependent mechanisms.

3. Splenic extramedullary hemopoiesis caused by a dysfunctional mutation in the NF-κB-inducing kinase gene

Miho Shinzawa, Yuya Maruyama, Junwen Qin, Nobuko Akiyama, Maki Miyauchi, Hiromi Yanai, Masamichi Takami[5], Jun-ichiro Inoue, and Taishin Akiyama: [5]Department of Biochemistry, School of Dentistry, Showa University.

The NF-κB family of transcription factors regulates genes involved in inflammation, development, immune response, tumorigenesis, and cell survival. Various extracellular stimuli activate the NF-κB family via intracellular signaling pathways. Two NF-κB-activating pathways are currently known; the classical pathway and the alternative pathway. These signaling pathways are associated with several signal-transducing molecules, such as the TNF receptor-associated factor (TRAF) family members and serine/threonine kinases. NF-κB-inducing kinase (NIK) was originally identified as a serine/threonine kinase bound to TRAF2 and was found to be required for the alternative pathway from some of the TNF receptor family members. Alymphoplasia (aly/aly) mice have a natural point mutation in the NIK gene that causes a defect in the activation of the alternative pathway, thereby causing defects in the development of lymph nodes and Peyer's patches, and microarchitecture of the thymus and spleen. We developed a novel method to determine the aly mutation by genetic typing using PCR. This method facilitated the easy establishment of a congeneic aly/aly mouse line. Indeed, we generated a mouse line with aly mutation on a BALB/cA background (BALB/cA-aly/aly). BALB/cA-aly/aly mice showed significant splenomegaly with extramedullary hemopoiesis, which was not significant in aly/aly mice on a C57BL/6 background. Interestingly, the splenomegaly and extramedullary hemopoiesis caused by the aly mutation was gender-dependent. These data suggest that the alternative pathway is involved in the suppression of extramedullary hemopoiesis in adult mice.

4. Molecular mechanism of RANK signaling in osteoclastogenesis

Yuu Taguchi, Kazuaki Tsumura, Jin Gohda[2], and Jun-ichiro Inoue

Osteoclasts are responsible for bone resorption and play a crucial role in bone homeostasis in concert with osteoblasts, which mediate bone formation. Excess formation or activity of osteoclasts results in pathological bone resorption, such as postmenopausal osteoporosis and rheumatoid arthritis. Therefore, precise elucidation of the regulatory mechanisms of osteoclastogenesis is essential for understanding skeletal diseases and for developing drugs to treat such diseases.

Osteoclastogenesis is tightly regulated by the RANK/RANKL-signaling in progenitor cells. Intracellular signaling pathways of RANK are mediated by an adaptor molecule, TRAF6. The RANK/RANKL-signaling activates NF-κB and AP-1, and induces PLCγ2-mediated Ca^{2+} oscillation, which is required for induction of NFATc1, a master transcriptional factor in osteoclastogenesis. However, the molecular mechanisms by which the RANK/RANKL-signaling mediates osteoclastogenic signals are not fully understood. We have recently identified a novel domain in cytoplasmic region of RANK, HCR (highly conserved domain in RANK), which is essential for osteoclast differentiation in addition to the TRAF6 binding site. HCR is required for RANK-induced long-term activation of both NF-κB and PLCγ2. Furthermore, we found that HCR directly binds to Gab2, an adaptor protein, and then recruits TRAF6 and PLCγ2 in a stimulation-dependent manner. These strongly suggest that HCR provides a platform for forming a signal complex including Gab2, PLCγ2, and TRAF6 upon RANKL stimulation, and maintains long-term activation of RANK signaling. We also found that ectopic expression of the HCR-fragment composed of 62 amino acids inhibits RANKL-induced activation of NF-κB/MAPKs and RANKL-induced formation of osteoclasts. These results indicate that the HCR-fragment is a potent inhibitor of osteoclastogenesis presumably by sequestering signal complexes from HCR in endogenous RANK.

This year, we uncover the inhibitory mechanisms of osteoclastogenesis by ectopically expressed HCR-fragment. 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. In contrast, expression of the GY-peptide, which is a part of the C-peptide, did not significantly affect RANK signaling in prefusion step, but did inhibit cell-cell fusion, which is the essential event for forming mature osteoclasts. Moreover, Gab2 bound the C-peptide but not the N-peptide, suggesting that the C- and the N-peptides sequester TRAF6 in Gab2 dependent and independent manners, respectively. In contrast, we showed that the GY-peptide did not bind Gab2 but could bind Vav3, which mediates signaling for cell-cell fusion. Taken together, we propose that the HCR-fragment exerts two inhibitory mechanisms, one is inhibition of RANK -signaling in prefusion step by blocking TRAF6-mediated signaling, and the other is inhibition of cell-cell fusion by blocking Vav3-mediated signaling, respectively.

To further elucidate the mechanisms of the HCR-mediated-signaling and the HCR-mediated inhibition of osteoclastogenesis, we are trying to identify the novel binding protein to the HCR-fragment.

5. Identification of the target genes of constitutively activated NF-κB in basal-like subtype breast cancer cells

Taku Ito-Kureha, Noritaka Yamaguchi, Kentaro Semba[6] and Jun-ichiro Inoue: [6]Institute for Biomedical Engineering, Consolidated Research Institute for Advanced Science and Medical Care, Waseda University.

Our previous study showed that NF-κB is constitutively activated and crucial for proliferation of basal-like subtype breast cancer, the most malignant form of breast cancer. To understand the molecular function of NF-κB in the basal-like subtype cancer cells, we analyzed NF-κB target genes in these cells by expression of the non-degradable IκBα super-repressor, which can specifically inhibit NF-κB activation. Several promising candidates, which were thought to be involved in invasion, metastasis, and cell survival, have been identified. This year, we focused on one candidate gene, which encodes protein Y, an actin binding protein, because overexpression of the protein Y in breast cancer cells enhanced tumor progression in mouse xenograft model. Although proliferation of control cells and protein Y-expressing cells was not changed in 2D-culture condition, growth of protein Y-expressing cells increased in collagen gel 3D-culture. Expression of matrix metalloproteinase 13 (MMP13), an enzyme that regulates remodeling of extracellular matrix, was up-regulated in protein Y-expressing cells. Knockdown of the protein Y suppressed MMP13 expression and cell proliferation in collagen gel 3D-culture, suggesting that the protein Y induces MMP13 expression and leads to remodeling of extracellular matrix and increment of cell growth in the collagen gel. Protein level of β-catenin, which regulates MMP13 expression, was also reduced in the protein Y-knockdown cell. These results suggest that the NF-κB-protein Y-β-catenin-MMP13 pathway is a novel therapeutic target of basal-like breast cancer.

6. The role of NF-κB activity in breast cancer tumor-initiating cells

Mizuki Yamamoto, Dong Li, Eerdunfu, Noritaka Yamaguchi, Kentaro Semba[6] and Junichiro Inoue

Recent studies indicate that breast cancer arise from rare self-renewing tumor-initiating cells (TICs), which exhibit CD24-low, CD44-high, and EpCAM-positive phenotype. Because TICs have particular profiles such as resistibility against variant stress as well as normal stem cells, it is thought that TICs also involve recurrence and metastasis. However, it remains unclear which signaling pathway is important for the maintenance and functions of TICs. Recently, we have found that NF-κB is constitutively activated and promotes cell growth in basal-like subtype breast cancer cells. Because these cells exhibit CD24-low and CD44-high phenotype and form undifferentiated carcinomas in xenograft models, it is thought that TICs have a property similar to that of basal-like subtype cancer cells. In this study, to analyze significance of NF-κB in breast cancer TICs, we modulated NF-κB activity in basal-like subtype cells using retrovirus vector encoding IKKβ or IκBα super-repressor and examined population of TICs by FACS analysis, sphere culture assay, and xenograft model of NOD/SCID mice. The population of TICs was increased or decreased depending on the level of the NF-κB activity, suggesting that NF-κB is involved in the maintenance of TICs. Currently, we are analyzing the molecular function of NF-κB for maintenance of breast cancer TICs and found that the cell-cell interaction between TICs and bulk cells are required for efficient maintenance of TICs.

7. Analysis of the IL-1 signalosome with tandem affinity purification method

Noritaka Yamaguchi, Hiroyuki Takayama, Hiroko Kozuka-Hata[1], Masaaki Oyama[1] and Jun-ichiro Inoue

Our recent study revealed that poly-ubiquitination of TAK1 by TRAF6 is crucial for activation of the IL-1 pathway. To understand the molecular function of TAK1 ubiquitination, we analyzed TAK1 binding proteins in IL-1-stimulated cells using a tandem affinity purification of the TAK1 complex and subsequent proteomic analysis. By comparing data between non-stimulated cells and IL-1-stimulated cells, we identified several candidate proteins that specifically bind to poly-ubiquitinated TAK1. We isolated cDNAs encoding the candidate proteins and generated cell lines stably expressing them. Some stable lines showed enhanced or suppressed NF-κB activation upon IL-1 stimulation, suggesting that some candidate proteins are involved in NF-κB activation in IL-1 pathway. After confirmation of association of these candidates with TAK1 by immunnoprecipitation assay, we will analyze their molecular function in this pathway.

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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 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.

Tezuka, T., Kawamoto, Y., Nakatani, N., Inoue, A., Ikegami, 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. We are particularly interested in the molecular mechanisms underlying these cell-type specific requirements.

2. Pathophysiological mechanisms underlying *DOK7* myasthenia.

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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. The effects of these mutations in vivo are under 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 neuromuscular synapse formation due to decreased ability of Dok-7 to activate MuSK in myotubes. 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.

4. Novel autoantibodies 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 pa-

tients who are negative for MuSK and AChR antibodies. Given the essential role and postsynaptic localization of Lrp4 in the NMJ, we hypothesized that Lrp4 autoantibodies might be a pathogenic factor in MG. In the current study, 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*., in press; *Arch. Neurol*., in press). We are further investigating the etiology and pathology of Lrp4 antibody-positive MG.

5. Negative regulation of PTK-mediated signaling by Dok-family proteins in macrophages and osteoclasts.

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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. Indeed we previously demonstrated that Dok-1 and Dok-2 are key negative regulators of hematopoietic growth and survival signaling as well as TLR4-mediated innate immune signaling. In the current study, we found that Dok-1, Dok-2, and Dok-3 cooperatively inhibit macrophage proliferation and that *Dok-1*$^{-/-}$*Dok-2*$^{-/-}$*Dok-3*$^{-/-}$ mice develop histiocytic sarcoma, an aggressive malignancy of macrophages, but do not exhibit elevated incidence of other types of tumors. These mutant mice showed earlier mortality than wild-type or mice deficient in only 1 or 2 of these genes, and this mortality was associated with histiocytic sarcoma. About 80% of tumor-bearing *Dok-1*$^{-/-}$*Dok-2*$^{-/-}$*Dok-3*$^{-/-}$ mice showed multiple organ spread, with osteolytic lesions and orthotopic invasion from bone marrow to skeletal muscle. These findings commend *Dok-1*$^{-/-}$*Dok-2*$^{-/-}$*Dok-3*$^{-/-}$ mice as a useful model for the study of histiocytic sarcoma. Recently, we found that Dok-1 and Dok-2 double deficiency enhanced sensitivity of osteoclast precursors to M-CSF and that this deficiency enhanced the number and activity of osteoclasts, leading to osteopenia in mice lacking these proteins. We are investigating the molecular etiology and pathology of these neoplastic disorders and bone metabolism defects along with inflammatory diseases in mice lacking Dok-1, -2, and/or -3.

6. Proteomic 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 mass spectrometry-based proteomic analyses. We are investigating the roles of candidate proteins that appear to be involved in each of these biological events.

Publications

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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 cancer 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 activation.

1. Spatio-temporal dynamics of MT1-MMP for the degradation of ECM

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Invadopodia are small protrusions observed on the ventral surface of invasive cancer cells in vitro. When cancer cells were cultured on the layer of fibronectin/gelatin, degradation of these proteins was observed at sites of invadopodia. Thus, invadopodia are hypothesized as machinery to degrade ECM at the initial stage of cancer cell invasion. In accord with these, the number of invadopodia was shown to be positively correlated with the number of ECM degradation sites. MT1-MMP is a type I membrane proteinase involved in the degradation of ECM, and is enriched in invadopodia. The suppression of MT1-MMP by siRNA leads to the reduced ECM degradation. The accumulation of MT1-MMP to invadopodia is followed by the degradation of ECM at the same sites. The surface MT1-MMP was reported to be internalized and recycled. MT1-MMP is colocalized with clathrin, and the internalization proceeded through a clathrin-dependent pathway or a caveolae-mediated manner. Inhibition of dynamin 2 was shown to reduce the ECM degradation at invadopodia. In addition, exocytosis is implicated to be involved in the ECM degradation, because the v-SNARE TI-VAMP/VAMP7 was found to regulate MT1-MMP-dependent ECM degradation, and exocyst was found to play a pivotal role in invadopodial activity. However, the turnover of MT1-MMP at invadopodia is still not clear to date, much less the reason for its requirement. Therefore, we

first measured the turnover of MT1-MMP at a single invadopodium by FRAP experiment. Next we constructed a model for the dynamics of MT1-MMP at invadopodia using the observed turnover kinetics to investigate the role of the turnover in the degradation of ECM. The inhibition of the turnover of MT1-MMP blocked the ECM degradation at invadopodia. Simulations showed that without the turnover of MT1-MMP no appreciable ECM degradation was seen. These results demonstrate the crucial role of the turnover of MT1-MMP for the ECM degradation at invadopodia both from experiments and simulations.

2. Short transient peak in the activity of MT1-MMP

Ayako Watanabe, Daisuke Hoshino, Naohiko Koshikawa, Takashi Suzuki, Motoharu Seiki and Kazuhisa Ichikawa

Focal degradation of ECM is the first step in the invasion of cancer cells. MT1-MMP is a potent membrane protease employed by aggressive cancer cells. In our previous study, we found that the quick turnover was essential for the degradation of ECM at invadopodia. Here we characterize and analyze the ECM-degrading activity of MT1-MMP. First, our model resembled a transient sharp transient peak in the activity of MT1-MMP followed by steady activity. Such a transient peak was not seen in the previously published model. Existence of this transient peak depends on the concentration of TIMP-2, an endogenous inhibitor of MT1-MMP: transient peak is much prominent at higher TIMP-2 concentration, and almost abolished at lower concentrations. Next, we evaluate the role of this transient peak in the ECM degradation. When the transient peak was forced to suppress in computer simulations, the ECM degradation was also heavily suppressed showing the essential role of this transient peak. Third, we compared the effect of pulsatile turnover of MT1-MMP in the invadopodia. In our previous report, ECM degradation by MT1-MMP was simulated assuming continuous turnover. However, MT1-MMP is transported to invadopodia by vesicle trafficking, which will resemble a pulsatile insertion. The pulsatile insertion model showed basically the consistent results with continuous insertion model in the ECM degradation. The present analysis and characterization of ECM degradation by MT1-MMP strongly indicate the importance of dynamic activity of MT1-MMP in the ECM degradation.

3. Dynamics of transcription factor NF-κB

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Transcription factor NF-κB shuttles between cytoplasm and nucleus, and it is known that the nuclear NF-κB oscillates. This stimulated studies by computer simulation, and nearly forty reports have been published to date on the computational model of the oscillation of NF-κB. These reports showed fairly good agreement with experimentally observed oscillation of nuclear NF-κB, and some of these showed experimentally different gene expression by the change in the NF-κB oscillation pattern as predicted by their simulations. The computational models reported to date, 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) have not been involved or limited. Since these spatial parameters will be different in different cells, it will be valuable to know the relationship between spatial parameters and the oscillation pattern in three-dimensional (3D) cell. we constructed a 3D computational model for the oscillation of nuclear NF-κB using A-Cell software. First, we found that the biochemical kinetic constants used in the temporal mode 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, affected the oscillation pattern. Among them, N/C largely altered the oscillation pattern showing larger N/C and larger nuclear membrane area resulted in prolonged oscillation of nuclear NF-κB.

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