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. Genomic and epigenomic abnormalities involved in human tumors, including adult T-cell leukemia, cholangiocarcinoma, lung, breast, head and neck and urological cancers, are also being investigated.

1. The biological functions of cell adhesion in human tumor

Takeshi Ito, Yuki Kumagai, Zenichi Tanei, Yumi Tsuboi, Anri Saito, Shuejen Shiu, Hiroyuki Kogai, Ken Akashi, Atsushi Kanatani, Takehiro Tsuchiya, Masaru Kasai, Takuya Tabuchi, Tomoko Maruyama, Hiromi Ichihara, Atsuko Nakamura, Mika Sakurai-Yageta, Kaoru Kiguchi, Motoi Oba,[1] and Yoshinori Murakami; [1]Research Institute of Molecular and Cell Biology of Cancer, Showa University

Disruption of cell adhesion is a critical step to invasion and metastasis of human cancer and their acquired resistance to several anti-cancer and molecular targeting drugs. CADM1/TSLC1 is an immunoglobulin superfamily cell adhesion molecule and acts as a tumor suppressor in various cancers. In order to understand possible cross-talk of CADM1 with other oncogenic signals, we have examined mutation of the *KRAS2* and *TP53* genes and promoter methylation of the *CADM1* and *4.1B* genes in the same series of human non-small cell lung cancer (NSCLC) and have found that promoter methylation of the *CADM1* and *4.1B* genes occurs independently of the *EGFR* or the *KRAS2* mutation in non-small cell lung cancer (1). We, then, investigated dynamic regulation of the CADM 1-4.1B-MPP3 complex in mature cell adhesion by fluorescence recovery after photobleaching (FRAP) analysis. Traditional FRAP analysis were performed for relatively short period of around 10min. On the other hand, thanks to recent advances in the sensitive laser detector systems, we examine FRAP of CADM1 complex for longer period of 60 min and analyze the recovery with exponential curve-fitting to distinguish the fractions with different diffusion constants. This approach reveals that the fluorescence recovery of CADM1 is fitted to a single exponential function with a time constant (tau) of approximately 16 min, whereas 4.1B and MPP3 are fitted to a double exponential function with two taus of approximately 40-60 sec and 16 min. The longer tau is similar to that of CADM1, suggesting that 4.1B and MPP3 have two distinct fractions, one forming a complex with CADM1 and the other present as a free pool. Fluorescence loss in photobleaching analysis supports the presence of a free pool of these proteins near the plasma membrane. Furthermore, double exponential fitting makes it possible to estimate the ratio of 4.1B and MPP3 pre-

sent as a free pool and as a complex with CADM1 as approximately 3:2 and 3:1, respectively. These approaches demonstrate a central role of CADM1 in the stability of its complex formation with 4.1B and MPP3 and would provide more mechanistic aspects of the dynamics of protein complexes in random processes (2). We have also demonstrated the importance of immunoglubuin superfamily cell adhesion molecules other than CADM1 in collaboration with others (3). Additional roles of CADM1 in immunological recognition by CD4+ cytotoxic T lymphocyte was also investigated in collaboration with others (4).

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

Takeshi Ito, Yuki Kumagai, Daisuke Suzuki, Shintaro Mori, Tomoko Maruyama, Akiko Nakamura, 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) and small cell lung cancer (SCLC), conferring an invasive phenotype characteristic to ATL. To understand the oncogenic functions of CADM1 in ATL and SCLC that are distinct from tumor suppressor functions in many epithelial cancers, structural and functional features of *N*-linked and *O*-linked glycosylation of CADM1 protein are being examined using mass spectrometry in collaboration with Shimadzu Co. Ltd.. To establish sensitive diagnostic tools of SCLC through detecting CADM1, monoclonal antibodies against the fragment of CADM1 unique to ATL or SCLC are being generated and characterized in collaboration with researchers in the Institute of Advanced Science and Technology, the University of Tokyo. These antibodies would be useful to improve the FACS system to identify and characterize malignant ATL cells, whose prototype has been established in the IMSUT Hospital. Novel detection systems of SCLC and possible therapeutic strategies against ATL and SCLC are also being investigated by the support of several grants of applied medical sciences.

3. Analyses of genomic and epigenomic alterations of human cholangiocarcinoma and head and neck, lung, breast, and urological cancers.

Ken Akashi, Hiroyuki Kogai, Hasaya Dockduang, Ayako Sato, Takeshi Ito, Kaoru Kiguchi and Yoshinori Murakami.

To unveil the molecular mechanisms of multistage carcinogenesis, genetic and epigenetic alterations in key molecules in human tumorigenesis were examined in various cancers. Molecular targeting therapy to specific genetic alterations has not been established in head and neck squamous cell carcinoma (HNSCC) except for cetuximab treatment. To characterize alterations of actionable oncogenes in HNSCC, we examined the gain of copy and mutation of the *MET* gene in 54 Japanese HNSCC by droplet digital PCR (ddPCR). Copy gain of the *MET* was detected in 10 of 54 (19%) HNSCCs and more frequently observed in tumors of the hypopharynx (4 of 12; 33%) or larynx (5 of 13; 38%) than those of the oral cavity (1 of 21; 4%) or oropharynx (0 of 8; 0%), suggesting the existence of site-specific features in the oncogenic mechanisms of HNSCCs. Copy gain of the *MET* was also observed preferentially in older patients, although no correlation in other parameters, including clinical stages and overall or recurrence-free survival, was observed. These results suggest that copy gain of the *MET* could provide an indicator for treatment with tyrosine kinase inhibitors for MET in a subset of hypopharyngeal or laryngeal cancer (5). We also examined 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), in breast cancer, CCA, HNSCC and bladder cancer by comprehensive approaches using CNA-specific arrays, we identified and being characterized a number of candidate genes showing gain or loss of gene copies in each cancers. Cholangiocarcinoma is one of the representative cancers which are refractory to any therapeutic approaches. Using tyrosine kinase array and STATs profiling, we have reported the predominant activation of STAT3 in cholangiocarcinoma in collaboration with others (6).

4. Identification of a novel Mint3 binding protein.

Hiroki J. Nakaoka, Seiko Yoshino, Akane Kanamori, Miho Ishiura, Masaya Misumi, Yukari Aso, Takeharu Sakamoto

Unlike most cells, cancer cells activate hypoxia inducible factor-1 (HIF-1) to use glycolysis even at normal oxygen levels, or normoxia1. Therefore, HIF-1 is an attractive target in cancer therapy. However, the regulation of HIF-1 during normoxia is not well characterised, although Mint3 was recently found to activate HIF-1 in cancer cells and macrophages by suppressing the HIF-1 inhibitor, factor inhibiting HIF-1 (FIH-1). Thus, we analysed Mint3-binding proteins to investigate the mechanism by which Mint3 regulates HIF-1. Yeast two-hybrid screening using Mint3 as bait identified protein X as a novel factor regulating HIF-1 activity via

Mint3. Protein X bound to the phosphotyrosine-binding domain of Mint3, formed a ternary complex with Mint3 and FIH-1, and co-localised with Mint3 at the Golgi apparatus. Depletion of protein X decreased the expression of HIF-1 target genes and reduced glycolysis in normoxic cancer cells. Inhibition of protein X in cancer cells by expressing shRNAs reduced tumourigenicity (7, 8).

5. Molecular pathological analyses of human lung cancer

Daisuke Matsubara, Zenichi Tanei, Zhang Wen-Jing 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 and have identified protein arginine methyltransferase 5 (PRMT5) as a candidate histone methyltransferase gene whose expression was correlated with EMT by microarray analysis. We have also reported previously that ZEB1, one of the master regulators of EMT, is another candidate gene involved in EMT of lung cancer. Then, we extensively examined possible aberrations of the switch/sucrose non-fermenting (SWI/SNF) complex genes, including BRG1, BRM, BAF47, ARID1A, and ARID1B, in human NSCLC and showed high incidence of altered expression of these genes in a subset of NSCLC, especially in large cell carcinomas and pleomorphic carcinomas in collaboration with others (9).

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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 and adult T cell leukemia (ATL).

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

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Transcription factor NF-κB binds specifically to a decameric motif of nucleotide, κB site, and activates transcription. The activation of 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. In canonical NF-κB pathway, 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 (K 48)-linked ubiquitination and proteasome-dependent degradation of IκB. NF-κB subsequently translocates to the nucleus to activate transcription of tar-

get genes. This project is to identify molecules that regulate signal from membrane receptors to NF-κB/IκB complex. We have previously identified upstream activators of 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. To understand the molecular mechanisms of TRAF6-mediated NF-κB activation, we try to identify proteins that are ubiquitinated by TRAF6 upon stimulation. 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. We are also interested in noncanonical NF-κB pathway, which is crucial for immunity by establishing lymphoid organogenesis and B-cell and dendritic cell (DC) maturation. RelB is a major NF-κB subunit in the pathway. To elucidate the mechanism of the RelB-mediated immune cell maturation, a precise understanding of the relationship between cell maturation and RelB expression and activation at the single-cell level is required. Therefore, we generated knock-in mice expressing a fusion protein between RelB and fluorescent protein (RelB-Venus) from the *Relb* locus. The $Relb^{Venus/Venus}$ mice developed without any abnormalities observed in the $Relb^{-/-}$ mice, allowing us to monitor RelB-Venus expression and nuclear localization as RelB expression and activation. $Relb^{Venus/Venus}$ DC analyses revealed that DCs consist of $RelB^{-}$, $RelB^{low}$and $RelB^{high}$ populations. The $RelB^{high}$ population, which included mature DCs with projections, displayed RelB nuclear localization, whereas RelB in the $RelB^{low}$ population was in the cytoplasm. Although both the $RelB^{low}$ and $RelB^{-}$ populations barely showed projections, MHC II and co-stimulatory molecule expression were higher in the $RelB^{low}$ than in the $RelB^{-}$ splenic conventional DCs. Taken together, our results identify the $RelB^{low}$ population as a possible novel intermediate maturation stage of cDCs and the $Relb^{Venus/Venus}$ mice as a useful tool to analyze the dynamic regulation of the non-canonical NF-κB pathway.

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 targets polyubiquitinated NEMO for lysosomal degradation, thereby negatively regulating NF-κB activation. We also have shown that the expression of p47 is reduced in adult T-cell leukemia (ATL) patient-derived cell lines, in which NF-κB is constitutively activated. Our results suggest that the altered p47 expression may trigger development of various cancers. 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. We then obtained p47-deficient MEFs by introducing Cre recombinase into $p47^{fl/fl}$ MEFs. p47-deficient MEFs exhibited defective Golgi stucyutr. We also crossed p47-loxP-flanked mice 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

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Gohda[2], and Jun-ichiro Inoue

Osteoclasts are large multinucleated cells which have ability to degrade bone matrixes, and play a crucial role in bone homeostasis in concert with osteoblast which have function of bone formation. As a result of excess formation or activation of osteoclasts, pathological bone resorption is observed in postmenopausal osteoporosis and rheumatoid arthritis. Therefore, elucidating the molecular mechanism of osteoclastogenesis is important for understanding bone diseases and developing novel strategies to treat such diseases.

Osteoclasts are differentiated from hematopoietic stem cells upon stimulation with cytokines, for example macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor-κB (RANK) ligand (RANKL). It is known that activation of signal transduction pathway emanating from receptor RANK is essential for osteoclastogenesis. This RANK signaling activates transcriptional factors, NF-κB and AP-1, through the E3 ubiquitin ligase TRAF6, and also induces PLCγ2-mediated Ca^{2+} oscillation. These signals lead to the induction of NFATc1 that is a master transcriptional factor in osteoclastogenesis. We have previously identified a novel domain in the cytoplasmic region of RANK, and named Highly Conserved domain in RANK (HCR), which does not have any homology of amino-acid sequence with other proteins. The HCR acts as a platform for formation of signal complex including TRAF6, PLCγ2 and adaptor protein Gab2. This formation is involved in sustaining activation of RANK signaling, and is essential for the NFATc1 induction and osteoclastogenesis. To elucidate the other functions and the precise molecular mechanism of HCR, we performed yeast two-hybrid screening to identify the interacting protein to HCR. We are investigating whether some candidate proteins associate to HCR in osteoclast precursor cells and are involved in HCR functions and osteoclastogenesis. Moreover, to find the novel molecules involved in osteoclastogenesis, we investigated the change of gene expression levels during osteoclastogenesis by analyzing microarray data. Since three genes were selected as candidates, we are now investigating whether these genes are important for osteoclastogenesis *in vivo*. Furthermore, we also tried to elucidate the precise molecular mechanism at the subsequent step of NFATc1 induction in osteoclastogenesis from the point of view of TRAF6 functions, and revealed that TRAF6 is involved in cell-cell fusion and actin ring formation in addition to NFATc1 induction. We are currently trying to identify the novel target protein ubiquitinated by TRAF6.

5. TRAF6 regulates pregnancy-induced mammary gland development and maintenance of epithelial stem cells

Mizuki Yamamoto, and Jun-ichiro Inoue

Mammary gland development is characterized by the unique process by which the epithelium invades the stroma. During puberty, tubule formation is coupled with branching morphogenesis which establishes the basic arboreal network emanating from the nipple. During pregnancy, the ductal cells undergo rapid proliferation and form alveolar structures within the branches for milk production. Upon weaning of the pups, lactation stops and the mammary gland undergoes rapid involution. In the RANK signaling TRAF6 activates MAPK and canonical NF-κB independently with IKKα-mediated non-canonical NF-κB pathway. Previously, it has been reported that deficiency of RANK and its downstream kinase IKKα inhibited pregnancy-induced alveolar formation via *Ccnd1* expression. However the role of TRAF6 in mammary gland development remains unclear. To elucidate the role of TRAF6, we first analyzed TRAF6-deficient mammary gland structure in virgin and pregnant mice and found that that TRAF6-deficiency, like RANK-deficiency, inhibited pregnancy-induced alveolar formation but did not lead to any morphological defect in non-pregnant gland.

6. Catalytic subunits of the phosphatase calcineurin interact with NF-κB-inducing kinase (NIK) and attenuate NIK-dependent gene expression

Miho Shinzawa, Hiroyasu Konno, Junwen Qin, Nobuko Akiyama, Maki Miyauchi, Hiroyuki Ohashi[5], Etsuko Miyamoto-Sato[5,6], Hiroshi Yanagawa[7], Taishin Akiyama and Jun-ichiro Inoue: [5]Division of Interactome Medical Sciences, The Institute of Medical Science, The University of Tokyo, Minato-ku, Tokyo, Japan; [6]Division of Molecular Biology, Research Institute for Biomedical Sciences, Tokyo University of Science, Yamazaki, Noda-shi, Chiba, Japan; [7]Department of Biosciences and Informatics, Faculty of Science and Technology, Keio University, Yokohama, Japan

Nuclear factor (NF)-κB-inducing kinase (NIK) is a signal transducer that regulates a wide variety of immune systems by activating NF-κB pathways. Aberrant NIK activation reportedly provokes tumor malignancy. Therefore, a precise regulation of NIK activity should be required. In this study, we explored novel interacting proteins of NIK by *in vitro* virus screening. We then identified the catalytic subunit Aα isoform of serine/threonine phosphatase calcineurin (CnAα) as a novel NIK-interacting pro-

tein. Co-immunoprecipitation experiments confirmed the interaction of NIK with CnAα in living cells. In addition to CnAα, calcineurin catalytic subunit Aβ isoform (CnAβ) also interacted with NIK. We further found that both the kinase domain and C-terminal region of NIK binds to CnAα and CnAβ. Furthermore, the phosphatase domain of CnAα is necessary for the binding to NIK. Interestingly, TRAF3, a critical regulator of NIK activity, also interacts with CnAα and CnAβ. Knockdown of CnAα and CnAβ significantly enhanced expression of the NIK-dependent gene *Spi-B* and activation of RelA and RelB induced by lymphotoxin-β receptor (LtβR) signaling. These data suggest that CnAα and CnAβ attenuate NF-κB activation pathway mediated by LtβR-NIK signaling. Taken together, this study suggest that CnAα and CnAβ fine-tune NIK functions.

7. Hypergravity provokes a temporary reduction in $CD4^+CD8^+$ thymocyte number and a persistent decrease in medullary thymic epithelial cell frequency in mice

Ryosuke Tateishi, Nobuko Akiyama, Maki Miyauchi, Riko Yoshinaga, Hiroki Sasanuma[8], Takashi Kudo[9], Miki Shimbo[9], Masahiro Shinohara[10], Koji Obata[11], Jun-ichiro Inoue, Masaki Shirakawa[12], Dai Shiba[12], Hiroshi Asahara[10], Nobuaki Yoshida[8], Satoru Takahashi[9], Hironobu Morita[11], and Taishin Akiyama: [8]Laboratory of Developmental Genetics, Center for Experimental Medicine and Systems Biology, The University of Tokyo; [9]Department of Anatomy and Embryology, Faculty of Medicine, University of Tsukuba; [10]Department of Systems BioMedicine, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University; [11]Department of Physiology, Gifu University Graduate School of Medicine; [12]Japan Aerospace Exploration Agency (JAXA), Tsukuba, Japan

Gravity change influences a wide variety of immunological systems. In this study, we investigated the effects of hypergravity (2G) on thymic cells in mice. Exposure of mice to 2G for relatively short period (3 days) decreased the frequency of $CD4^+$ $CD8^+$ thymocytes (DP) and mature medullary thymic epithelial cells (mTECs). Moreover, keratin-5 and keratin-8 double-positive ($K5^+K8^+$) TECs, which reportedly contain TEC progenitors, were increased. The reduction of DP was recovered by an exposure to 2G for 14 days. On the other hand, the reduction of mature mTECs and the increment of $K5^+K8^+$ TEC persisted in this period of 2G-exposure. Intriguingly, a surgical deletion of the inner ear's vestibular apparatus suppressed these hypergravity effects. Gene expression analysis by quantitative PCR analysis revealed that Aire and RANK, which play critical role in mTEC function and development, were up-regulated by the 3-day exposure and subsequently down-regulated by the 14-day exposure to 2G. Unexpectedly, a surgical lesion of the inner ear's vestibular apparatus did not affect the change in mTEC gene expression. Overall, this study suggests that exposure of mice to hypergravity provokes a transient reduction of DP and a long-lasting reduction of mature mTECs in a vestibular system-dependent manner. Moreover, the 2G exposure causes an aberrant expression of mTEC genes in the vestibular system-independent fashion. These data might provide insight on the effect of gravity change on immune system during spaceflight and living.

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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 in neuromuscular synaptogenesis.

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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. Interestingly, mice lacking Lrp4, which forms a complex with MuSK and acts as an essential agrin-binding module, do not show MuSK-dependent AChR prepatterning or NMJ formation. This suggests that Lrp4 is required for MuSK activation under physiological conditions, in contrast to our observation that Dok-7 can activate MuSK in the absence of Lrp4 or its ligand agrin, at least in vitro. Thus, we examined the effects of forced expression of Dok-7 in skeletal muscle on NMJ formation in the absence of Lrp4 and found that it indeed induces MuSK activation in mice lacking Lrp4. However, the activation level of MuSK was significantly lower in the absence than in the presence of Lrp4. Together, these data indicate that Lrp4 is required for efficient activation of MuSK by Dok-7 in the muscle. Since Lrp4 is also essential for presynaptic differentiation of motor nerve terminals (*Nature* 489: 438-442, 2012), this apparent cooperation between Lrp4 and Dok-7 in MuSK activation may be complicated. We are investigating mechanisms of this cooperation in vivo.

2. Agrin's role aside from MuSK activation in the postnatal maintenance of NMJs.

Tezuka, T., Hoshi, T., Burgess, RW.[1], Ueta, R., and Yamanashi, Y.: [1]The Jackson Laboratory.

Although NMJ formation requires agrin under physiological conditions, it is dispensable for NMJ formation experimentally in the absence of the neurotransmitter acetylcholine, which inhibits postsynaptic specialization. Thus, it was hypothesized that MuSK needs agrin together with Lrp4 and Dok-7 to achieve sufficient activation to surmount inhibition by acetylcholine. To test this hypothesis, we examined the effects of forced expression of Dok-7 in skeletal muscle on NMJ formation in the absence of agrin and found that it indeed restores NMJ formation in agrin-deficient embryos. However, these NMJs rapidly disappeared after birth, whereas exogenous Dok-7-mediated MuSK activation was maintained. These findings indicate that the MuSK activator agrin plays another role essential for the postnatal maintenance, but not for embryonic formation, of NMJs. Because a pathogenic mutation of agrin in patients with congenital myasthenic syndromes (see below) did not show impaired ability to activate MuSK at least in vitro (*Am. J. Hum. Genet.*, 85: 155-167, 2009), the novel role of agrin may be relevant to pathogenicity of the mutation. We are investigating how agrin plays the role in the postnatal maintenance of NMJs.

3. 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 established knock-in mice (Dok-7 KI mice) that have a mutation associated with the majority of patients with *DOK7* myasthenia. As expected, Dok-7 KI mice showed characteristic features of severe muscle weakness and died between postnatal day 13 and 20. Furthermore, they showed abnormally small

NMJs lacking postsynaptic folding, a pathological feature seen in patients with *DOK7* myasthenia. Consistent with this, Dok-7 KI mice exhibited decreased MuSK activity in skeletal muscle, indicating that the Dok-7 KI mice develop defects similar to those found in patients with *DOK7* myasthenia, although the mice exhibit a more severe phenotype. We are investigating other defects in NMJ function and detailed pathophysiology in the Dok-7 KI mice.

4. *DOK7* gene therapy that enlarges NMJs.

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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 obvious defects in motor activity, overexpression of Dok-7 in the skeletal muscle of patients with *DOK7* myasthenia might ameliorate NMJ formation and muscle weakness. To test this possibility, we generated an Adeno-associated virus-based vector (AAV-D7), which strongly expressed human Dok-7 in myotubes and induced AChR cluster formation. Indeed, therapeutic administration of AAV-D7 to Dok-7 KI mice described above resulted in enlargement of NMJs and substantial increases in muscle strength and life span. Furthermore, when applied to model mice of another neuromuscular disorder, autosomal dominant Emery-Dreifuss muscular dystrophy, therapeutic administration of AAV-D7 likewise resulted in enlargement of NMJs as well as positive effects on motor activity and life span. These results suggest that therapies aimed at enlarging the NMJ may be useful for a range of neuromuscular disorders. We are investigating the effects of AAV-D7 administration on other types of neuromuscular disease models. Because we used cytomegalovirus promoter, which showed high efficacy but little tissue-specificity of Dok-7 expression, for AAV-D7 in the above experiments, we are developing another type of AAV-D7, which contains muscle-specific promoter for the safer therapeutic approach.

5. 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, implicating 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). Also it was reported that antibodies to Lrp4 induced MG in model animals (*J. Clin. Invest.*, 123: 5190-5202, 2013). Given that Lrp4 antibodies are reported to be involved in amyotrophic lateral sclerosis (ALS) (*Ann. Clin. Transl. Neurol.*, 1: 80-87, 2014), which is associated with NMJ defects, we have expanded our research area with regard to Lrp4 antibodies from MG to neuromuscular disorders characterized by NMJ defects.

6. 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 further investigating roles of Dok-1 to Dok-6, including those in tumor malignancy, inflammatory disorders, and other types of intractable diseases.

7. Omic analyses.

Tezuka, T., Eguchi, T., Honda, M., Ueta, R., Arimura, S., Miyoshi. S., Hoshi, T., Iemura, S.[1], Natsume, T.[2], Oyama, M.[3], Sagara, H.[3], and Yamanashi, Y.: [1]Translational Research Center, Fukushima Medical University. [2]National Institute of Advanced Science and Technology, Molecular Profiling Research Center for Drug Discovery. [3]Medical Proteomics Laboratory, IMSUT.

To gain insights into signaling mechanisms underlying a variety of physiological and pathophysiological events, including NMJ formation, tumorigenesis, and tumor metastasis, we have performed proteomic and transcriptomic analyses. We are investigating the roles of candidate proteins and genes that appear to be involved in each of these biological events. In addition, we have prepared experimental settings for other omic approaches such as glycomic and metabolomic analyses.

For instance, we previously 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. We are investigating roles for glycosylation of Lrp4 in its pleiotropic activities.

8. Screening of chemical compound and siRNA libraries.

Ueta, R., Yukimura R., Waseda, M., Honda, M., Kito M., Sasaki, Y., Nagatoishi, S.[1], Tsumoto, K.[1], and Yamanashi, Y.: [1]Medical Proteomics Laboratory, IMSUT.

In addition to the omic analyses described above, we performed high throughput screenings of chemical compound and siRNA 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 and candidate genes that may regulate important signalings.

Publications

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