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-cell interaction in invasion, metastasis and immunological responses of cancer. Genomic and epigenomic abnormalities involved in human tumors, including adult T-cell leukemia, cholangiocarcinoma, lung, breast, head and neck and urological cancers, and various common diseases are also being investigated.

1. The biological functions of cell-cell interaction in human oncogenesis

Takeshi Ito, Yumi Tsuboi, Yuki Kumagai, Masaru Kasai, Atsuko Nakamura, Toko Funaki, Yuki Azuma, Yoshiaki Kanamura, Yota Mitobe, Tomoko Masuda, Hiromi Ichihara, Kaoru Kiguchi, Motoi Oba[1], Daisuke Matsubara 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/TSLC is an immunoglobulin superfamily cell adhesion molecule (IgCAMs) and acts as a tumor suppressor in various cancers. By contrast, CADM1 rather promotes cell invasion and metastasis in adult T-cell leukemia (ATL) or small cell lung cancer (SCLC). In order to elucidate molecular pathways involved in this CADM1-mediated dual functions in oncogenesis, mass spectrometry (MS) analysis was performed to identify a series of proteins associated with CADM1 in epithelial cells and SCLC. In epithelial cells, we have identified several molecules in the tyrosine kinase pathways and found that interaction of CADM1 with these tyrosine kinases could modify the growth associated signaling triggered by tyrosine kinases. In colon cancer, a molecule identified by MS analysis showed critical roles in cell mobility and tumorigenicity in nude mice of colon cancer cells through interrupting tyrosine-kinase pathways. In SCLC, MS analysis identified a series of proteins also important for human tumorigenesis and possibly for response against anti-cancer drugs.

We are also investigating possible cross-talk of IgCAMs and its biological and immunological significance comprehensively by cloning more than 300 IgCAMs expressed in human cells and analyzing molecule-molecule interactions using the surface plasmon resonance imaging (SPRi) and the amplified luminescence proximity homogenous assay (ALPHA). As a pilot study using SPRi approach, we quantified the affinity of interaction of CADM1 with other known IgCAM molecules. The cell adhesion molecule (CADM) family of the immunoglobulin superfamily (IgSF) comprises four members, CADM1－CADM4, and participates in the formation of epithelial and synaptic adhesion through cell－cell homophilic and heterophilic interactions. To identify the partners interacting with each mem-

ber of the CADM family proteins, we set up a platform for multiple detection of the extracellular protein − protein interactions using SPRi and analyzed the interactions between the CADM family proteins and 10 IgSF of their structurally related cell adhesion molecules. SPRi analysis identified a new interaction between CADM1 and CADM4, where this heterophilic interaction was shown to be involved in morphological spreading of adult T-cell leukemia cells expressing CADM1 when incubated on CADM4-coated glass. Moreover, class-I MHC-restricted T-cell-associated molecule (CRTAM) was identified to show the highest affinity to CADM1 among its binding partners by comparing the dissociation constants calculated from the SPR sensorgrams. These results suggest that the SPRi platform would provide a novel screening tool to characterize extracellular protein − protein interactions among cell surface- and secreted proteins, including IgSF molecules (1, 12).

2. Studies for establishing novel diagnostic and therapeutic approaches to a subset of human cancer

Takeshi Ito, Ayaka Sato, Yuki Kumagai, Yumi Tsuboi, Tomoko Masuda, Daisuke Matsubara, Zenichi Tanei[2], Masako Ikemura[2], Toshio Niki[3], Yasuyuki Seto[4], Masashi Fukayama[2] and Yoshinori Murakami: [2]Department of Pathology and [4]Department of Breast and Endocrine Surgery, Graduate School of Medicine, The University of Tokyo, [3]Department of Integrative Pathology, Jichi Medical University

CADM1 is overexpressed in adult T-cell leukemia (ATL) and small cell lung cancer (SCLC), conferring highly invasive or metastatic phenotypes characteristic to ATL or SCLC. To establish sensitive diagnostic tools of ATL or SCLC through detecting CADM1, monoclonal antibodies against the fragments of CADM1 overexpressed in ATL or SCLC are being generated and characterized in collaboration with scientists in the Institute of Advanced Science and Technology, the University of Tokyo. Detection systems of ATL and SCLC by these antibodies are being validated using the serum from patients of ATL and SCLC in collaboration with clinical oncologists in the University of Tokyo Hospital and National Cancer Research Center Hospital. These antibodies would be also promising to generate several therapeutic approaches, including radioisotope-conjugated antibodies and chimeric antigen receptor-T cell therapy.

Circulating tumor DNA (ctDNA) was also analyzed in plasma from patients of early-stage breast cancer surgically resected at the University of Tokyo Hospital, Tokyo, Japan using the *PICK3CA* mutation as an indicator (2). Similar analysis was carried out to establish a diagnostic approach to early-stage or early-phase recurrence of thyroid cancer using V600E mutation of the *BRAF* gene. Comparative analysis of pre- and post-operative plasm from thyroid cancer patients would provide a promising information to predict locally invasive phenotype and recurrence of thyroid cancer.

To unveil additional molecular mechanisms underlying multistage carcinogenesis, genomic, epigenomic, and transcriptional alterations in key molecules, including CADM1, in human tumorigenesis were examined in breast and other cancers in collaboration with others (3, 4). In lung carcinogenesis, emphysema appears to be a kind of precancerous lesion and we identified that loss of CADM1 function is involved in emphysema formation in collaboration with others (5).

3. Genomic-epidemiological studies of various human diseases on the basis of Biobank Japan.

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A large number of genomic DNA from normal peripheral lymphocytes as well as serum samples from more than 267,000 cases with 51 diseases was collected and preserved in BioBank Japan in the Institute of Medical Science, the University of Tokyo. These DNA samples and clinical information were shown to be valuable for genome-wide association study (GWAS) of susceptibility of various human diseases, including gastric cancer, colorectal cancer, ovarian cancer, hypospadias and diabetes, in collaboration with a large study group of order-made medicine and basic and clinical geneticists in Japan (6-10, 13, 14). Serum samples collected from a large number of patients and collected in Biobank Japan was also shown to be in high quality for various proteomic and metabolomic analyses in collaboration with others (11).

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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). In addition, we are investigating novel molecular mechanisms how tumor microenvironments and inflammation are regulated.

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 (K48)-linked ubiquitination and proteasome-dependent degradation of IκB. 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 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. Molecular mechanism of RANK signaling in osteoclastogenesis

Yuu Taguchi, Yui Iwamae, Yuki Nakano, Mikako Suzuki, Saya Bando, Youko Hirayama, Jin Gohda[1], and Jun-ichiro Inoue

Bone is an important organ, which supports body structure and hematopoiesis. 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 generates bone matrix. As a result of excess formation or activation of osteoclasts, pathological bone resorption is observed in postmenopausal osteoporosis, rheumatoid arthritis and bone metastasis. 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 macrophage colony-stimulating factor (M-CSF) and receptor activator of NF-κB ligand (RANKL). It is known that the activation of signal transduction pathway emanating from receptor RANK is essential for osteoclastogenesis. The RANK signal activates transcriptional factors, NF-κB and AP-1, through the E3 ubiquitin ligase TRAF6, and induces activation of PLCγ2-mediated Ca^{2+} signaling pathway. These signals lead to the induction of NFATc1, a master transcriptional factor in osteoclastogenesis. We have previously demonstrated that RANK has a functional amino acid sequences, 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 of signal complex is involved in sustaining activation of RANK signaling, and is essential for the NFATc1 induction and osteoclastogenesis. To elucidate other functions and the precise molecular mechanism of HCR, we have performed yeast two-hybrid screening and protein-array to identify the interacting protein to receptor RANK including HCR. Some candidate proteins were associated with RANK and HCR, and were involved in the induction of osteoclast-specific gene expression, suggesting that HCR has an additional function other than NFATc1 induction. We are currently investigating the molecular mechanisms of these candidate proteins in osteoclastogenesis. Moreover, to reveal the novel mechanisms involved in osteoclastogenesis, we performed microarray analysis of gene expression levels during osteoclastogenesis. Since some genes were dramatically downregulated in response to RANKL stimulation, we are currently investigating whether these genes are involved in the regulation of osteoclastogenesis *in vitro* and *in vivo*. We also obtained candidate genes involved in osteoclastogenesis by using CRISPR/Cas9-gRNA library screening system. Furthermore, to develop drugs for treatment to osteoporosis, we tried to search candidate compounds which have an ability to suppress osteoclastogenesis. Because some compounds showed inhibitory effect to osteoclastogenesis *in vitro* without arresting cell growth, we are now trying to elucidate mechanisms of inhibition.

3. Intratumoral bidirectional transitions between epithelial and mesenchymal cells in triple-negative breast cancer

Mizuki Yamamoto, Chiho Abe, Aya Watanabe and Jun-ichiro Inoue

Epithelial-mesenchymal transition (EMT) and its reverse process, MET, are crucial in several stages of cancer metastasis. EMT allows cancer cells to move to proximal blood vessels for intravasation. However, because EMT and MET processes are dynamic, mesenchymal cancer cells are likely to undergo MET transiently and subsequently re-undergo EMT to restart the metastatic process. Therefore, spatiotemporally-coordinated mutual regulation between EMT and MET could occur during metastasis.

To elucidate such regulation, we chose HCC38, a human triple-negative breast cancer cell line, because HCC38 is composed of epithelial and mesenchymal populations at a fixed ratio even though mesenchymal cells proliferate significantly more slowly than epithelial cells. We established E-cadherin- and Vimentin-reporter expressing HCC38 cells to analyze EMT status using FACS analysis and live cell imaging. Using this cell, we performed CRISPR/Cas9-mediated screening for intratumoral EMT-regulating genes and found several candidates.

4. Molecular mechanism of the Flavi virus E-protein-mediated membrane fusion.

Mizuki Yamamoto, Yusuke Fujinami, Aya Watanabe and Jun-ichiro Inoue

We have developed a cell-based fusion assay for prME protein of Flavi virus in a low pH-dependent manner, using Aedes albopictus cell line C6/36 cells expressing Renilla luciferase (RL)-based split reporter proteins. Using this assay, we are investigating molecular mechanisms for the E-protein-mediated membrane fusion.

5. Mint3 depletion attenuates proliferation in pancreatic cancer cells

Akane Kanamori, Jun-ichiro Inoue and Takeharu Sakamoto

Pancreatic cancer is one of the deadliest cancers. Although severe hypoxia is characteristic for pancreatic cancer, most cancer cells grow in normoxic and modest hypoxic areas. Hypoxia inducible factor-1 (HIF-1) is a master transcriptional factor for hypoxic response and thought to promote the malignancy of pancreatic cancer not only in severe hypoxic area but also in normoxic and modest hypoxic areas where cancer cells grow. However, the importance of HIF-1 in pancreatic cancer proliferation under the normoxic condition remains unclear. To address this, we focused on Mint3 which activates HIF-1 even in normoxia in cancer cells. Mint3 depletion attenuated proliferation in human pancreatic cancer AsPC1, BxPC3, Panc-1, and MIA-PaCa2 cells. Further analyses revealed that Mint3 depletion caused cell cycle arrest with increased p21 and p27 expression in pancreatic cancer cells in a HIF-1-dependent manner. Mint3 depletion also attenuated orthotopic tumor growth of AsPC1 cells in immunodeficient mice. Thus, Mint3 is a possible target for pancreas cancer treatment.

6. Pathophysiological analyses of the genes related to oxygen-dependent cancer cell proliferation using genetically modified mice

Yuya Fukui, Yoohwa Chung, Jun-ichiro Inoue and Takeharu Sakamoto

Responses to outside stimuli are essential for body homeostasis and dysregulation of these responses can cause pathological conditions such as inflammatory diseases and cancer. We previously surveyed the genes related to oxygen-dependent cancer cell proliferation using genome-wide shRNA libraries and identified 13 genes. Among them, we further focused on two genes, *X* and *Y*, whose pathophysiological roles remain unclear and established conventional and conditional knockout mice of these genes. Gene *X* was expressed ubiquitously in adult mouse tissues and Gene *X* knockout mice showed embryonic lethality. Interestingly, depletion of Gene *X* in myeloid cells promoted acute inflammation in TPA-induced ear inflammation, DSS-induced colitis, and LPS-induced septic shock models without affecting myeloid cell differentiation in the steady state. Meanwhile, Gene *Y* was predominantly expressed in testes among adult mouse tissues. Gene *Y* knockout mice were born less frequently than expected Mendelian ratio. Gene *Y* knockout mice exhibited male infertility. Thus, Gene *Y* contributes to embryogenesis to some extent and male fertility *in vivo*. We further examined the role of Gene *Y* in the tumor microenvironment. Tamoxifen-induced Gene *Y* conditional knockout mice showed attenuated tumor growth of injected murine breast cancer E0771 cells compared with control mice. Further analyses revealed that Gene *Y* was expressed in some of $CD45^+$ cells in E0771 tumor tissues. Thus, Gene *Y* expression in some $CD45^+$ cells might affect tumor growth.

Publications

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Department of Cancer Biology

Division of Genetics
腫瘍抑制分野

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

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, dysregulated 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 N-terminal pleckstrin homology (PH) and phosphotyrosine binding (PTB) domains, followed by Src homology 2 (SH2) target motifs in the C-terminal moiety, suggesting an adaptor function. Indeed, as described below, Dok-1 and Dok-2 recruit p120 ras-GAP 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 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 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. In addition, we have recently found that postnatal knockdown of *dok-7* gene expression in mice causes structural defects in NMJs and myasthenic pathology, suggesting an essential role for Dok-7 not only in the embryonic formation but also in the postnatal maintenance of NMJs.

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 in the embryonic NMJ formation (*Nature* 489: 438-442, 2012), this apparent cooperation between Lrp4 and Dok-7 in MuSK activation may be complicated.

Although we previously failed to detect MuSK activation in cultured myotubes by Dok-7 that lacks the C-terminal region (Dok-7-ΔC), we have recently found that purified, recombinant Dok-7-ΔC shows marginal ability to activate MuSK's cytoplasmic portion, carrying the kinase domain. Consistently, forced expression of Dok-7-ΔC rescued Dok-7 knockout mice from neonatal lethality caused by the lack of NMJs, indicating restored MuSK activation and NMJ formation. However, these mice showed only marginal activation of MuSK and died by 3 weeks of age apparently due to an abnormally small number and size of NMJs. Therefore, Dok-7's C-terminal region plays a key, but not fully essential, role in MuSK activation and NMJ formation.

Interestingly, overexpression of Dok-7 in skeletal muscle abnormally activates MuSK, leading to the formation of abnormally large NMJs in mice. However, these mice with abnormally large NMJs show no obvious motor dysfunction. We are investigating electrophysiological and ultrastructural properties of the abnormally large NMJs in vivo.

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

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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 minimal regions of agrin required for the role to understand molecular mechanisms underlying the agrin-mediated 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 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 functions and detailed pathophysiology, including electrophysiology and ultrastructural physiology in the Dok-7 KI mice with or without potential therapeutic treatments.

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. Indeed, we have recently found that therapeutic administration of AAV-D7 is beneficial to other mouse models of neuromuscular disorders, including amyotrophic lateral sclerosis (ALS), a progressive, multifactorial motor neurodegenerative disease with severe muscle atrophy. We are further investigating the effects of AAV-D7 administration in other types of muscle weakness.

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, and that these Lrp4 antibodies inhibited agrin and MuSK-mediated AChR clustering in cultured myotubes (*J. Neurol*., 259: 427-435, 2012; *Arch. Neurol*., 69: 445-451, 2012). Also it was reported that antibodies to Lrp4 inhibited agrin/MuSK signaling and induced MG in model animals (*J. Clin. Invest*., 123: 5190-5202, 2013). Thus, LRP4 antibodies appear to be a new biomarker of MG (*Ann. N.Y. Acad. Sci.* 1413: 126-135, 2018). We are investigating pathogenicity of Lrp4 antibodies.

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

Inoue-Yamauchi, A., Jozawa, H., Arimura, S., Kajikawa, S., Kanno, T., Takasaka, K., Shimura, E.[1], Hayata, T.[2], Ezura, Y.[3], Taguchi, Y.[4], Ueta, R., Oda, H.[5], Nakae, S.[1], Inoue, J.[4], Yoshida, N.[6], Noda, M.[3], and Yamanashi, Y.: [1]Laboratory of Systems Biology, IMSUT. [2]Department of Biological Signaling and Regulation, Faculty of Medicine, University of Tsukuba. [3]Department of Skeletal Molecular Pharmacology, Medical Research Institute, Tokyo Medical and Dental University. [4]Division of Cellular and Molecular Biology, IMSUT. [5]Department of Pathology, Tokyo Women's Medical University. [6]Laboratory of Developmental Genetics, IMSUT.

Dok-family proteins can be classified into three subgroups based on their structural similarities and expression patterns; namely, 1) Dok-1, -2, and -3, which are preferentially expressed in hematopoietic cells, 2) Dok-4, -5, and -6, which are preferentially expressed in non-hematopoietic cells, and 3) Dok-7, which is preferentially expressed in muscle cells. As mentioned above, Dok-1 and its closest paralog, Dok-2, recruit p120 rasGAP upon tyrosine phosphorylation to suppress Ras-Erk signaling. Although Dok-3 does not bind with p120 rasGAP, it also inhibits Ras-Erk signaling. Consistently, we 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. Also, we found that Dok-1 and Dok-2 negatively regulate intestinal inflammation in the dextran sulfate sodium-induced colitis model, apparently through the induction of IL-17A and IL-22 expression. However, we have recently found that Dok-3 and Dok-1/-2 play distinctive roles in cell fusion and proliferation during osteoclastogenesis and cooperatively protect mice from osteopenia. We are further investigating roles of Dok-1 to Dok-6, including those in tumor malignancy, inflammatory disorders, bone homeostasis, and other types of intractable diseases.

7. Omic analyses.

Ueta, R., Eguchi, T., Jozawa, H., Saito A., Tezuka, T., Arimura, S., Miyoshi. S., Takada Y.[1], Iemura, S.[2], Natsume, T.[3], Kozuka-Hata, H.[4], Oyama, M.[4], and Yamanashi, Y.: [1]Laboratory of Glyco-bioengineering, The Noguchi Institute, [2]Translational Research Center, Fukushima Medical University. [3]National Institute of Advanced Science and Technology, Molecular Profiling Research Center for Drug Discovery. [4]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 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, downregulation 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 glycomic, transcriptomic and metabolomic data from skeletal muscle in order to understand molecular mechanisms underlying muscle weakness.

8. Screening of chemical compound and siRNA libraries.

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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 are investigating in vivo effects of hit compounds or down-regulation of candidate genes, and continue the ongoing screenings to further collect appropriate hit compounds and candidate genes that may regulate important signalings. We are also investigating target proteins for the hit compounds to understand their modes of actions.

Publications

Kajikawa S., Taguchi Y., Hayata T., Ezura Y., Ueta R., Arimura S., Inoue J.I., Noda M., and Yamanashi Y. Dok-3 and Dok-1/-2 adaptors play distinctive roles in cell fusion and proliferation during osteoclastogenesis and cooperatively protect mice from osteopenia. *Biochemical and Biophysical Research Communications* 498: 967-974 (2018)

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In response to genetic and epigenetic insults, normal human cells execute various cellular responses such as transient cell cycle arrest, apoptosis, and cellular senescence as an anti-tumorigenesis barrier. Our research interests are to elucidate the mechanisms underlying these cellular responses. On the basis of these mechanisms, our final goal is to develop innovative cancer therapies and prevention. We are currently working on regulatory mechanisms of senescence and their implications in aging and carcinogenesis in vivo. Mechanisms underlying maintenance of genomic and epigenomic integrities such as DNA methylation maintenance and spindle assembly checkpoints are also under investigation.

1. Regulatory mechanisms of aging and carcinogenesis by senescent cells *in vivo*

Yoshikazu Johmura, Chieko Konishi, Sayaka Yamane, Yoshie Chiba, Dan Li, Wang Tehwei, Takehiro Yamanaka, Kisho Yokote, Narumi Suzuki, Shizuka Takeyama, Honoka Hagiwara, Satotaka Ohmori, Tomomi Kanai, Akane Kenjo, and Makoto Nakanishi:

Several lines of evidences have underpinned a prominent role of senescent cells in aging, life span and pathogenesis of age-associated disorders. Durable cessation of proliferation in response to any growth signals is an essential hallmark of senescence, but other characters including apoptosis resistance, mitochondrial dysfunction, and secretory phenotypes (SASP) are also notable. In addition to the above physiological aspects, senescent cells showed various unique morphological peculiarity in metabolic organelles. These morphological characters are typically observed independent of senescence-inducting stimuli and thus might underlie common metabolic reorganizations that ensure their survival and physiological traits.

Accumulation of senescent cells strongly associates with a variety of age-related pathologies, such as atherosclerosis, type II diabetes, and cancers. Recently, selective elimination of p16-positive senescent cells (senolysis) by transgenic approach from progeroid and normally aged mice has been reported to improve the healthy lifespan and ameliorates the consequences of various age-related disorders. Because of the characteristic heterogeneity of senescence *in vivo* depending on a variety of the inducing factors such as DNA damage, oncogene activation, oxidative stress as well as telomere shortening, the compounds and molecular targets that effectively induce a lethality into all types of senescent cells have not yet been identified. In order to develop such drugs, we uncovered the metabolic vulnerability in senescence in which glutaminolysis was addicted for lysosome-mediated intracellular low pH. Inhibition of glutaminolysis effectively induced senolysis both *in vitro* and *in vivo*.

2. Fbxo22 as a prognostic value for luminal A-type breast cancers

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nishi, Sayaka Yamane, Yoshie Chiba, Dan Li, Wang Tehwei, Takehiro Yamanaka, Kisho Yokote, Shizuka Takeyama, Honoka Hagiwara, Satotaka Ohmori, Tomomi Kanai, Akane Kenjo, Tomohiko Ohta[1], and Makoto Nakanishi: [1]Department of Translational Oncology, St. Marianna University Graduate School of Medicine

Breast cancer is the most frequently diagnosed cancer in women. Approximately 70% of breast cancers are positive for estrogen receptor-α (ER), and tamoxifen (TAM) is the standard drug for treatment of ER-positive breast cancer, especially for premenopausal women. Treatment with TAM as an adjuvant decreases the annual breast cancer mortality by approximately 30%. However, up to 25% of patients with early stage breast cancer treated with TAM experience relapse of the disease within 15 years. Hence, modification of the treatment is absolutely required for some populations. We demonstrated an unidentified series of regulatory mechanisms controlling cofactor dynamics on ER and TAM function whose activities require F-box protein 22 (Fbxo22). Skp, Cullin, F-box containing complex ($SCF)^{Fbxo22}$ ubiquitylates lysine demethylase 4B (KDM4B) complexed with TAM-bound ER, whose degradation releases steroid receptor coactivator (SRC) from ER. Depletion of Fbxo22 results in ER-dependent transcriptional activation via transactivation function 1 (AF1) function even in the presence of SERMs. In living cells, tamoxifen releases SRC and KDM4B from ER in a Fbxo22-dependent manner. SRC release by tamoxifen requires Fbxo22 on almost all ER-SRC-bound enhancer/promoters. Tamoxifen fails to prevent growth of Fbxo22-depleted ER-positive breast cancers both *in vitro* and *in vivo*. Clinically, a low level of Fbxo22 in tumor tissues predicts a poorer outcome in ER-positive/Human Epidermal Growth Factor Receptor Type 2 (HER-2)-negative breast cancers with high hazard ratios independently of other markers such as Ki-67 and node status. We propose that the level of Fbxo22 in tumor tissues defines a new subclass of ER-positive breast cancers for which patients SCF^{Fbxo22}-mediated KDM4B degradation can be a therapeutic target by the next generation of SERMs.

3. Regulation of maintenance DNA methylation by dual-mono ubiquitylated PAF15

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DNA cytosine methylation is a conserved epigenetic modification essential for embryonic development, transcriptional regulation, and genome stability. In higher eukaryotes, individual differentiated cells possess unique DNA methylation patterns that determine their own cellular phenotypes. Therefore, the DNA methylation pattern must be precisely maintained in coordination with DNA replication during S phase. We and others have recently reported that UHRF1-mediated dual mono-ubiquitylation of histone H3 (H3Ub2) on lysine residues 14, 18 and 23 plays a role in the recruitment of DNMT1 and its enzymatic activation, ensuring the high fidelity of maintenance DNA methylation. We have recently identified another mechanism coupled with DNA replication machinery for the recruitment of DNMT1 to replicating chromatin in which UHRF1-mediated dual mono-ubiquitylation of PCNA-associated factor 15 (PAF15) plays an essential role. In *Xenopus* cell- free extracts, PAF15 accumulates on chromatin with dual mono-ubiquitylation (PAF15Ub2) in a PCNA-, UHRF1- and DNA replication-dependent manner. PAF15Ub2 interacts with DNMT1 with a structural mode similar to that of H3Ub2. Suppression of DNMT1 interaction with H3Ub2 in the extract shows that PAF15 and histone H3 have non-redundant roles in the recruitment of DNMT1 and subsequent DNA methylation. Consistent with this, in mammals, PAF15 is also subjected to UHRF1-dependent dual mono-ubiquitylation and is capable of binding to DNMT1. Mouse embryonic stem cells expressing PAF15 carrying substitutions of lysine to arginine at ubiquitylation sites demonstrate a marked reduction in the DNA methylation level. Together, our study reveals that PAF15 and histone H3 have non-redundant roles in the regulation of DNA methylation maintenance.

4. Regulatory mechanisms of chromosome segregation by mitotic rounding

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During mitosis, animal cells undergo dynamic reorganization of cell shape, from flat to round. To generate force for mitotic rounding, cells increase their cortical tension and intracellular pressure. Mitotic cell rounding is critical for chromosome segregation, development, tissue organization, and tumor-suppression. Mitotic cell rounding requires at least three key modules: 1) F-actin regulated by RhoA and an actin nucleator formin DIAPH1, 2) Myosin II regulated by Rac1 and Cdc42, and 3) the

Ezrin, Radixin and Moesin (ERM) family proteins.

DIAPH1 is a member of actin nucleator formin family proteins, whose mutations are associated with various diseases including nonsyndromic deafness and microcephaly. Formin family proteins are defined by the formin homology 1 (FH1) and formin homology 2 (FH2) domains. The formin homology 1 (FH1) domain is required for the interaction to the actin monomer-binding protein profilin, whereas FH2 domain is responsible for actin filament nucleation. Diaphanous-related formins (DRFs) compose a subgroup activated by the binding of Rho-type small GTPases. DRFs are involved in organizing various cytoskeletal structures such as filopodia, lamellipodia and cytokinetic contractile rings. Among them, DIAPH1 is required for actin stress fiber formation and maintenance of cortical force during mitotic cell rounding. Therefore, we hypothesized that RhoA-DIAPH1-PFN1 axis could be minutely regulated during mitosis. We are now investigating mechanisms of how Cdk1, a master regulator of mitosis, regulates this axis and coordinates between mitotic rounding and chromosome segregation.

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