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 oncogenesis

Takeshi Ito, Yuki Kumagai, Zenichi Tanei, Yumi Tsuboi, Masaru Kasai, Atsuko Nakamura, Daisuke Suzuki, Toko Funaki, Yutaro Shiraishi, Tomoko Maruyama, Hiromi Ichihara, Kaoru Kiguchi, Motoi Oba,[1] Akiteru Goto[2] and Yoshinori Murakami; [1]Research Institute of Molecular and Cell Biology of Cancer, Showa University, [2]Department of Pathology, Akita University Graduate School of Medicine

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 the roles of CADM1 in lung adenocarcinoma cells with the mutated *EGFR* or various amplified receptor tyrosine kinase (RTK) genes. Northern and western blotting analyses showed that CADM1 expression was frequently lost in these lung adenocarcinoma cells. When CADM1 expression was restored, proliferative activity was strongly suppressed in lung cancer cells with amplification of a subset of RTK genes, while no suppression was observed in lung cancer cells with the *EGFR* mutation, suggesting that CADM1 could suppress specific oncogenic pathways by cross talking on the cell membrane. We have also demonstrated the importance of CADM1 and its ligand, CRTAM, in CD4+ cytotoxic T lymphocyte lineage in collaboration with others (1). Additional roles of immunoglobulin superfamily cell adhesion molecules in pancreatic cancer was also investigated in collaboration with others (2).

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 highly invasive or metastatic phenotypes characteristic to ATL or SCLC. By generating a cell-based assay to measure the activity of trans-homophilic signaling of CADM1 as cell spreading activity of ATL cells, we found that PI3K, as well as Akt and Rac1, act in the downstream of CADM1 signaling. On the other hand, down-regulation of PI3K, Akt and Rac1 significantly suppressed CADM1-mediated adhesion and spreading of ATL cells in the cell based assay. These findings suggest that PI3K, Akt and Rac1

could provide potential targets for suppressing invasive or metastatic phenotype of ATL or SCLC cells. To establish sensitive diagnostic tools of SCLC through detecting CADM1, monoclonal antibodies against the fragment 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. These antibodies would be useful to improve the FACS system to identify and characterize malignant ATL cells, whose prototype has already been established in the IMSUT Hospital. These antibodies would be also promising to generate several therapeutic approaches, including radioisotope-conjugated antibodies and chimeric antigen receptor-T cell therapy. Novel detection systems of SCLC are also being investigated by the support of several grants of applied medical sciences.

2. Molecular pathological analyses of human lung cancer

Takeshi Ito, Daisuke Matsubara[3], Atsuko Nakamura, Toshiro Niki[2] and Yoshinori Murakami; [2]Department of Integrative Pathology, [3]Jichi Medical University

The hippo pathway appears to be important in tumor development and progression because it is associated with contact inhibition of normal cells caused by cell attachment signaling. In the course of analyzing the Hippo pathway, we are interested in overexpression of YAP1, the main Hippo pathway effector and a potent oncogene, in non-small-cell lung cancer (NSCLC); however, the YAP1 expression pattern in small-cell lung cancer (SCLC) has not yet been elucidated in detail. We report that the loss of YAP1 is a special feature of high-grade neuroendocrine lung tumors. A hierarchical cluster analysis of 15 high-grade neuroendocrine tumor cell lines containing 14 SCLC cell lines that depended on the genes of Hippo pathway molecules and neuroendocrine markers clearly classified these lines into two groups: the YAP1-negative and neuroendocrine marker-positive group (n = 11), and the YAP1-positive and neuroendocrine marker-negative group (n = 4). Among the 41 NSCLC cell lines examined, the loss of YAP1 was only observed in one cell line showing the strong expression of neuroendocrine markers. Immunostaining for YAP1, using the sections of 189 NSCLC, 41 SCLC, and 30 large cell neuroendocrine carcinoma (LCNEC) cases, revealed that the loss of YAP1 was common in SCLC (40/41, 98%) and LCNEC (18/30, 60%), but was rare in NSCLC (6/189, 3%). Among the SCLC and LCNEC cases tested, the loss of YAP1 correlated with the expression of neuroendocrine markers, and a survival analysis revealed that YAP1-negative cases were more chemosensitive than YAP1-positive cases. Chemosensitivity test for cisplatin using YAP1-positive/YAP1-negative SCLC cell lines also showed compatible results. YAP1-sh-mediated knockdown induced the neuroendocrine marker RAB3a, which suggested the possible involvement of YAP1 in the regulation of neuroendocrine differentiation. Thus, we showed that the loss of YAP1 has potential as a clinical marker for predicting neuroendocrine features and chemosensitivity (3).

3. Analyses of genomic and epigenomic alterations of human lung and breast cancer and other common diseases.

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To unveil additional molecular mechanisms underying multistage carcinogenesis, genomic, epigenomic, and transcriptional alterations in key molecules in human tumorigenesis were also examined in various cancers. Circulating tumor DNAs were also being analyzed to obtain possible markers for predicting therapeutic effects of breast cancer. A large number of genomic DNA from normal peripheral lymphocytes as well as serum samples from more than 200,000 cases with 47 diseases collected and stored in BioBank Japan was shown to be valuable to obtain the precise view of clinical features and genetic polymorphisms associated with the onset of human diseases in collaboration with a large study group of order-made medicine in Japan (4-6).

4. Analyses of novel signalling pathways in cancer cells and macrophages that are associated with cell survival in various stresses, including hypoxic condition..

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Unlike most cells, cancer cells activate hypoxia inducible factor-1 (HIF-1) to use glycolysis even at normal oxygen levels, or normoxia. Therefore, HIF-1 is an attractive target in cancer therapy. However, the regulation of HIF-1 during normoxia is not well characterised. We have recently demonstrated that Mint3 activates HIF-1 in cancer cells and macrophages by suppressing the HIF-1 inhibitor, factor inhibiting HIF-1 (FIH-1). We have identified N-terminal EF-hand calcium binding protein 3

(NECAB3) as a novel binding factor of Mint3 that regulates HIF-1 activity by yeast two-hybrid screening. NECAB3 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 NECAB3 decreased the expression of HIF-1 target genes and reduced glycolysis in normoxic cancer cells. NECAB3 mutants that binds Mint3 but lacks an intact monooxygenase domain also inhibited HIF-1 activation. Inhibition of NECAB3 in cancer cells by either expressing shRNAs or generating a dominant negative mutant reduced tumourigenicity. Taken together, the data indicate that NECAB3 is a promising new target for cancer therapy (7).

Since Mint3 promotes ATP production via glycolysis by activating HIF-1 in macrophages, we have also examined possible roles of Mint3 in lung pathogenesis and anti-viral defence upon IFV infection. We demonstrated that Mint3-deficient mice exhibited improved influenza pneumonia with reduced inflammatory cytokines/chemokine levels and neutrophil infiltration in the IFV-infected lungs without alteration in viral burden, type-I interferon production, or acquired immunity. These results suggest that Mint3 might represent one of the likely therapeutic targets for the treatment of severe influenza pneumonia without affecting host anti-viral defence through suppressing macrophage cytokine/chemokine production (8). Additional functions of Mint3 in immune system was examined in collaboration with others (9).

Furthermore, to understand the mechanisms of cancer cells to survive in the hypoxic condition, we have previously identified several genes whose expressions were induced in hypoxia. A ring finger protein 126 (RNF126) is one of these genes encoding an E3 ubiquitin ligase and plays a pivotal role in the resistance of cancer cells to the stress associated with non-adherent conditions. Loss of anchorage to the extracellular matrix leads to apoptosis (anoikis) in normal cells, but cancerous cells are usually resistant to such stress. Non-adherent cancer cells exhibited increased flux through the tricarboxylic acid cycle via increased conversion of pyruvate to acetyl CoA. RNF126 was found to act as a ubiquitin ligase for pyruvate dehydrogenase kinases (PDKs), resulting in their proteasomal degradation. This decrease in PDK levels allowed pyruvate dehydrogenases to catalyze the conversion of pyruvate to acetyl CoA. Moreover, depletion of RNF126 or increased expression of PDK1 in cancer cells suppressed colony formation in soft agar as well as tumorigenicity in mice. RNF126 expression in cancer cells was found to be under the control of the ERK signaling pathway, which is essential for anoikis resistance. These finidngs suggest that RNF126 is an attractive molecule for treating cancer by selectively targeting anchorage-independent growth (10).

Publications

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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 (K48)-linked ubiquitination and proteasome-depend-

ent 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. HTLV-1 Tax induces formation of the active macromolecular IKK complex by generating Lys63- and Met-1-linked hybrid polyubiquitin chains

Yuri Shibata, Ginga Komatsu, Jin Gohda[2] and Jun-ichiro Inoue

Activation of NF-κB by human T-cell leukemia virus type 1 (HTLV-1) Tax is thought to be crucial in T-cell transformation and the onset of adult T-cell leukemia (ATL). Therefore, a better understanding of the precise mechanism underlying aberrant NF-κB activation is essential to develop new therapeutic approaches. It is well known that Tax activates NF-κB through activation of the IKK complex by generating Lys63-linked polyubiquitin chains. However, the molecular mechanism underlying Tax-induced IKK activation is not fully understood. In this study, we demonstrate that Tax recruits linear (Met1-linked) ubiquitin chain assembly complex (LUBAC) to the IKK complex and that Tax fails to induce IKK activation in cells that lack LUBAC activity. The ubiquitin absolute quantification (ubiquitin-AQUA) analyses revealed that both Lys63-linked and Met1-linked polyubiquitin chains are associated with the IKK complex. Furthermore, treatment of the IKK-associated polyubiquitin chains with Met1-linked-chain-specific deubiquitinase (OTULIN) resulted in the reduction of high molecular weight polyubiquitin chains and the generation of short Lys63-linked ubiquitin chains, indicating that Tax can induce the generation of Lys63- and Met1-linked hybrid polyubiquitin chains. We also demonstrate that Tax induces formation of the active macromolecular IKK complex and that the blocking of Tax-induced polyubiquitin chain synthesis inhibited formation of the macromolecular complex. Taken together, Tax triggers Lys63- and Met1-linked hybrid polyubiquitin chains by recruiting LUBAC to the IKK complex, leading to the formation of the active macromolecular IKK complex.

3. Molecular mechanism of RANK signaling in osteoclastogenesis

Yuu Taguchi, Yo Yumiketa, Yui Iwamae, Kichi Chou, Youko Hirayama, Masaaki Oyama[1], Hiroko Kozuka-Hata[1], Jin Gohda[2], 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 nuclear factor-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 also 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, termed 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 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 expression of some osteoclast-specific genes, 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 vivo* by using CRISPR/Cas9 system. Furthermore, we tried to elucidate the TRAF6-dependent molecular mechanism at the subsequent step of NFATc1 induction in osteoclastogenesis such as cell-cell fusion and actin ring formation.

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

Mizuki Yamamoto, Kota Sakane 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.

RANK signaling triggered by progesterone-induced RANKL leads to mammary stem cell (MaSC) generation and promotes pregnancy-induced epithelial cell differentiation and expansion to enable mammary gland development. RANK activates three pathways, including the canonical and non-canonical NF-κB pathways and the pathway that induces Id2 nuclear translocation. While the Id2 pathway leads to cell survival and maturation, the distribution of roles played by the two NF-κB pathways remains to be elucidated. To determine the function of TRAF6-canonical NF-κB pathway on mammary gland development, we are analyzing TRAF6-deficient mammary gland structure and gene expression profiles, and found that TRAF6-canonical NF-κB pathway regulates pregnancy-induced epithelial cell expansion.

5. Identification of embryonic precursor cells that differentiate into Aire-expressing thymic epithelial cells

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Medullary thymic epithelial cells expressing autoimmune regulator ($Aire^+$ mTECs) play essential role on preventing the onset of autoimmunity. However, the differentiation program of $Aire^+$ mTECs is not fully understood. We previously reported that receptor activator of nuclear factor-κB (RANK) signaling is essential for differentiation of $Aire^+$ mTECs in embryo. In this study, we found the embryonic precursors of $Aire^+$ mTECs (pMECs) by monitoring the expression of receptor activator of nuclear factor-κB (RANK). We evidenced that pMECs give rise to $Aire^+$ mTECs in re-aggregation thymic organ culture (RTOC) in vitro and in vivo. Surprisingly, although pMECs differentiate into mTECs but not into cortical TECs, pMECs express cortical TEC molecules in addition to mTEC markers UEA-1 ligand and RANK. Introduction of pMECs in the embryonic thymus efficiently inhibited the autoimmunity onset provoked by deficiency of $Aire^+$ mTEC. We further found that RANK signaling mediated by signal transducer TRAF6 promotes differentiation of pMECs into $Aire^+$ mTECs. Moreover, non-classical nuclear factor-κB activation activated by RANK and lymphotoxin-β-receptor signaling controls pMEC induction from progenitors (pro-pMECs) that express lower level of RANK and higher level of CD24 expression. As result, this study clarified two novel stages in the program of $Aire^+$ mTECs differentiation.

Publications

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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.

Ueta, R., Eguchi, T., Tezuka, T., Izawa, Y., Miyoshi, S., Weatherbee, SD.[1], Nagatoishi, S.[2], Tsumoto, K.[2], and Yamanashi, Y.: [1]Department of Genetics, Yale University. [2]Medical Proteomics Laboratory, IMSUT.

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 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. 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. We are investigating how the C-terminal region acts 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 agrin-interacting proteins for their involvements in 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 gra-

vis) 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 functions and detailed pathophysiology, including electrophysiology, 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. Indeed, we have recently found that therapeutic administration of AAV-D7 is beneficial to other mouse models of neuromuscular disorders. We are further investigating the effects of AAV-D7 administration in detail.

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). Given that Lrp4 antibodies are found in patients of amyotrophic lateral sclerosis (ALS) (*Ann. Clin. Transl. Neurol.*, 1: 80-87, 2014), which is associated with NMJ defects, Lrp4 antibodies may be involved in a range of neuromuscular disorders that feature defects in NMJs, including those of unknown etiology. We are investigating pathogenicity of Lrp4 antibodies.

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 have recently 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. 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., Miyoshi. S., Arimura, S., Jozawa, H., Ueta, R., Iemura, S.[1], Natsume, T.[2], Sagara, H.[3], Kozuka-Hata, H.[3], Oyama, M.[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, 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. Given that many genes encoding glycosylation enzymes have been found to be involved in the pathogenesis of CMS, we are investigating roles for glycosylation of Lrp4 and other NMJ-related proteins in their activities.

8. Screening of chemical compound and siRNA libraries.

Ueta, R., Kosuge, M., Yamazaki, S., Waseda, M., Kito, M., Yukimura, R., 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

Ueta R., Tezuka T., Izawa Y., Miyoshi S., Nagatoishi S., Tsumoto K., and Yamanashi Y. The carboxyl-terminal region of Dok-7 plays a key, but not essential, role in activation of muscle-specific receptor kinase MuSK and neuromuscular synapse formation. *J. Biochem.*, in press

Waseda M., Arimura S., Shimura E., Nakae S., and Yamanashi Y. Loss of Dok-1 and Dok-2 in mice causes severe experimental colitis accompanied by reduced expression of IL-17A and IL-22. *Biochem. Biophys. Res. Comm.,* 478: 135-142 (2016)

Eguchi T., Tezuka T., Miyoshi S., and Yamanashi Y. Postnatal knockdown of *dok-7* gene expression in mice causes structural defects in neuromuscular synapses and myasthenic pathology. *Genes Cells*, 21: 670-676 (2016)

Ohashi T., Yamamoto T., Yamanashi Y., and Ohsugi M. Human TUBG2 gene is expressed as two splice variant mRNA and involved in cell growth. *FEBS Letters,* 590: 1053-1063 (2016)

Department of Cancer Biology

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Normal human cells possess various anti-tumor barriers such as cell cycle checkpoints, apoptosis, and cellular senescence against genetic and epigenetic alterations. Our research interests are to elucidate the mechanisms underlying these cellular responses. On the basis of basic findings, our final goal is to develop innovative cancer therapies and prevention. We are currently working on regulatory mechanisms of senescence induction, maintenance, and their clearance. Maintenance of epigenome information including DNA methylation during cell proliferation whose abnormalities occur at early phase of human carcinogenesis is also under investigation.

1. Mechanism of senescence induction and maintenance

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One important hallmark of senescence is the inability to proliferate in response to physiological mitotic stimuli. The limited lifespan of human cells is governed by telomere shortening as well as various genotoxic stressors, all of which ultimately activate DNA damage responses. We and others have recently uncovered the molecular mechanisms involved in permanent cell cycle arrest during the senescence process in which p53 activation at G2 plays a necessary and sufficient role by inducing a mitosis skip. Another hallmark of senescence is the appearance of senescence-associated secretory phenotypes (SASP), such as robust secretion of numerous growth factors, cytokines, proteases, and other proteins, that can cause deleterious effects on the tissue microenvironment. On the other hand, SASP also has positive effects on the repair of damaged tissue, at least at a young age. Induction of these two hallmarks of senescence is often coordinated, but their respective mechanisms do not always overlap. Most notably, p38MAPK is critically required for SASP through activating NF-κB independently of canonical DDR, but p53 restrains p38MAPK, leading to the suppression of SASP in senescent cells. There appear to be missing links that could more fully explain the antagonistic effects of p53 on the induction of these two representative hallmarks of senescence.

The key to the regulation of p53 activity is control of the stability of its protein, which is mainly orchestrated through a network of ubiquitylation reactions, although other mechanisms such as regulation of its localization are also involved. While numerous E3 ubiquitin ligases for p53 have been reported, data are less clear regarding the *in vivo* relevance of these E3 ligases in p53 regulation except for murine double minute 2 (Mdm2) Mdm2 is itself a transcriptional target of p53, and acts to create a negative feedback loop. Importantly, in mice with a disrupted p53-Mdm2 feedback loop, the degradation profile of p53 upon DNA damage ap-

peared to be normal, calling the role of Mdm2 as the sole E3 ubiquitin ligase for stress-induced p53 into question. In order to uncover the mechanisms underlying negative regulation of p53 during senescence maintenance, we performed gene expression analysis using normal and sorted senescent cells and found that Fbxo22 was highly expressed in senescent cells in a p53-dependent manner. Moreover, SCF^{Fbxo22} ubiquitylated p53 and formed a complex with a lysine demethylase, KDM4A. Ectopic expression of a catalytic mutant of KDM4A stabilized p53 and enhanced p53 interaction with PHF20 in the presence of Fbxo22. SCF^{Fbxo22}-KDM4A was required for the induction of p16 and senescence-associated secretory phenotypes at the late phase of senescence. *Fbxo22*$^{-/-}$ mice were almost half the size of *Fbxo22*$^{+/-}$ mice due to the accumulation of p53. These results indicate that SCF^{Fbxo22}-KDM4A is an E3 ubiquitin ligase that targets methylated p53 and regulates key senescent processes.

2. Mechanisms of maintenance DNA methylation during cell proliferation

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Methylation of the cytosine residue at CpG sites has a crucial role in early embryonic development and cellular differentiation in vertebrates. Maintenance DNA methylation is mainly regulated by Dnmt1, which converts hemi-methylated DNA to its fully methylated form. We have recently unraveled a mechanism underlying Dnmt1 recruitment to hemi-methylated DNA sites by Uhrf1 (Ubiquitin-like, containing PHD and RING finger domains 1) in which Uhrf1-dependent ubiquitylation of histone H3 (H3) plays an essential role. Recently, ubiquitin interacting motif (UIM) within replication foci targeting sequence domain (RFTS) of Dnmt1 was proposed as an ubiquitylated H3 (H3Ub) binding region. However, for the rigorous inheritance of DNA methylation patterns, recognition of hemi-methylated DNA region by Dnmt1 must be of high affinity and specific, suggesting the existence of a unique and unidentified module of H3Ub recognition by Dnmt1.

Fine-tuned regulation of DNA methyltransferase activities is also required for rigorous inheritance of DNA methylation patterns. Recently, unexpected regulatory principles of DNA methyltransferases (DNMTs) were identified, in which their catalytic activities are auto-inhibited by their intramolecular domain-domain interactions. In the case of Dnmt1, crystal structure of nearly full-length Dnmt1 revealed that the RFTS is deeply inserted into the DNA-binding pocket of the catalytic domain, indicating its auto-inhibitory mode. Thus, above two distinct functions of RFTS suggest the molecular coupling between targeting and activation of Dnmt1 at DNA replication sites.

To address this important issue, we identified two mono ubiquitylated histone H3 as a unique and specific structure that is preferentially recognized by Dnmt1. In addition, crystal structure of RFTS of Dnmt1 in complex with H3-K18/23Ub2 revealed that the two ubiquitins were simultaneously bound to RFTS via canonical hydrophobic and atypical hydrophilic interactions. The C-lobe of RFTS together with K23Ub surface also recognized N-terminal tail of H3. Binding of H3-K18/23Ub2 also underwent spatial rearrangement of two lobes in RFTS, suggesting the opening of its active site. Incubation of Dnmt1 with H3-K18/23Ub2 drastically increased its catalytic activity *in vitro*. Our results thus shed light on the essential role of previously unidentified and unique module of Dnmt1, which recognizes H3Ub2 in rigorous maintenance of DNA methylation.

3. Mechanisms of proper chromosomal segregation during mitosis

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Successful chromosome segregation is coordinated by phosphorylation and dephosphorylation of a set of proteins that localize to chromosomes during mitosis. The chromosomal passenger complex (CPC) is a highly conserved complex that orchestrates various mitotic events. Aurora B kinase within the CPC plays a central role in triggering mitotic processes through phosphorylation of substantive substrates. Therefore, the dynamic localization and activation of CPC should be rigorously regulated with respect to time and space to ensure accurate chromosome segregation. However, the mechanism leading to the culminated and circumscribed activation of Aurora B at centromeres has not been fully understood. In order to address this important issue, we analyzed a role of histone H2AX, a variant of H2A, in Aurora B activation. We found that Aurora B-mediated phosphorylation of histone H2AX at serine 121 (H2AX-pS121) promoted Aurora B autophosphorylation and was essential for proper chromosome segregation. Aurora B-mediated H2AX-pS121 was specifically detected at the centromere during mitosis. H2AX depletion resulted in a severe defect in activation and deposition of Aurora B at this locus. A phosphomimic mutant of H2AX at S121 interacted with activated

Aurora B more efficiently than wild-type *in vitro*. Taken together, these results proposed a model in which Aurora B-mediated H2AX-pS121 likely provided a platform for Aurora B auto-activation circuitry at centromeres and thus played a pivotal role in proper chromosome segregation.

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