Department of Cancer Biology

Division of Oncology
癌細胞シグナル研究分野

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Structural and functional analysis of the protooncogenes reveals that a large majority of them encode protein-tyrosine kinases or transcription factors. The characterization of protooncogenes is important not only for understanding the development of malignant tumors but also for elucidating the mechanism of the regulation of growth and function of normal cells. Our current interest is to characterize the cellular signaling mediated by protein tyrosine phosphorylation in the central nervous system and cancer cells. We are also interested in the function of antioncogene products and serine/threonine phosphorylation-mediated signaling that are involved in the regulation of cell cycle progression.

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

Toru Suzuki, Junko Tsuzuku, Mitsuhiro Yoneda, Atsuko Negishi, Masahiro Morita, Takamitsu Nishikawa, and Tadashi Yamamoto

By screening a cDNA expression library with autophosphorylated c-ErbB-2 protein, we isolated cDNA clones coding for its possible interaction molecules. One of the genes thus identified, termed *tob*, encodes a 45kDa protein with homology to the growth suppressing proteins, Btg1 and Btg2/PC3. We also cloned novel genes homologous to *tob* that were termed *tob2* and *ana*. These proteins compose a functionally related anti-proliferative protein family, called the Tob/Btg family

To elucidate the physiological function of the Tob/Btg family proteins, we generated mice lacking *tob*, *tob2*, or *ana*. *tob*-deficient mice ($tob^{-/-}$) had a greater bone mass resulting from increased number of osteoblasts. We also found that aged $tob^{-/-}$ mice develop a variety of tumors. To circumvent functional redundancy between Tob and Tob2, double deficient animals ($tob^{-/-}tob2^{-/-}$ mice) were generated. Analysis of their phenotypes is underway. Unlike other Tob family proteins, Ana is specifically expressed in type II alveolar epithelial cells. Since lung adenocarcinoma is thought to be mainly derived from type II alveolar epithelial cells, Ana may be involved in development of lung tumor. Indeed, *ana*-deficient mice developed spontaneous tumors including lung adenocarcinoma. We also found that expression of *ana* gene was largely reduced in almost all of the lung cancer cell lines and clinical samples of lung adenocarcinoma examined. These data suggested that downregulation of *ana* gene might be responsible for lung adenocarcinoma progression. Now, we investigate the molecular mechanism by which Ana regulates progression of lung tumor.

Recently, we found that Tob is involved in DNA damage response. Tob protein and mRNA is rapidly induced after DNA damage such as

UV irradiation. Increase of Tob after DNA damage is independent of tumor suppressor p53, in contrast to *btg2*. Severe DNA damage promoted rapid degradation of Tob and subsequent apoptotic cell death, whereas mild DNA damage activated transcription of the *tob* gene without inducing apoptosis. Thus, Tob may be involved in the mechanism which determines whether damaged cells undergo apoptosis or cease to grow.

Other studies to establish biological significance of the *tob* family members include the followings: (1) *Generation of cnot7-deficient (cnot7$^{-/-}$) mice*. We have already identified CNot7 as a Tob-interacting molecule by yeast two-hybrid screening. *cnot7$^{-/-}$* males are sterile owing to oligo-astheno-teratozoospermia, suggesting that CNot7 is essential for spermatogenesis. To investigate the functional relationships between CNot7 and Tob family proteins, we examined the biological significance of CNot7 in bone formation. Like *tob$^{-/-}$* mice, micro-CT analysis of the bone volume revealed enhancement of bone volume per tissue in *cnot7$^{-/-}$* mice as compared to control mice. In order to examine the osteoblastic properties of the cells, bone marrow cells were cultured in the presence or absence of β-glycerophosphate and ascorbate. CNot7 deficiency enhanced alizarin red positive nodule formation compared to control cells. In contrast, TRAP-positive osteoclast-like cell development in bone marrow cells was similar regardless of genotype, suggesting that deficiency of CNot7 mainly affects cells in the osteoblast lineage. (2) *Search for other molecules that interact with Tob*. We purified Tob-containing complexes from Hela cells that stably express Tob tagged with the Flag epitope by immunoprecipitation with anti-Flag antibody. Mass spectrometric analysis showed that CNot1, CNot2, CNot3, CNot6/CCR 4 and CNot7 were all included in the Tob-containing complex. Although the CCR4/Not complex possesses mRNA deadenylase activity, thus regulating the stability of mRNAs, biological roles of the complex in mammals remain unknown. We also performed yeast two-hybrid screening using Tob as a bait, and then identified a serine/threonine kinase. Interestingly, a yeast homolog of this kinase is reported to associate with the CCR4/Not complex. Now, we are examining the biological functions of CCR4/Not complex by generating mice lacking each components of the complex. (3) *Analysis of CCR4/Not complex*. We newly identified and characterized CCR4b/CNot6L, which is homologous to yeast CCR4 mRNA deadenylase. CCR4b is localized mainly in the cytoplasm and displays deadenylase activity both *in vitro* and *in vivo*. CCR4b forms a multi-subunit complex similar to the yeast CCR4/Not complex. Suppression of CCR4 b by RNA interference (RNAi) results in growth retardation of NIH3T3 cells accompanied by elevation of both *p27^{Kip1}* mRNA and p27^{Kip1} protein. Reintroduction of wild-type CCR4b but not mutant CCR4b lacking deadenylase activity restores the growth of CCR4b-depleted NIH3T3 cells. The data suggest that CCR4b regulates the cell growth in a manner dependent on its deadenylase activity. Our findings suggest that CCR4b deadenylase is a constituent of mammalian CCR 4/Not complex and regulates the turnover rate of specific target mRNAs. Now, we examine the effect of RNAi-mediated knock down of the other components of the CCR4/Not complex on cell proliferation and other biological phenomenon.

2. Role of chromokinesin Kid and mitotic kinases in execution of cell division

Miho Ohsugi, Noriko Tokai-Nishizumi, Keiko Haraguchi, Chenyu Bai, Naoki Oshimori, Natsuko Masuda, and Tadashi Yamamoto

Mitosis is a process whereby a complete copy of the genetic information is distributed to each new cell. This process is critically important, with even small errors leading to aneuploidy or cell death. The chromosomal and/or centrosomal abnormalities are often observed in tumor cells and those abnormalities may often be the first events in the development of a cancer. It is well known that microtubule-based motor proteins are involved in spindle formation and chromosome movements in mitosis. In addition, orchestrated regulation by mitotic kinases is important for the progression of each step of mitosis.

i) Chromokinesin Kid

The human chromokinesin Kid/Kinesin-10 is a member of the chromosome-associated kinesin family identified in our lab in 1996. Kid is a plus end-directed microtubule-based motor protein with DNA-binding domains, and is localized to entire length of chromosomes and spindles during prometaphase and metaphase. We found that depletion of Kid from HeLa cells using RNA interference (RNAi) causes multiple phenotypes, including misalignment of metaphase chromosomes, shortening of the spindle pole-to-pole distance, and defects in anaphase chromosome segregation. These phenotypes reflect the multiple roles of Kid during mitosis. First, Kid is required for the proper chromosome arm alignment at the metaphase plate. Second, independent of its role in chromosome movement, Kid contributes to spindle morphogenesis by inducing microtubule bundling to stabilize spindle microtubules. In addition, at

anaphase, Kid shows unique localization in the interstices between adjacent chromosomes. In Kid-depleted HeLa cells, anaphase chromosomes appeared to less tightly adhere to each other. Our data suggest that Kid may play a role in connecting adjacent chromosomes to hold individual anaphase chromosomes together by acting as a glue able to bind to both MTs and chromosomes. As for the regulatory mechanism of Kid, we defined two functional nuclear localization signals in Kid and identified Importin α as a Kid binding proteins. Our ongoing study suggests that Importin α/β complex controls both localization and functions of Kid. Furthermore, we have generated *Kid*-deficient mice, and found that more than half of the $Kid^{-/-}$ embryos die before implantation. Further analysis of *Kid*-deficient mice will facilitate our understanding of the physiological role and importance of Kid.

ii) Mitotic kinases

Plk1 (polo-like kinase 1) is a highly conserved serine/threonine kinase that plays multiple pivotal roles in mitosis, meiosis, and also in oncogenesis. However, the exact mechanisms of Plk 1's actions remain to be elucidated. To address this issue, we searched for novel substrates of Plk-1 by solid-phase phosphorylation screen. We have identified several possible substrates of Plk 1, including a novel centrosomal protein Kizuna (Kiz). RNAi-mediated depletion of Kiz causes fragmentation and dissociation of pericentriolar material from centrioles at prometaphase, resulting in multipolar spindles. Our data demonstrate that Kiz is critical for establishing mitotic centrosome architecture that can endure the forces that converge on the centrosome during spindle formation and chromosome congression. Our data also suggest that Plk1 maintains the integrity of the spindle pole by phosphorylating Kiz. We are currently investigating other newly identified substrates of Plk1, which will uncover the molecular mechanisms underlying the Plk1-mediated control of mitosis, meiosis and oncogenesis.

hLATS1 and *hLATS2* are other mitotic serine/threonine kinases which share significant homology with a *Drosophila* tumor suppressor gene *lats*. FISH analysis revealed that the *hLATS1* and *hLATS2* genes are localized to chromosome 6q24 -25.1 and 13q11-12, respectively, in which loss of heterozygosity is observed in various cancers. To examine the role of hLATS2 in tumorigenesis and cell cycle regulation, we searched for hLATS2-binding proteins and identified several LIM domain-containing proteins including Ajuba and Zrp1/Trip-6. We found that Ajuba interacts with beta-catenin and negatively regulates beta-catenin-TCF-induced transactivation. We showed that Ajuba promotes GSK-3beta mediated phosphorylation of beta-catenin and subsequent degradation of beta-catenin. Our data suggest a novel function of Ajuba as a negative regulator of the Wnt signaling pathway. We also explore the role of Zrp1/Trip-6 in focal adhesions and cell-cell contact sites, which are disassembled during mitosis, and found that Zrp1 plays an important role in the signaling pathway that regulates activities of RhoGTPase families.

3. Roles of protein-tyrosine kinases and phosphatases in the central nervous system

Tohru Tezuka, Takanobu Nakazawa, Kazumasa Yokoyama, Shin-ichiro Kina, June Goto, Mina Delawary, Sachiko Taniguchi, Naosuke Hoshina, Miho Ohsugi, and Tadashi Yamamoto

The Src-family protein-tyrosine kinases (PTKs) are implicated in various neural functions such as formation of neural network, myelination, and synaptic plasticity. To analyze the roles of Src and Fyn, we have been focusing on the various substrates of these kinases, including N-methyl-D-aspartate (NMDA) type of ionotropic glutamate receptors, p250GAP, TCGAP, and Nogo-A.

NMDA receptors play important roles in synaptogenesis, synaptic plasticity, and excitotoxity. Our own studies have shown that NR2A and NR2B subunits of NMDA receptors are the major substrates of Fyn in neuron. To elucidate the biological significance of Fyn-mediated phosphorylation of NR2A and NR2B at the molecular level, we have determined Fyn-mediated phosphorylation sites on NR2A and NR2B subunits. To establish the biological significance of Tyr-1472 phosphorylation, which is the major tyrosine phosphorylation site on NR2B, we generated mice with a knock-in mutation of the Tyr -1472 site to phenylalanine (YF mice) and showed that Tyr-1472 phosphorylation is essential for amygdala-dependent learning. Furthermore, YF mice showed increased anxiety, which may be also due to the impaired amygdala functions. We thus identify Tyr-1472 phosphorylation as a key mediator of synaptic plasticity, fear-related learning, and emotional behavior in the amygdala. We have also found that Tyr-1325 on NR2A is a major tyrosine phosphorylation site. Tyr-1325 phosphorylation is important for modulating NMDA receptor-mediated currents in recombinant NR1-NR2A channels. Roles of Tyr-1325 phosphorylation of NR2A are going to be analyzed using similar strategices emploged

for Tyr-1472 phosphorylation of NR2B.

In parallel of these studies, to uncover the signaling pathways in which Src and Fyn are involved, we have been trying to identify binding partners, substrates, and transcriptional target genes of these kinases in the brain. We have already identified a number of novel proteins that could be associate with or are phosphorylated by the Src-family kinases. Characterization of these putative binding partners and substrates that include RhoGAPs, RhoGEFs, and proteins without known motifs is in progress. One of them is Nogo-A, a neurite outgrowth inhibitor protein associated with myelin. Fyn phosphorylated Nogo-A in cells that expressed these proteins exogenously. We also found that Nogo-A was phosphorylated at Tyr-694 in the N-terminal region of Nogo-A. Tyr-694 phosphorylation of Nogo-A may regulate Nogo-A function such as the *in vitro* inhibitory activity. Alternatively, given that Nogo-A functions as a receptor that mediates signals from neurons to oligodendrocytes, Tyr-694 phosphorylation of Nogo-A may regulate intracellular signaling events.

Accumulating evidence shows that not only tyrosine phosphorylation but also tyrosine dephosphorylation regulates various brain functions. We have shown that protein-tyrosine phosphatase PTPMEG binds to glutamate receptors, NMDA receptor and GluRδ2, via its PDZ domain. PTPMEG is a family member of band 4.1 domain-containing protein-tyrosine phosphatases, and is expressed prominently in thalamus and cerebellum in the brain. To know the physiological roles of PTPMEG, we generated PTPMEG$^{-/-}$ mice. The mice showed impaired cerebellum-dependent learning and synaptic plasticity, suggesting that tyrosine dephosphorylation events regulated by PTPMEG are critical for functions of the cerebellum.

The Cbl-family ubiquitin ligases are also associated with and phosphorylated by the Src-family kinases. Among the family members, Cbl and Cbl-b are expressed in the brain with distinct expression profiles. We found that Cbl-family proteins suppress the signaling through various tyrosine kinases such as Src, ErbB-2, and ErbB-4 through ubiquitination of these kinases. In addition, we found that Cbl also ubiqutinates tyrosine-phosphorylated mDab1, an essential protein for brain architecture. Roles of Cbl and Cbl-b in neural functions are under investigation.

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Interaction of cell surface molecules and their ligand mediates complex arrays of cell signaling. These cell surface events are profoundly affected by various types of post-translational modifications. Proteolysis on cell surface is one of such post-translational mechanisms regulating cellular functions in physiological and pathological situations. Most of the extracellular proteinases belong either to serine proteinase or metalloproteinase. We have been studying membrane-anchored type of matrix metalloproteinases called membrane-type matrix metalloproteinases (MT-MMPs). Our current issue is to understand how these MT-MMPs are regulated in physiological condition or what kinds of roles they play in the cellular events such as tumor invasion and proliferation.

1. Multifunctional roles of MT1-MMP in myofiber formation and morphostatic maintenance of skeletal muscle

Ohtake, Y. Tojo, H. Seiki, M.

Sequential activation of muscle-specific transcription factors is the critical basis for myogenic differentiation. However, the complexity of this process does not exclude the possibility that other molecules and systems are regulatory as well. We observed that myogenic differentiation proceeded through three distinct stages of proliferation, elongation and fusion, which are distinguishable by their cellular morphologies and gene expression patterns of proliferation- and differentiation-specific markers. Treatment of the differentiating myoblasts with inhibitors of matrix metalloproteinases (MMPs) revealed that MMP activity at the elongation stage is a critical prerequisite to complete the successive myoblast cell fusion. The MMP regulated the myogenic differentiation independently from the genetic program that governs expression of the myogenic genes. Membrane-type 1 matrix metalloproteinase (MT1-MMP) was identified as a major contributor to this checkpoint for morphological differentiation and degraded fibronectin, a possible inhibitory factor for myogenic cell fusion. A MT1-MMP deficiency caused similar myogenic impediments forming smaller myofibers in situ. Additionally, the mutant mice demonstrated some central nucleation of the myofibers typically found in muscular dystrophy and MT1-MMP was found to cleave laminin-2/4 in the basement membrane. Thus, MT1-MMP is a new multilateral regulator for muscle differentiation and maintenance through processing of stage-specific distinct ECM substrates.

2. Membrane type 1 matrix metalloproteinase (MT1-MMP/MMP-14) cleaves and releases a 22-kDa extracellular matrix metalloproteinase inducer (EMMPRIN) fragment from tumor cells

Egawa, N. Koshikawa, N. Tomari, T. Nabeshima, K. Isobe, T. Seiki, M.

Proteolytic shedding is an important step in the functional down-regulation and turnover of most membrane proteins at the cell surface. Extracellular matrix metalloproteinase inducer (EMMPRIN) is a multifunctional glycoprotein that has two Ig-like domains in its extracellular portion and functions in cell adhesion as an inducer of matrix metalloproteinase (MMP) expression in surrounding cells. Although the shedding of EMMPRIN is reportedly because of cleavage by metalloproteinases, the responsible proteases, cleavage sites, and stimulants are not yet known. In this study, we found that human tumor HT1080 and A431 cells shed a 22-kDa EMMPRIN fragment into the culture medium. The shedding was enhanced by phorbol 12-myristate 13-acetate and inhibited by TIMP-2 but not by TIMP-1, suggesting the involvement of membrane-type MMPs (MT-MMPs). Indeed, down-regulation of the MT1-MMP expression in A431 cells using small interfering RNA inhibited the shedding. The 22-kDa fragment was purified, and the C-terminal amino acid was determined. A synthetic peptide spanning the cutting site was cleaved by MT1-MMP in vitro. The cleavage site is located in the linker region connecting the two Ig-like domains. The N-terminal Ig-like domain is important for the MMP inducing activity of EMMPRIN and for cell-cell interactions, presumably through its ability to engage in homophilic interactions, and the 22-kDa fragment retained the ability to augment MMP-2 expression in human fibroblasts. Thus, the MT1-MMP-dependent cleavage eliminates the functional N-terminal domain of EMMPRIN from the cell surface, which is expected to down-regulate its function. At the same time, the released 22-kDa fragment may mediate the expression of MMPs in tumor tissues.

3. Cell surface collagenolysis requires homodimerization of the membrane-bound collagenase MT1-MMP

Itoh, Y. Ito, N. Nagase, H. Evans, R.D. Bird, S. A. Seiki, M.

Pericellular degradation of interstitial collagens is a crucial event for cells to migrate through the dense connective tissue matrices, where collagens exist as insoluble fibers. A key proteinase that participates in this process is considered to be membrane-type 1 matrix metalloproteinase (MT1-MMP or MMP-14), but little is known about the mechanism by which it cleaves the insoluble collagen. Here we report that homodimerization of MT1-MMP through its hemopexin (Hpx) domain is essential for cleaving type I collagen fibers at the cell surface. When dimerization was blocked by coexpressing either a membrane-bound or a soluble form of the Hpx domain, cell surface collagenolytic activity was inhibited in a dose-dependent manner. When MMP-13, a soluble collagenase active as a monomer in solution, was expressed as a membrane-anchored form on the cell surface, homodimerization was also required to cleave collagen. Our results introduce a new concept in that pericellular collagenolysis is regulated by correct molecular assembly of the membrane-anchored collagenase, thereby governing the directionality of the cell to migrate in tissue.

4. Regulated nucleo-cytoplasmic shuttling of human aci-reductone dioxygenase (hADI1) and its potential role in mRNA processing

Gotoh, I. Uekita, T. and Seiki, M.

Bacterial aci-reductone dioxygenase (ARD), a member of the cupin superfamily, has evolutionarily primitive protein folding and functions in the methionine recycling pathway. Recently, a human ARD orthologue (human ADI1, hADI1) has been identified and exhibits functions other than ARD activity. hADI1 localizes mainly to the cytoplasm, but a substantial fraction is nuclear, suggesting functions in both cellular compartments. In this study, we report that nucleo-cytoplasmic transport of hADI1 is regulated by a non-canonical nuclear export signal (NES) located in the N-terminal region of hADI1. The NES is composed of multiple basic amino-acid residues instead of the canonical leucine-rich sequence. Nuclear export of hADI1 was not mediated by CRM1, a major transporter that binds to leucine-rich NES. Substitution of the basic residues with alanines abolished NES activity. Mutant hADI1 accumulated in the nucleus and formed speckles frequently observed with splicing factors and some transcription factors. Indeed, hADI1 specifically co-localized with the splicing factor U1-70K to the nucleus but not with another splicing factor, SC35. U1-70K overexpression induced nuclear accumulation of hADI1. Nuclear hADI1 expression significantly altered the splicing pattern of the adenovirus E1 A mini-gene, which generates multiple alternatively spliced transcripts. Thus, hADI1 may have acquired a novel role in nuclear mRNA processing possibly by modulating U1-70K-related functions, an activity negatively regulated by a non-classical NES sequence.

5. Neovessel-Mural Cell Crosstalk Directs the Site-Specific Expression of MT1-MMP to Endothelial Tip Cells

Yana, I. Sagara, H. Takai, S. Takatsu, K. Nakamura, K. Nakao K. Katsuki M. Taniguchi, S. Aoki, T. Sato, H. Weiss, S. Seiki, M.

The membrane-anchored matrix metalloproteinase (MMP), MT1-MMP, plays a key role in the angiogenic process, but the mechanisms underlying its spatiotemporal regulation in the in vivo setting have not been defined. Using whole mount immunohistochemical analysis and a β-galactosidase transgene inserted into mt1-mmp, we demonstrate that MT1-MMP expression in vivo is confined to the sprouting tip of neocapillary structures where endothelial cell proliferation and collagen degradation are coordinately localized. During angiogenesis in vitro wherein endothelial cells are stimulated to undergo neovessel formation in the presence or absence of accessory mural cells, site-specific MT1-MMP expression is shown to be controlled by endothelial cell-vascular smooth muscle cell (VSMC) crosstalk. When vessel maturation induced by VSMCs is inhibited by introducing a soluble form of the receptor tyrosine kinase, tie2, MT1-MMP distribution is no longer restricted to the endothelial tip cells, but instead distributes throughout the neovessel network in vitro as well as ex vivo. Taken together, these data demonstrate that vascular maturation coordinated by endothelial cell/mural cell interactions redirects MT1-MMP expression to the neovessel tip where the protease regulates matrix remodeling at the leading edge of the developing vasculature.

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Division of Cancer Genomics
癌遺伝形質分野

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Cell motility is the basis of various physiological and pathological phenomena, such as inflammation, morphogenesis, and metastasis of cancer cells. In order for cells to move, cells dynamically reorganize their actin and microtubular cytoskeletons. We have been examining the regulatory mechanism of the cytoskeleton in response to extracellular stimuli. We are also investigating the Wnt and Hedgehog signals, which regulate morphogenesis and are involved in development of human cancers.

1. Polarity-regulating kinase Par1b/MARK2 negatively regulates development of dendrites on hippocampal neurons

Takeshi Terabayashi, and Hiroaki Miki

Neurons are highly polarized cells that possess two types of protrusions, axons and dendrites, that function in the transmission and reception of neural signals, respectively. Recent studies elucidated that Wnt signaling regulates dendrite development, but the molecular mechanism of how it specifically affects dendrites, but not axons, remains unknown. We report here that a polarity-regulating kinase, Par1b/MARK 2, specifically regulates dendrite development in hippocampal neurons. Expression of Par1b/MARK2 shortens the length and decreases the branching of dendrites, but not of axons. Knockdown of endogenous Par1b/MARK2 by RNA interference stimulates dendrite development. When neurons were stimulated with Wnt to induce dendrite development, Par1b/MARK2 was recruited to membranes. Expression of a Par1b/MARK2 mutant that accumulates exclusively in membranes does not affect dendrite development. In addition, Par1b/MARK2 efficiently phosphorylates MAP2, which is localized mainly in dendrites. These results implicate that Par1 b/MARK2 negatively regulates dendrite development downstream of Wnt signaling through phosphorylation of MAP2.

2. The thioredoxin-related redox-regulating protein nucleoredoxin inhibits Wnt-beta-catenin signalling through Dishevelled

Yosuke Funato, and Hiroaki Miki

Dishevelled (Dvl) transduces signals from the Wnt receptor, Frizzled, to downstream components, leading to the stabilization of beta-catenin and subsequent activation of the transcription factor T cell factor (TCF) and/or lymphoid enchancer factor (LEF). However, the mechanism of Dvl action remains unclear. Here, we report that nucleoredoxin (NRX), a thioredoxin (TRX) family protein, interacts with Dvl. Overexpression of NRX selectively suppresses the Wnt-beta-catenin pathway and ablation of NRX by RNA-interference (RNAi) results in activation of TCF, accelerated cell proliferation and enhancement of oncogenicity through cooperation with mitogen-activated extracellular signal regulated kinase kinase (MEK) or Ras. We find that cells respond to H2O2 stimulation by activating

TCF. Redox-dependent activation of the Wnt-beta-catenin pathway occurs independently of extracellular Wnts and is impaired by RNAi of NRX. In addition, association between Dvl and NRX is inhibited by H2O2 treatment. These data suggest a relationship between the Wnt-beta-catenin pathway and redox signalling through redox-sensitive association of NRX with Dvl.

3. Fused kinase is stabilized by Cdc37/Hsp90 and enhances Gli protein levels

Yoshiaki Kise, Kei Takenaka, and Hiroaki Miki

Serine/threonine kinase Fused (Fu) is an essential component of Hedgehog (Hh) signaling in *Drosophila*, but the biochemical functions of Fu remain unclear. Here, we have investigated proteins co-precipitated with mammalian Fu and identified a kinase-specific chaperone complex, Cdc37/Hsp90, as a novel-binding partner of Fu. Inhibition of Hsp90 function by geldanamycin (GA) induces rapid degradation of Fu through a ubiquitin-proteasome pathway. We next show that co-expression of Fu with transcription factors Gli1 and Gli2 significantly increases their protein levels and luciferase reporter activities, which are blocked by GA. These increases can be ascribed to Fu-mediated stabilization of Gli because co-expression of Fu prolongs half-life of Gli1 and reduces polyubiquitination of Gli1. Finally, we show that GA inhibits proliferation of PC3, a Hh signaling-activated prostate cancer cell line. This growth inhibition is partially rescued by expression of ectopic Gli1, suggesting that Fu may contribute to enhance Hh signaling activity in cancer cells.

4. GSK3beta positively regulates Hedgehog signaling through Sufu in mammalian cells.

Kei Takenaka, Yoshiaki Kise, and Hiroaki Miki

Hedgehog signaling plays important roles in embryonic patterning of multicellular organisms. This pathway is ultimately transmitted by the zinc-finger transcriptional factor Gli, of which activity is suppressed by Sufu, a negative regulator of this signaling. To clarify this regulation to more detail, we screened for Sufu-binding proteins. We identified GSK3beta as a specific binding partner of Sufu by mass spectrometric analysis. GSK3beta bound to Sufu both *in vitro* and *in vivo*. Down-regulation of GSK3beta expression by RNAi in Hedgehog-responsive cells attenuated Hedgehog signaling, suggesting that GSK3beta functions as a positive regulator of Hedgehog signaling. In addition, an *in vitro* kinase assay showed that GSK3beta phosphorylates Sufu and phosphorylation-mimicking mutant of Sufu showed significantly decreased ability to bind Gli1 and could not suppress the Gli-mediated expression of a reporter gene efficiently. These results strongly suggest that GSK3beta phosphorylates Sufu to positively regulate Hedgehog signaling in mammalian cells.

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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. Thus, we are mainly focusing on five such proteins including Tumor necrosis factor receptor-associated factor 6 (TRAF6), nuclear factor-κB (NF-κB), developmentally Regulated GTP-binding protein (DRG), epidermal growth factor receptor (EGFR) and erythroblastosis B2 (ERBB2). Our current studies indicate that these proteins play critical roles in cell proliferation and differentiation. Therefore, elucidation of how these proteins work is definitely required for understanding onset of diseases. We are also searching for novel oncogenes and analyzing signal transduction pathways more comprehensively by recent techniques of genomics and proteomics.

1. The molecular mechanism of the regulation of Rel/NFκB transcription factor

Jin Gohda, Takayuki Matsumura[1], Kosuke Yamazaki, Yuko Hata[2], Masaaki Oyama[2], Kentaro Semba and Jun-ichiro Inoue: [1]Institute for Biomedical Engineering, Consolidated Research Institute for Advanced Science and Medical Care, Waseda University, [2]Medical Proteomics Laboratory, IMSUT

Transcription factor Rel/NFκB binds specifically to a decameric motif of nucleotide, κB site, and activates transcription. The activation of Rel /NFκB has been demonstrated to be carried out post-translationally upon extracellular stimuli through membrane receptors such as interleukin -1 receptor, tumor necrosis factor (TNF) α receptor or CD40. Rel/NFκB 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 Lys48-linked ubiquitination and proteasome-dependent degradation. Rel/ NFκB subsequently translocates to the nucleus to activate transcription of target genes. This project is to identify a molecule that transduces signal from membrane receptor (IL-1 receptor, TNF receptor) to Rel/NFκB/IκB complex. We have previously identified upstream activators of Rel/NFκB, tumor necrosis factor receptor-associated factor (TRAF) 5 and TRAF6. We have also found that MAPKKK, TAK1 is downstream of TRAF6 and becomes activated upon dimerization of TRAF6 and that Ubc13/Uev1A complex, together with TRAF6, catalyses the formation of a Lys63 (K63)-linked polyubiquitin chain that conjugates TRAF6 and mediates IKK activation through unique proteasome-independent mechanism. Several lines of evidence suggest that TRAF6 is able to activate some kinase other

than TAK1. To identify such kinases, we are trying to identify the molecule(s) that function between TRAF6 and the IKK complex by biochemical protein purification.

2. Role of TRAF6 in T-cell self-torelance

Taishin Akiyama, Hiroyasu Konno, Junwen Qin, Hiehiko Motegi, Yusuke Shimo, Daisuke Ohshima, Hiromi Yanai and Jun-ichiro Inoue

Thymic microenvironments are essential for generation of a T cell repertoire28. Cortical thymic epithelial cells (cTECs) are involved in selection of thymocytes capable of recognizing self-MHC, whereas medullary TECs (mTECs) play a crucial role in self-tolerance by eliminating self-reactive T cells. In TRAF6-/- mice, size of the thymic medulla was reduced, and the corticomedullary junction was ill-defined. Immunohistochemical analysis revealed an abnormal distribution and impaired maturation of mTECs in TRAF6-/- thymus. Aire protein promotes ectopic expression of peripheral tissue-specific antigens (TSAs), thereby establishing central tolerance to TSAs. Expression of aire and TSAs are reduced significantly in 14-day-old TRAF6-/- thymus. The altered thymic organization and reduced aire expression strongly suggest that TRAF6-/- mice may possess an autoimmune phenotype. This speculation was supported by observations of inflammatory infiltrates in lung, liver, pancreas, and kidney of TRAF6-/- mice. Furthermore, the number of regulatory T cells (regT) was dramatically reduced in 14-day-old TRAF6-/- mice. To determine whether the autoimmune-like phenotypes of TRAF6-/- mice are related to altered thymic stroma, fetal thymi isolated from embryonic day 14 TRAF6-/- and control mice were grafted under the renal capsules of nude mice. Eight weeks after grafting, normal generation of thymocytes and distribution of mature T cells in spleen and lymph nodes were observed in recipients grafted with TRAF6-/- thymus (KO/nu) or control thymus (WT/nu). KO/nu mice had inflammatory infiltrates in lung, liver, pancreas, and kidney similar to those in TRAF6-/- mice. Furthermore, sera from KO/nu mice contained autoantibodies against whole liver, islets in Langerhans of the pancreas, and blood vessel walls in the lung. Taken together, these data indicate that the altered thymic stroma in TRAF6-/- mice is sufficient to induce autoimmunity.

A similarly altered thymic structure and multi -organ inflammation were reported as abnormalities in RelB-deficient mice and in alymphoplasia (aly) mice, which carry a mutation in NIK. RelB and its transcripts were not detected in TRAF6-/- fetal thymic stroma, indicating that RelB expression requires TRAF6 signaling. RelB forms heterodimers with p52, a processed product of p100. Processing of p100 is triggered by phosphorylation pf p100 catalyzed by IKKα, which is activated by NIK. In aly mice, RelB induction and p100 processing are impaired, while the ratio of p52 to p100, which is indicative of p100 processing, was not affected by TRAF6-deficiency. Therefore, at least two critical NFκB-related events may be essential for the initial stage of mTEC differentiation: induction of RelB expression, which requires both TRAF6 and NIK, and optimal processing of p100 to p 52, which requires NIK.

Autoimmunity induced by TRAF6-/- thymic stroma may be due to a defect in aire gene expression or a defect in the production of regT. Reduced regT production is also observed in aly mice. These results suggests that normal development of regT requires thymic microenvironments whose formation is directed by both TRAF6 and NIK-mediated signals.

3. Role of TRAF6 in development of lymphoid organs

Taishin Akiyama, Junwen Qin and Jun-ichiro Inoue

NF-κB activation is essential for microenvironment formation in secondary lymphoid organs. However, signal transduction pathways regulating NF-κB activation in the microenvironment formation remain to be determined. We found that TRAF6 directs the expression of B-cell attracting chemokine, CXCL13, in follicular dendritic cells (FDCs) to develop B-cell follicles. Non-canonical NF-κB pathway activated by the ligation of lymphtoxin-β-receptor (LtβR) was essential for the primary B-cell cluster formation with initial growth of FDCs at early stage in neonatal mice. In contrast, TRAF6 was dispensable in this stage but was required for converting B-cell clusters to B-cell follicles and maintaining FDCs in the later stage. Furthermore, TRAF6 was dispensable for NF-κB activation induced by the LtβR ligation. These results indicate that development and maintenance of B-cell follicles and FDCs require distinct and cooperative contribution of TRAF6 and lymphotoxin-mediated pathways.

4. Role of TRAF6 in osteoclastogenesis

Jin Gohda, Takayuki, Matsumura1, Yuu Taguchi, Sayaka Yamane and Jun-ichiro Inoue

Receptor activator of NFκB (RANK) and

RANK ligand (RANKL) play crucial roles in maintaining the homeostasis of bone remodeling, which is regulated by a balance of osteoblast-mediated bone formation and osteoclast-mediated bone resorption; stimulation of RANK in osteoclast progenitor cells is an essential step for osteoclast formation. Excess formation or activity of osteoclasts in human leads to pathological bone resorption, as observed in postmenopausal osteoporosis, rheumatoid arthritis, Paget's disease and tumor bone metastases. Therefore, precise elucidation of the regulatory mechanisms of osteoclast formation, particularly the molecular mechanisms of RANK signaling, is essential for understanding the onset of skeletal diseases and for developing drugs to treat these diseases.

Intracellular signaling pathways of RANK are mediated by members of the TRAF family. TRAF2, TRAF3, TRAF5 and TRAF6 bind to the cytoplasmic tail of RANK in vitro. We showed previously that TRAF6-deficient ($TRAF6^{-/-}$) mice exhibit severe osteopetrosis and are defective in osteoclast formation due to defective signaling from RANK upon binding of RANKL. CD40, another member of the TNF receptor superfamily, transmits signals through TRAF family members, including TRAF2, TRAF3, TRAF5 and TRAF6. We have previously shown that TRAF6 plays a crucial role in CD40-mediated NFkB and MAPK activation in osteoclast progenitor cells. Despite such similarities between roles of TRAF6 in RANK signaling and those in CD40 signaling, stimulation of CD40 in osteoclast progenitor cells does not result in osteoclast formation. Therefore, RANK, but not CD 40, is able to transmit specific signals leading to osteoclastogenesis. Elucidation of the molecular mechanisms of this RANK-specific function in osteoclastogenesis is essential for developing drugs for the treatment of pathological bone resorption. Thus, we generated and expressed chimeric receptors, h40/mRK in which the extracellular domain of human CD40 was fused to the transmembrane domain and cytoplasmic tail of mouse RANK, and we searched for specific structures in RANK critical for osteoclastogenesis. Our results strongly suggest that RANK may harbor a specific domain that amplifies TRAF6 signaling. We are currently narrowing down such domains.

5. Molecular mechanisms of NF-κB activation in HTLV-1 infected cells

Jin Gohda, Takashi Nishina, Yuri Shibata and Jun-ichiro Inoue

Infection of human T-cell leukemia virus type 1 (HTLV-1) to CD4-positive T cells transforms the host cells in a long term through multiple steps, and finally leads to the onset of Adult T-cell leukemia (ATL). A transcriptional factor, NFκB is constitutively activated during transformation of T cells, and such activation is caused by HTLV-1-derived oncoprotein, Tax. However, molecular mechanism by which NFκB is activated in HTLV-1-transformed cells remains elucidated. One of MAP3K members, TAK1 plays a critical role in NFκB activation mediated by cytokines, including TNFα and IL-1, and its activation is regulated by lysine 63-linked polyubiquitination. TAK1 is constitutively activated in Tax-positive HTLV-1-transfomed cell lines. Furthermore, Tax induced lysine 63-type polyubiquitination of NEMO, a noncatalytic subunit of IκB kinase complex. However, Tax itself could not induce TAK1 activation and TAK1 was dispensable for Tax-mediated NFκB activation. In addition, CYLD that has deubiquitinating activity against lysine 63-type ubiquitin chains failed to inhibit Tax-induced NFκB activation. Finally, knockdown of other MAP3Ks (MEKK1, MEKK3, and NIK) by RNA interference technique could not influence Tax-induced NFκB activation. These findings suggest that Tax induced NFκB activation by a novel mechanism that is independent of lysine-63 type polyubiquitinqtion.

6. Functional characterization of Developmentally Regulated GTP-binding protein (DRG)

Kosuke Ishikawa, Koichi Itoh[3] and Jun-ichiro Inoue: [3]Division of Molecularbiology, IMSUT

One subfamily of the GTPase superfamily, DRG (Developmentally regulated GTP-binding protein) is composed of two protein DRG1 and DRG2. Both are highly conserved in eukaryotes, and their expressions are high in developmental or growing cells in vertebrates, suggesting very fundamental role under growth. Recent comprehensive analysis in Yeast showed DRG1 ortholog is copurified with initiation factors or elongation factors, suggesting involvement in translational machinery. Yeast DRG1 ortholog also binds YDR152w. We identified YDR152w as a novel negative regulator for the above stress signal transduction from GCN1 to GCN2. We found expression of YDR152w leads to growth defect under amino acid starvation. Interaction analyses suggested that because YDR152w has GI domain, which is also found in GCN2 N-terminal region required to interact with GCN1, YDR152w may dominant-negatively catch GCN 1 interaction in substitute for GCN2. Future

work is characterizing the nature between growth control system by DRG subfamily and its special translational control via YDR152w.

7. Lentivirus vectors expressing short hairpin RNAs to inhibit HIV replication

Takuya Yamamoto[3], Jun-ichiro Inoue and Yasuko Tsunetsugu-Yokota[4]: [4]Department of Immunology, National Institute of Infectious Diseases

Successful attempts to inhibit HIV-1 replication in T cells using RNAi have been reported. However, the effect of HIV-specific RNAi on macrophages is not well known. Macrophages are key targets for anti-HIV-1 therapy because they are able to survive long after the initial infection with HIV and can spread the virus to T cells. Therefore, we first identified a putative RNAi target of HIV, consisting of the portion of the nef gene overlapping the U3 region (Nef 366), and generated a lentivirus-based short hairpin RNA (shRNA) expression vector (Lenti shNef366). We show that Lenti shNef366 inhibits HIV-1 replication in a monocytic cell line and in primary monocyte-derived macrophages (MDMs), reactivation of latent HIV-1 infection, and the production of secondary HIV-1 from MDMs harboring a genomic copy of Nef366. Moreover, we found that the up-regulated production of macrophage inflammatory protein 1β (MIP-1β), but not MIP-1α, in MDMs by Nef expression was considerably suppressed by Lenti shNef366. These results suggest that HIV-1 dissemination to T cells through its interaction with HIV-1-infected MDMs can also be controlled by Lenti shNef366. Thus, lentivirus-mediated shRNA expression targeting the U3-overlapping region of HIV nef represents a feasible approach to genetic vaccine therapy for HIV-1.

8. Identification of NFκB-target genes in tumor cells

Noritaka Yamaguchi[1], Sakura Azuma, Shinya Watanabe[5], Kentaro Semba and Jun-ichiro Inoue: [5]Department of Clinical Informatics, Tokyo Medical Dental University School of Medicine

NFκB plays an important role in tumor development. In contrast to normal cells in which NFκB activity is transiently induced by several extracellular stimuli, many types of tumors and tumor cell lines show constitutive activation of NFκB. Overexpression of an IκBα mutant (IκB super repressor) which lacks phosphorylation sites by IKK revealed that NFκB activity contributed to the survival or growth of tumor cells, tumor invasion and metastasis and angiogenesis. Tumor cells that show constitutive NFκB activity are also highly resistant to anticancer drugs or ionizing radiation and inhibition of NFκB activity increases their sensitivity to such treatments. Therefore, it is important to elucidate gene expression network regulated by NFκB for anticancer therapy.

For this purpose, we first made gene expression profiles of one hundred and twenty-five human tumor cell lines from various tissue origins including colon, lung, pancreatic, esophagus, stomach, breast and ovary. Clustering analysis of the cell lines by NFκB status identified genes whose expression is correlated with NFκB status. We tested whether expression of those genes is repressed in breast cancer cell lines that express IκB super repressor. This analysis showed that expression of the 25 genes was correlated with NFκB status of 35 breast cancer cell lines and repressed by IκB super repressor. Ten out of them are known NFκB target genes and fifteen genes are novel. Analysis of these novel genes is in progress.

9. Analysis of EGFR-family signal network in non-small cell lung cancer by proteomic approach

Takashi Miyasaka[5], Yuko Hata[2], Masaaki Oyama[2], Jun-ichiro Inoue, Kentaro Semba and Tadashi Yamamoto[2,6]: [6]Division of Advanced Clinical Proteomics, IMSUT

Lung cancer is the leading cause of cancer mortality all over the world including Japan. Recent finding of EGFR mutation in non-small cell lung cancer (NSCLC) has dramatic impact on lung cancer therapy with EGFR inhibitor, gefitinib: Retrospective studies of NSCLC patients with gefitinib treatment showed close association between EGFR mutations and clinical response. Mutant EGFR-mediated signal transduction pathways in NSCLC are, however, just beginning to be understood. Recent proteomics techniques have enabled us to analyze signal transduction pathways more comprehensively and more quantitatively. In order to understand the complete signal transduction network mediated by the gefitinib-sensitive mutant EGF receptor (EGFR) in human non-small cell lung cancer (NSCLC), we are currently taking two approaches. First, we purified phosphotyrosine-containing protein complexes from cellular lysates of NCI-H3255 that express L858R mutant EGFR by anti-phosphotyrosine antibodies and followed by SDS-PAGE. This cell line overexpresses the constitutive activated mutant EGFR

and its growth is totally dependent on EGFR-mediated signaling. Gel purified proteins are digested with trypsin and subjected to nanoLC-MS/MS. This conventional proteomic approach has identified novel links with EGFR and several proteins including receptor tyrosine kinases, signal transduction mediators, chaperones and RNA-binding proteins. We further confirmed that at least eight proteins were tyrosine phosphorylated upon EGF stimulation. Analysis of these proteins is in progress.

10. Clustering analysis on the basis of gene expression profiling of ERBB2-amplified breast cancer cells

Kentaro Semba, Shinya Watanabe[5], Sakura Azuma and Jun-ichiro Inoue

ErbB2/Her2/Neu is a receptor-type protein tyrosine kinase, whose amplification and increased expression is closely correlated with prognosis of breast cancer. Aberrant signal transduction mediated by overexpressed ErbB2 is thought to be involved in growth and metastatic potential of breast cancer cells. Although a humanized monoclonal anti-ErbB2 antibody (trastuzumab) has shown benefits in metastatic patients, the molecular mechanism of breast cancer development, especially what genes contribute to breast tumorigenesis cooperatively with ErbB2 has not been studied extensively. To elucidate the characteristics of breast cancer cells with overexpression of ErbB2, we analyzed expression profiles of 32,000 genes in thirty-five human breast cancer cell lines. Clustering analysis based on ErbB2 mRNA level showed that a set of sixty-four genes classified breast cancer cell lines into two major groups and that tumor cells with overexpression of ErbB2 might have arisen from one of the two groups. Interestingly, at least two of the sixty-four genes were amplified in a part of breast cancer cell lines, suggesting that they may contribute to tumor progression. We also found that one of the sixty-four genes encoding a transcription factor is required for proliferation of tumor cells.

11. Identification of novel oncogenes at the amplified gene loci

Noritaka Yamaguchi[1], Sakura Azuma, Jun-ichiro Inoue, Kentaro Semba and Shinya Watanabe[5]

Aberrant gene expression due to genomic imbalances are thought to underlie tumor progression. Recent genome-wide analysis of copy number and expression have identified causative genes in various types of tumors. Alignment of the expression profiles of one hundred and twenty-five human tumor cell lines according to the chromosomal localization has enabled us to identify candidates of amplified loci (amplicons). Breast cancer is the most common cancer in women worldwide. We constructed high-resolution expression maps of 35 human breast cancer cell lines by aligning relative expression ratios of 17 373 transcripts according to their chromosomal positions. These expression maps and subsequent Southern blot analyses enabled us to identify novel amplicons on chromosomes 8q21, 17q11, 17q21, 17q25 and Xp11 in a subset of breast cancer cell lines and breast cancer specimens. The highly expressed genes identified in this study may contribute to more aggressive breast cancer phenotypes and thus may be targets for specific anticancer therapies against individual tumor subtypes.

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Our major goals are to clarify (1) signal transduction systems in cell growth, reorganization of cytoskeleton and morphogenesis and (2) roles of inositol phospholipids as biomodulators. Using variety of knockout mice, we are currently focusing on the mechanism of cellular movement based on cortical actin reorganization, as well as membrane trafficking and other physiological events mediated by inositol phospholipids signaling.

1. A novel membrane tubulation- and PI(4,5)P 2-binding domain involved in endocytosis

Tsujita K, Sasaki N, Furutani M, Oikawa T, Suetsugu S, Takenawa T

A variety of endocytic and actin regulatory proteins, such as CIP4, FBP17, syndapin/pacsins and cdc15, share FCH domain which is highly conserved from yeast to mammal. However, alignment of these proteins further showed that there is a wider conserved area than FCH domain including adjacent coiled coil region. We correctively named this entire region as extended FC (EFC) domain. Here we found that EFC domain of FBP17, CIP4, FER, PSTPIP1 and PSTPIP2 strongly binds to phosphatidiylinositol 4,5-bisphosphate [PI(4,5)P2]. Furthermore, the EFC domain tubulated plasma *in vivo* and deformed PI(4,5)P2-containing liposomes into narrow tubules *in vitro*. Mutagenesis analysis showed that evolutionally conserved basic amino acid residues were critical for lipid binding and tubulation of membranes both *in vivo* and *in vitro*. In addition, overexpression of FBP17 inhibited endocytosis of EGF receptorin an EFC domain-dependent manner. Knock down of FBP 17 also inhibited EGF uptake. FBP17 could recruit N-WASP to the plasma membrane where they activate actin polymerization. Finally, when invaginating membrane tubules were induced by FBP17 expression, EGF receptor, dynamin and N-WASP were co-localized at the membrane tubules, suggesting that these proteins form the functional complex in membrane invagination steps.

2. Optimization of WAVE2 complex-induced actin polymerization by membrane-bound IRSp53, PIP(3) and Rac.

Suetsugu S, Kurisu S, Oikawa T, Yamazaki D, Oda A, Takenawa T.

WAVE2 activates the actin-related protein (Arp) 2/3 complex for Rac-induced actin polymerization during lamellipodium formation and exists as a large protein complex with Sra1/PIR 121, Nap1, Abi1, and HSPC300. IRSp53 binds to both Rac and Cdc42 and is proposed to link Rac to WAVE2. We found that the knockdown of IRSp53 by RNA interference decreased lamel-

lipodium formation without a decrease in the amount of WAVE2 complex. Localization of WAVE2 at the cell periphery was retained in IRSp53 knockdown cells. Moreover, activated Cdc42, but not Rac, weakened the association between WAVE2 and IRSp53. When we measured Arp2/3 activation *in vitro*, the WAVE2 complex isolated from the membrane fraction was fully active in an IRSp53-dependent manner whereas WAVE2 isolated from the cytosol was not. Purified WAVE2 proteins, with or without its complex partners, were activated by the mixture of IRSp53 and PIP(3)-containing liposomes in a Rac-dependent manner. Therefore, IRSp53 optimizes the activity of the WAVE2 complex in the presence of activated Rac and PIP(3).

3. Application of phosphoinositide-binding domains for the detection and quantification of specific phosphoinositides.

Furutani M, Tsujita K, Itoh T, Ijuin T, Takenawa T.

In mammals, seven phosphoinositides are known to play crucial roles as signaling molecules in a variety of cellular processes. Their synthesis and degradation are thought to be strictly controlled by metabolic enzymes such as phosphoinositide kinases and phosphatases, and their aberrant activities cause diseases. Thus, there is a great interest in convenient and high-throughput measurement of such enzymatic activities for the screening of drugs that promote or inhibit them. To date, radioactive labeling and colorimetric detection of released inorganic phosphates are mainly applied to measure phosphoinositide kinase and phosphatase activities. Here, we describe a novel method for detecting and quantifying individual phosphoinositides via phosphoinositide-binding domains that exhibit high specificity and affinity toward this lipid. Enzyme-linked immunosorbent assay wells are modified with alkyl chains (C16), which enables more uniform and quantitative immobilization of phosphoinositide-containing liposomes onto the well surfaces. Phosphoinositides, as the substrate or the product, are detected by pleckstrin homology domains that specifically bind to each phosphoinositide. By this method, phosphoinositide contents are measured with higher sensitivities than those by conventional methods. More importantly, both phosphoinositide kinase and phosphatase activities can be measured for purified enzymes and crude cellular lysates. This assay is easy, sensitive, and quantitative and thus may have a variety of applications in the development of diagnostic tests or the screening of therapeutic treatments for diseases such as cancer and diabetes which may be caused by abnormal phosphoinositide metabolism.

4. Caenorhabditis elegans WASP-interacting protein homologue WIP-1 is involved in morphogenesis through maintenance of WSP-1 protein levels

Sawa M, Takenawa T.

Mammalian WASP and N-WASP are involved in reorganization of the actin cytoskeleton through activation of the Arp2/3 complex and in regulation of cell motility or cell shape changes. In the present study, we identified WASP-interacting protein homologue (WIP)-1 in Caenorhabditis elegans. WIP-1 contains the domains and sequences conserved among mammalian WIP family proteins. Yeast two-hybrid analysis detected a physical interaction between WIP-1 and WSP-1, the sole homologue of WASP /N-WASP in C. elegans. Western analysis of embryo lysates showed that RNA interference (RNAi) treatment for wip-1 decreased levels of WSP-1 protein, and wsp-1(RNAi) treatment decreased levels of WIP-1 protein. However, wsp-1 mRNA levels were not decreased in wip-1 (RNAi)-treated embryos, and wip-1 mRNA levels were not decreased in wsp-1(RNAi)-treated embryos. Furthermore, disruption of WIP-1 by RNAi resulted in embryonic lethality with morphologic defects in hypodermal cell migration, a process known as ventral enclosure. This phenotype was similar to that observed in RNAi experiments for wsp-1. Immunostaining showed that WIP-1 was expressed in migrating hypodermal cells, as was WSP-1. This expression during ventral enclosure was reduced in wip-1(RNAi)-treated embryos and wsp-1(RNAi)-treated embryos. Our results suggest that C. elegans WIP-1 may function in hypodermal cell migration during ventral enclosure by maintaining levels of WSP-1.

5. Thin layer chromatography-blotting, a novel method for the detection of phosphoinositides.

Furutani M, Itoh T, Ijuin T, Tsujita K, Takenawa T.

Phosphoinositides are believed to be involved in fundamental cellular events such as signal transduction and vesicular trafficking. Aberrant metabolisms of this lipid, caused by mutations in phosphoinositide kinases, phosphatases and lipases are known to be related to variety of human disorders such as diabetes and cancer.

While the majority of such information is obtained by analyzing genetic and biochemical properties of phosphoinositide-metabolic enzymes, direct measurement of cellular content of the lipid is hindered by the lack of a simple method that is sensitive enough to measure phosphoinositides present in trace amounts *in vivo*. Here, we describe a novel, thin layer chromatography (TLC)-based method by which cellular phosphoinositides are separated, transferred and detected by specific phosphoinositide -binding domains. This method was applied to follow the generation of minor phosphoinositides, such as PtdIns(3,4,5)P3 and PtdIns(3,4)P2 in response to insulin and to compare PtdIns(4,5)P 2 and PtdIns(3,4,5)P3 levels in several cancer cell lines. The method has potential application not only in investigating the physiological roles of phosphoinositides, but also in diagnosing metabolic disease and cancer by directly assessing phosphoinositide levels in samples obtained from patients.

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Carbohydrates bound to the polypeptide chains widely occur in the body, and their structures change during development and differentiation of the cells and under pathological conditions. Our major research interest is to elucidate direct and indirect roles of the carbohydrates in cell-cell interaction. For this end, we are currently focusing on structure and function of carbohydrate binding proteins and their ligands, regulation of protein structures and functions by glycosylation, and development of new methods and tools for protein-carbohydrate interaction.

1. Cell adhesion molecules involved in normal and pathological processes

Etsuko Mori, Dong Cun Jin and Seiichi Takasaki

The interaction between the two gametes requires several steps that result in gamete fusion to produce a zygote. Cell adhesion is a major part of these unique processes. We are investigating three distinct adhesion phenomena: 1) adhesion between the oviduct epithelial cells and sperm, 2) adhesion/signaling between the sperm membrane and the extracellular coat of the egg, and 3) adhesion/fusion between egg and sperm membranes. To solve the mechanism underlying these processes, identification of functional molecules on the surface of both gametes is essential. Therefore, we are now focusing on the potential adhesion molecules on the sperm membrane. Our previous studies showed that the zona pellucida (ZP) glycoproteins on porcine egg express functional oligosaccharides with N-acetyllactosamine and Lewis X structures, and that the sperm express carbohydrate binding proteins on their plasma membrane recognizing the ZP oligosaccharides. Therefore, we tried to purify the carbohydrate binding proteins of boar sperm solubilized with detergent using a column of Sepharose beads coupled with desialylated α1-acid glycoprotein which contains N-acetyllactosamine-type oligosaccharides, and succeeded in recovering the potential proteins in the retarded fraction. The proteins were shown to have molecular masses of 70 kDa and 40 kDa, respectively. Interestingly, mass spectrometric analysis of tryptic peptides from the 70 and 40 kDa proteins and homology search of determined partial amino acid sequences indicated that both of the proteins are members of the ADAM protein, a gene family encoding membrane proteins with a disintegrin and metalloprotease domain. This protein family are unique in possessing both a potential cell adhesion activity as well as a potential protease activity. We cloned cDNA encoding a large parts of the 70kDa protein, and produced its recombinant protein in *E. coli* and in yeast. The protein was shown to have adhesion activity to cell surface integrin molecules. We also cloned cDNA encoding the 40kDa protein. Its recombinant protein containing disintegrin and Cys-rich domains was produced in E. coli. Quite interestingly, the purified protein was shown to bind to asialo-α1-acid glycoprotein, but not to agalacto-α1-acid glycoprotein, indicating that the protein has an affinity to the exposed β-galactosyl residues. The protein also bound to asialo-fetuin-

beads, and this binding was inhibited by addition of oligosaccharides containing terminal β-galactosyl residues. These results clearly indicate that the 40 kDa protein has carbohydrate binding activity. In view of its binding specificity, the 40 kDa protein is suggested to work as a Zona-binding protein. The finding not only provides a new insight into the recognition mechanism working in sperm-egg interaction, but also indicates a novel function of the ADAM family proteins. It is of great interest to examine the carbohydrate binding activity of other members of ADAM, because ADAMs are suggested to work in various biological events as sheddases and modulators of cell-cell and cell-matrix interactions.

2. Analysis of cancer-related sialic acid and antibody

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Ferritin is an iron-storage protein and its serum level is known to increase in the patient with inflammation or malignant tumor. The previous observation also indicates that ferritin in the malignant tumor tissue shows more acidic property in electrophoresis than that of normal tissue. To further elucidate the difference between ferritins from normal human liver tissue and that of cancer cells, their sialic acids were analyzed. The Western blot analysis and the cytochemical staining using an anti-*N*-glycolylneuraminic acid (NeuGc) antiserum indicated that ferritin from the human hepatocarcinoma tissue contains NeuGc, but that from the normal liver does not. The result was also confirmed by HPLC analysis and MALDI-TOF/MS analysis of sialic acids which were derivatized by the DMB method. It was also shown that the sialic acid content in hepatocarcinoma feritin was much higher than that in the normal liver ferritin. These results suggest that normal and cancerous liver ferritins are qualitatively and quantitatively different in sialylation. It is well known that a gene encoding CMP-NeuAc hydroxylase that converts CMP-NeuAc to CMP-NeuGc resulting in incorporation of NeuGc to glycans has an exon deletion/frame shift mutation in humans. The present consensus is that the expression of NeuGc in human cells is due to uptake of non-human NeuGc from dietary sources and its incorporation into cells. Therefore, we examined if the expression of NeuGc by K562 cells is affected by the culture media. The result indicated that K562 cells express NeuGc even if the cells are cultured in serum-free media which lack NeuGc. In view of the lack of CMP-Neu5Ac hydroxylase in human cells, alternative metabolic pathways for the biosynthesis of glycoconjugate-bound Neu5Gc are considered. However, we can not exclude the idea that expression of NeuGc in cancer tissue in vivo is at least partly due to the mechanism like the sialin-mediated incorporation from external milieu.

The exact mechanism by which human cancer cells express NeuGc is not clear, but expression of NeuGc in cancerous tissue strongly suggests the presence of antibodies against NeuGc-containing glycoconjugates. Therefore, sera of cancer patients were tested by ELISA using plates coated with NeuGc-GM3 or NeuGc-containing ferritin purified from K562 cells. The result showed that 32% of sera from 50 patients with solid tumors in liver, colon, pancreas, lung and ovary contain anti-NeuGc IgG. Similarly, 30% of sera from patients with acute myelogenous leukemia, chronic myelogenous leukemia, acute lymphoid leukemia, chronic lymphoid leukemia, acute leukemia, chronic leukemia, myelodysplastic syndromes, and multiple myeloma were shown to contain anti-NeuGc IgG. Thus, the quantification of anti-NeuGc antibodies and /or NeuGc-containing glycoconjugates may be of diagnostic and prognostic importance.

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Our major research interest is to elucidate the molecular mechanisms involved in signal transduction for malignant transformation and for tumor angiogenesis. We have been mainly focusing on the functions of tyrosine kinases [VEGF receptors-1 (Flt-1), VEGFR-2 (Flt-1/KDR), FGF receptor and EGF receptor], an adaptor/docking protein FRS2, and VEGF-independent regulation of angiogenesis. This year we found that VEGF-receptor-1 (VEGFR-1) is important not only for endothelial cell proliferation/differentiation but also for promotion of inflammatory diseases such as rheumatoid arthritis. Furthermore, we showed that VEGFR-2-specific ligand VEGF-E and its humanized molecules stimulated angiogenesis without macrophage recruitment and edema. We also found that an FGF4-induced Cdx2-mediated Bmp4 signaling pathway regulates a stem cell niche in vivo.

1. Signaling of vascular endothelial growth factor receptor-1 tyrosine kinase promotes rheumatoid arthritis through activation of monocyte/macrophages.

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Vascular endothelial growth factor (VEGF) and its receptor-1 (VEGFR-1/Flt-1) were shown to be involved in pathological angiogenesis, particularly rheumatoid arthritis (RA). However, the molecular basis of its actions is not fully understood. Here we found that in a murine model of RA, deletion of the tyrosine kinase (TK) domain of VEGFR-1 decreased the incidence and clinical symptoms of RA in a gene-dosage-dependent manner. Pathological symptoms, such as synovial hyperplasia, inflammatory infiltrates, pannus formation and cartilage/bone destruction became milder in *Vegfr-1 tk-/-* mice compared with *Wild-type (Wt)* mice in the *Human T-cell Leukemia Virus-1 (HTLV-1)* induced chronic models. VEGFR-1 TK-deficient bone marrow cells showed a suppression of multilineage colony formation. Furthermore, their macrophages differentiated *in vitro* showed a decrease in immunological reaction such as phagocytosis and cytokine secretion of Interleukin-6 (IL-6) and VEGF-A. Treatment of RA model mice with a small molecule inhibitor for VEGFRs TK, KRN951, also attenuated the arthritis. These results indicate that the VEGFR-1 TK signaling modulates the proliferation of bone marrow hematopoietic cells and immunity of monocyte/macrophages, and promotes chronic inflammation, which may be a new target for

RA treatment.

2. Chimeric VEGF-E_{NZ7}/PlGF Promotes Angiogenesis via VEGFR-2 without Significant Enhancement of Vascular Permeability and Inflammation

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VEGF plays critical roles in the regulation of angiogenesis and lymphangiogenesis. However, tissue edema, hemorrhage and inflammation occurred when VEGF-A was used for angiogenic therapy. To design a novel angiogenic factor without severe side effects, we examined the biological function of chimeric VEGF-E_{NZ7}/PlGF, which composed of Orf-$Virus_{NZ7}$-derived VEGF-E_{NZ7} and human PlGF1, in a transgenic (Tg) mouse model.

A strong angiogenic response was observed in both VEGF-E_{NZ7}/PlGF and VEGF-A_{165} Tg mice. Notably, the vascular leakage of VEGF-E_{NZ7}/PlGF-induced blood vessels was 4-fold lower than that of VEGF-A_{165}-induced blood vessels. Furthermore, the monocyte/macrophage recruitment in the skin of VEGF-E_{NZ7}/PlGF Tg mice was about 8-fold decreased compared with that of VEGF-A_{165} Tg mice. In addition, the lymphatic vessels in VEGF-E_{NZ7}/PlGF Tg mice were structurally normal, whereas they were markedly dilated in VEGF-A_{165} Tg mice possibly due to the high vascular leakage. Receptor binding assay demonstrated that VEGF-E_{NZ7}/PlGF was the ligand only activating VEGFR-2. These results indicated that neither the hyper-permeability in response to simultaneous stimulation of VEGFR-1 and VEGFR-2 nor VEGFR-1-mediated severe inflammation was associated with VEGF-E_{NZ7}/PlGF-induced angiogenesis. The unique receptor binding property may indicate VEGF-E_{NZ7}/PlGF as a novel candidate for therapeutic angiogenesis.

3. Chimeric VEGF-E_{NZ7}/PlGF Specifically Binding to VEGFR-2 Accelerates Skin Wound Healing via Enhancement of Neovascularization

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As indicated above, VEGF-E_{NZ7}/PlGF molecules composed of Orf virus-derived VEGF-E_{NZ7} and human PlGF1 are potent angiogenic factors stimulating angiogenesis without significant enhancement of vascular leakage and inflammation *in vivo*. For its future clinical application, we need to understand the beneficial effects of VEGF-E_{NZ7}/PlGF during wound healing in adulthood. In this study, several angiogenic factors were administrated to skin punched wounds of both wild-type and diabetic mice. The treatment with VEGF-E_{NZ7}/PlGF accelerated wound closure accompanied with enhanced angiogenesis, the process was occurring slightly faster than that in VEGF-A_{164} group. Moreover, the macrophage infiltration and lymphangiogenesis level in healed wounds were strikingly lower in VEGF-E_{NZ7}/PlGF group than VEGF-A_{164} group, suggesting that the increased inflammation was the key issue preventing speedy wound healing of VEGF-A_{164}-treated skin. Considering clinical safety, we further examined the antigenicity of chimeric VEGF-E_{NZ7}/PlGF. Compared with the original VEGF-E_{NZ7}, the immunogenicity of VEGF-E_{NZ7}/PlGF molecules was markedly decreased in mice and squirrel monkeys with the increase of PlGF1 humanized ratio. These results indicate that VEGF-E_{NZ7}/PlGF molecules are superior to VEGF-A for the acceleration of either normal or delayed skin wound healing and might be regarded as potential drugs in therapeutic angiogenesis.

4. An FGF4-induced Cdx2-mediated BMP4 signaling pathway regulates a stem cell niche in mammalian embryos.

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A variety of stem cells are controlled by the action of multiple growth factors in vitro. However, it remains largely unclear how growth factors control the proliferation and differentiation of stem cells in vivo. Here, we demonstrate a novel paracrine mechanism critical for maintaining a stem cell niche in mammalian early embryos, which involves communication between

the inner cell mass and the trophectoderm, from which embryonic stem (ES) cells and trophoblast stem (TS) cells can be derived, respectively. It is known that ES cells produce fibroblast growth factor 4 (FGF4) and TS cells produce bone morphogenic protein 4 (Bmp4), respectively. We found that activation of ERK stimulates expression of the transcription factor Cdx2 in TS cells in response to FGF4. By analyzing the 5′ flanking region of Bmp4, we identified an enhancer element that has a consensus sequence of Cdx2 binding sites in TS cells. We further showed by applying an electrophoretic mobility shift assay that Cdx2 binds to this sequence. We also analyzed cultured blastocysts derived from embryos deficient in Frs2alpha; a docking protein that links FGF signaling with ERK activation. We found that blastocysts derived from Frs2alpha embryos gave rise to a smaller inner cell mass as compared to those from wild type embryos. Moreover, this defect was rescued by exogenous addition of Bmp4 to the culture medium. These findings suggest that FGF4 derived from the inner cell mass activates a FRS2alpha-ERK pathway resulting in enhanced expression of Cdx2 in the trophoectoderm. This in turn leads to transcriptional activation of Bmp4, a paracrine factor that mediates the growth of the inner cell mass. These findings highlight a critical role of Cdx2 for maintaining the stem cell niche.

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