Department of Cancer Biology

Division of Oncology
癌細胞シグナル研究分野

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A large majority of protooncogenes encodes protein-tyrosine kinases or transcription factors. Protooncogenes are important not only for the development of malignant tumors but also for the regulation of growth and function of normal cells. Our current interest is to characterize the cellular signaling mediated by protein-tyrosine phosphorylation in cancer cells and the central nervous system. We are also interested in the protein 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, Masahiro Morita, Kentaro Ito, Kazuya Kitazawa, Yusuke Nakajima, 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. Unlike other Tob family proteins, Ana is specifically expressed in type II alveolar epithelial cells. Because 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 down regulation 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 and apoptosis is infrequent. Tob suppresses DNA damage-induced apoptosis by blocking the mitochondria-

dependent pro-apoptotic pathway. Consistent with the data, Tob is localized to the mitochondria as well as the nucleus. Furthermore, both Tob degradation following severe DNA damage and *tob* gene increase following mild DNA damage belong to the early events in the cellular response to DNA damage. Thus, Tob may be involved in the mechanism which determines whether damaged cells undergo apoptosis or growth arrest.

To further establish biological significance of the *tob* family members, we searched for molecules that interact with Tob by applying proteomics. 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/CCR4 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 identified Ndr2 kinase, a member of LATS family of serine/threonine kinases. A yeast homolog of Ndr2 is reported to associate with the yeast CCR4/Not complex. We are currently examining the biological functions of CCR4/Not complex by generating mice lacking respectively *cnot3*, *cnot6*, *cnot6L*, *cnot7*, *cnot8*, *cnot9* and *ndr2*. From molecular and cellular analysis, we showed that CCR4b/CNot6L, which is homologous to yeast CCR4 mRNA deadenylase, is localized mainly in the cytoplasm and displays deadenylase activity both *in vitro* and *in vivo*. Suppression of CCR4b/CNot6L by RNA interference (RNAi) results in growth retardation of NIH3T3 cells accompanied by elevation of both $p27^{Kip1}$ mRNA and $p27^{Kip1}$ protein. Reintroduction of wild-type CCR4b/CNot6L but not mutant CCR4b/CNot6L lacking deadenylase activity restores the growth of CCR4b/CNot6L-depleted NIH3T3 cells. The data suggest that CCR4b/CNot6L regulates the cell growth in a manner dependent on its deadenylase activity. We are further examining the effect of RNAi-mediated knock down of the other components of the CCR4/Not complex on cell proliferation and other biological phenomenon. We found that depletion of several components of the complex results in cell growth arrest or in extensive cell death. The cell death occurred caspase-dependent manner, indicating induction of apoptosis, the underlying mechanisms of which are under investigation.

2. Roles of protein-tyrosine kinases in the central nervous system

Takanobu Nakazawa, Kazumasa Yokoyama, Seiji Kawa, Takeshi Inoue, Naosuke Hoshina, Tohru Tezuka, and Tadashi Yamamoto

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

NMDA receptors play important roles in learning and memory. Our own studies have shown that NR2A and NR2B subunits of NMDA receptors are the major substrates of Fyn and Src. We have identified Tyr-1472 phosphorylation on NR2B, which is the major tyrosine phosphorylation site on NR2B, as a key mediator of fear-related learning and emotional behavior in the amygdala. On NR2A, we have identified Tyr-1325 as a major tyrosine phosphorylation site. We showed that Src-mediated Tyr-1325 phosphorylation potentiated NMDA receptor-mediated currents in recombinant NR1-NR2A channels. To establish the biological significance of Tyr-1325 phosphorylation, we generated mice with a knock-in mutation of the Tyr -1325 site to phenylalanine and showed that Tyr -1325 phosphorylation was essential for antidepressant-like behaviors. Molecular mechanisms of Tyr-1325 phosphorylation-mediated regulation of the behaviors are under investigation.

In parallel of these studies, to uncover Src- and Fyn-mediated signaling pathways, we have been trying to identify binding partners and substrates of these kinases in the brain. We have identified a number of putative mediators of Fyn- and Src-mediated signaling, including p250 GAP, TCGAP, Nogo-A, and RhoGEFs. Characterization of these proteins is in progress. One of them is FAK, a cytoplasmic tyrosine kinase. Tyrosine phosphorylation of FAK was enhanced during differentiation of CG4 oligodendrocyte cell lines in a Fyn-dependent manner. We found that FAK regulated laminin-induced oligodendroglial process outgrowth. Given that Fyn is essential for the morphological differentiation of oligodendrocytes and that Fyn activity was reduced in FAK-knockdown CG4 cells, FAK and Fyn appear to act cooperatively to evoke downstream signaling events required for differentiation of oligodendrocytes.

We previously found that p250GAP, an

NMDA receptor-associated RhoGAP, is phosphorylated by Fyn. We showed that p250GAP regulates spine morphogenesis by modulating RhoA activity. Knock-down of p250GAP increased spine width and elevated the endogenous RhoA activity in primary hippocampal neurons. The increased spine width by p250 GAP knock-down was suppressed by the expression of a dominant-negative form of RhoA. Furthermore, p250GAP knock-down occluded NMDA receptor-mediated RhoA activation. In response to NMDA receptor activation, exogenously expressed GFP-tagged p250GAP was redistributed. Thus, these data suggest that p250 GAP plays an important role in NMDA receptor -mediated regulation of RhoA activity leading to spine morphological plasticity. A Role of Fyn-mediated phosphorylation of p250GAP is under investigation.

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.

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

Miho Ohsugi, Noriko Tokai-Nishizumi, Keiko Haraguchi, Naoki Oshimori, Natsuko Masuda, Xue Li, 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) The chromokinesin Kid

The human chromokinesin Kid/Kinesin-10 is a member of the chromosome-associated kinesin family identified in our lab in 1996. Kid has been implicated in generating the polar ejection force that pushes the chromosome arms away from the spindle poles toward the spindle equator, as well as in the maintenance of spindle length during prometaphase and metaphase. We defined two functional nuclear localization signals (NLSs) in Kid and found that Importin α directly associates with Kid via NLSs. When both NLSs were mutated, not only nuclear localization of Kid during interphase, but also mitotic chromosomal localization was reduced. Importin α/β complex controls both localization and functions of Kid. Our results indicate that the mitotic chromosome loading of Kid is mediated by importin α/β and that Ran-GTP-mediated dissociation of importin α/β from Kid promotes the accumulation of Kid on chromosomes.

During anaphase and telophase, Kid shows unique localization in the interstices between adjacent chromosomes. By analyzing Kid-depleted HeLa cells and Kid-deficient mice, we found that Kid contributes to the compact clustering of the anaphase chromosome mass, so that all chromosomes are encapsulated into a single nucleus at the end of mitosis. Our results suggest that Kid-mediated anaphase chromosome compaction is specifically important during very early embryonic stages.

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. Previously, we identified the centrosomal Plk1 substrate, Kizuna (Kiz), which when depleted causes fragmentation and dissociation of the pericentriolar material from centrioles at prometaphase, resulting in multipolar spindles. Using a yeast two-hybrid screen for Kiz-interacting proteins, we identified an uncharacterized centrosome protein named Ckip. RNA interferance (RNAi) experiments demonstrate that Ckip is required for centrosomal localization of Kiz. In addition, our data suggest that Ckip also plays other important roles in regulating microtubule nucleation activity of the centrosomes. We are currently investigating other newly identified substrates of Plk1, which will uncover the molecular mechanisms underlying the Plk1-mediated control of cell divisions and oncogenesis.

hLATS1 and *hLATS2* are other mitotic serine/threonine kinases which share significant homology with a *Drosophila* tumor suppressor gene *lats*. Previously, we identified the hLATS2-binding proteins Ajuba and Zrp1/Trip-6. We investigated the role of Ajuba in the Wnt signaling pathway and found that Ajuba interacts with β-catenin. Association of Ajuba with β-

catenin reinforces the association between β-catenin and GSK-3β and promoted GSK-3β-mediated phosphorylation of β-catenin and subsequent destabilization of β-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, both of which are disassembled during mitosis. RNAi-mediated depletion of ZRP-1 from HeLa cells revealed that ZRP-1 was essential not only for the stress fiber formation and mature focal adhesion assembly but also for the actin reorganization at cell-cell contact sites and for proper cell-cell adhesion, and thus for collective cell migration. Stress fibers in ZRP-1-depleted cells were ameliorated by exogenous expression of RhoA. We also found that total Rac1 activity was elevated in ZRP-1 depleted cells, resulting in abnormal burst of actin polymerization and dynamic membrane protrusions. Taken together, our data suggest that ZRP-1 localizes to cell-matrix and cell-cell contact sites and plays critical role in coupling the cell-matrix/cell-cell contact signals with Rho GTPases-mediated actin remodeling, which is essential for proper cell-matrix and cell -cell contact formation.

Publications

Ohsugi, M., Adach, K., Horai, R., Kakuta, S., Sudo, K., Kotaki, H., Tokai-Nishizum,i N., Sagara, H., Iwakura, Y., and Yamamoto, T. Chromokinesin Kid-Mediated Anaphase Chromosome Compaction Safeguards Mouse Early Embryos Against Multinuclear Formation. Cell in press.

Tahara, K., Takagi, M., Ohsugi, M., Sone, T., Nishiumi, F., Maeshima, K., Horiuchi, Y., Tokai-Nishizumi, N., Imamoto, F., Yamamoto, T., Kose, S., and Imamoto, N. "Importin-β and the small GTPase Ran mediate chromosome loading of human chromokinesin Kid" J. Cell Biol. in press.

Miyasaka, T., Morita, M., Ito, K., Suzuki, T., Fukuda, H., Takeda, S., Inoue, J., Semba, K. and Yamamoto, T. Interaction of anti-proliferative protein Tob with the CCR4-NOT deadenylase complex. Cancer Sci. in press.

Kina, S., Tezuka, T., Kusakawa, S., Kishimoto, Y., Kakizawa, S., Hashimoto, K., Ohsugi, M., Kiyama, Y., Horai, R., Sudo, K., Kakuta, S., Iwakura, Y., Iino, M., Kano, M., Manabe, T., Yamamoto, T. Involvement of protein-tyrosine phosphatase PTPMEG in motor learning and cerebellar long-term depression. Eur J Neurosci. 26: 2269-78, 2007.

Washio-Oikawa, K., Nakamura, T., Usui, M., Yoneda, M., Ezura, Y., Ishikawa, I., Kazuhisa, N., Noda, T., Yamamoto, T. and Noda, M. Cnot7 null mice exhibit high bone mass phenotype and modulation of BMP action. J, Borne. Mineral Res. 22: 1217-23, 2007.

Hoshina, N., Tezuka, T., Yokoyama, K., Kozuka-Hata, H., Oyama, M. and Yamamoto, T. Focal adhesion kinase regulates laminin-induced oligodendroglial process outgrowth. Genes Cells 12: 1245-1254, 2007.

Yamaguchi, R., Wada, E., Kamichi, S., Yamada, D., Maeno, H., Delawary, M., Nakazawa, T., Yamamoto, T. and Wada, K. Neurotensin type 2 receptor in astrocytes is involved in fear memory in mice. J. Neurochem. 102: 1669-1676, 2007.

Inoue, A., Yasuda, T., Yamamoto, T., Yamanashi, Y. Dok is a positive regulator of IL-4 signaling and IgE response, J Biochem. 142: 257-263, 2007.

Morita, M., Suzuki, T., Nakamura, T., Yokoyama, K., Miyasaka, T. and Yamamoto, T. Depletion of mammalian CCR4b deadenylase-triggers increment of the $p27^{Kip1}$ mRNA level and impairs cell growth. Mol. Cell. Biol. 27: 4980-4990, 2007.

Bai, C., Ohsugi, M., Abe, Y., Yamamoto, T. ZRP-1 controls Rho GTPase-mediated actin reorganization by localizing at cell-matrix and cell-cell adhesions. J. Cell Sci. 120: 2828-2837, 2007.

Haraguchi, K., Ohsugi, M., Abe, Y., Semba, K., Akiyama, T. and Yamamoto, T. Ajuba negatively regulates the Wnt signaling pathway by promoting GSK-3β-mediated phosphorylation of β-catenin. Oncogene, Epub, 2007.

Saito, A., Nagasaki, M., Oyama, M., Kozuka-Hata, H., Semba, K., Sugano, S., Yamamoto, T. and Miyano, S. AYUMS: an algorithm for completely automatic quantitation based on LC-MS/MS proteome data and its application to the analysis of signal transduction. BMC Bioinformatics 8: 15, 2007.

Oyama, M., Kozuka-Hata, H., Suzuki, Y., Semba, K., Yamamoto, T. and Sugano, S. Diversity of translation start sites may define increased complexity of the human short ORFeome. Mol. Cell. Proteomics 6: 1000-1006, 2007.

Delawary, M., Nakazawa, T., Tezuka, T., Sawa, M., Iino, Y., Takenawa, T. and Yamamoto, T. Molecular characterization of a novel RhoGAP, RRC-1 of the nematode Caenorhabditis elegans. Biochem. Biophys. Res. Com-

mun. 357: 377-82, 2007.
Chikamori, M., Fujimoto, J., Tokai-Nishizumi, N. and Yamamoto, T. Identification of multiple SNT-binding sites on NPM-ALK oncoprotein and their involvement in cell transformation. Oncogene 26: 2950-2954, 2007.
中澤敬信「グルタミン酸受容体のチロシンリン酸化を介する記憶形成の制御」化学と生物，第45巻11号(2007)．
大杉美穂，押森直木，山本雅「Kizunaによる中心体の構造安定化の重要性」蛋白質核酸酵素，Vol. 52，№8 (2007)．

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Membrane proteins and their ligands including signaling molecules and extracellular matrix proteins mediate complex arrays of cell signaling. Fate and activities of these proteins are often regulated by proteases in the extracellular space. We are currently focused on studying biological roles of membrane-anchored type of matrix metalloproteinases (MT-MMPs) which are potent modulators of pericellular microenvironment and eventually regulate varieties of cellular functions such as proliferation, migration, apoptosis, and differentiation. Since uncontrolled expression of MT-MMPs in tumors contributes to their malignant characteristics, they are potential targets for cancer therapy.

1. Crosstalk between neovessels and mural cells directs the site-specific expression of MT1-MMP to endothelial tip cells

Ikuo Yana, Hiroshi Sagara, Satoshi Takaki, Kiyoshi Takatsu, Kenji Nakamura, Kazuki Nakao , Motoya Katsuki , Shun-ichiro Taniguchi, Takanori Aoki, Hiroshi Sato, Stephen J. Weiss, and Motoharu Seiki.

The membrane-anchored matrix metalloproteinase MT1-MMP (also known as MMP14) 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 the *lacZ* gene inserted into the *Mmp14* gene, we demonstrate that MT1-MMP vascular expression in vivo is confined largely 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 crosstalk between endothelial cells and vascular smooth muscle cells (VSMC). When vessel maturation induced by VSMCs is inhibited by introducing a soluble form of the receptor tyrosine kinase Tek, 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.

2. Development of a new tracking tool for the human monomeric laminin γ2 chain *in vitro and in vivo*

Naohiko Koshikawa, Tomoko Minegishi, Kazuki Nabeshima and Motoharu Seiki

Laminin-5 (Ln-5), a heterotrimer composed of three different laminin chains (α3, β3, and γ2), is a major component of the basement membrane in most adult tissues. One of the chains, laminin γ2 (Ln-γ2), is a specific marker of invasive tumors since it is frequently expressed as a monomer in malignant tumors. However, there is no simple and direct method to detect the monomeric form of Ln-γ2 selectively in the presence of Ln-5, because all available antibodies recognize both monomeric and heterotrimeric forms of Ln-γ2. In this study, we developed a new monoclonal antibody (mAb) termed 1H3 that reacts specifically with human Ln-γ2 monomers during immunoprecipitation, ELISA, Western blotting, and immunostaining. Ln-5 was not recognized by mAb 1H3 after denaturation with detergents under non-reducing conditions, but reactivity was recovered when denaturation was done under reducing conditions. The epitope of the antibody was mapped to region on the coiled-coil structure formed between Ln-γ2 and its partner chains Ln-α3 and Ln-β3 in Ln-5, whose structure is further stabilized by disulfide bonds. In normal tissue samples, the basement membrane was stained with conventional antibody against Ln-γ2 but not by mAb 1H3. In contrast, tumor cells in tissue sections could be stained with mAb 1H3 as efficiently as with conventional antibody. Thus, mAb 1H3 holds promise as a powerful tracking tool for the specific detection of monomeric Ln-γ2 *in vivo* and *in vitro*, and is potentially useful as a diagnostic tool for detecting tumors and as a vehicle for drug delivery to cancer tissues.

3. Stroma-derived matrix metalloproteinase (MMP)-2 promotes membrane type 1-MMP-dependent tumor growth in mice

Kaori Taniwaki, Hiroshi Fukamachi, Kiyoshi Komori , Takahiro Nonaka , Takeharu Sakamoto, Takayuki Shiomi, Yasunori Okada, Takeshi Itoh, Shigeyoshi Itohara, Motoharu Seiki, and Ikuo Yana

Matrix metalloproteinase-2 (MMP-2) is a stroma-derived MMP belonging to the type IV collagenase family. It is believed to mediate tumor cell behavior by degrading deposits of type IV collagen, a major component of the basement membrane. The membrane type 1-MMP (MT1-MMP) is a highly potent activator of MMP-2 and is expressed in many tumor and stromal cells. However, the roles played by stromal MMP-2 in tumor progression *in vivo* remain poorly understood. We established a colon epithelial cell line from an *Mt1-mmp*$^{-/-}$ mouse strain and transfected these cells with an inducible expression system for MT1-MMP (MT1rev cells). Following s.c. implantation into *Mmp-2*$^{+/+}$ mice and induction of MT1-MMP expression, MT1rev cells grew rapidly, whereas they grew very slowly in *Mmp-2*$^{-/-}$ mice, even in the presence of MT1-MMP. This MT1-MMP-dependent tumor growth of MT1rev cells was enhanced in *Mmp-2*$^{-/-}$ mice as long as MMP-2 was supplied via transfection or coimplantation of MMP-2-positive fibroblasts. MT1rev cells cultured in vitro in a three-dimensional collagen gel matrix also required the MT1-MMP/MMP-2 axis for rapid proliferation. MT1rev cells deposit type IV collagen primarily at the cell-collagen interface, and these deposits seem scarce at sites of invasion and proliferation. These data suggest that cooperation between stroma-derived MMP-2 and tumor-derived MT1-MMP may play a role in tumor invasion and proliferation via remodeling of the tumor-associated basement membrane. To our knowledge, this is the first study demonstrating that MT1-MMP-dependent tumor growth *in vivo* requires stromal-derived MMP-2. It also suggests that MMP-2 represents a potential target for tumor therapeutics.

4. Establishment of an MT4-MMP-deficient mouse strain representing an efficient tracking system for MT4-MMP/MMP-17 expression *in vivo* using beta-galactosidase

Akiko Rikimaru, Kiyoshi Komori, Takeharu Sakamoto, Hirotake Ichise, Nobuaki, Yoshida, Ikuo Yana, and Motoharu Seiki

Biological functions of membrane-type 4 matrix metalloproteinase (MT4-MMP/MMP-17) have been poorly evaluated because of lack of sensitive tracking system for its expression *in vivo*. We established a mutant mouse (*Mt4-mmp*$^{-/-}$) in which *Mt4-mmp* gene was replaced with a reporter gene encoding β-galactosidase (LacZ). *Mt4-mmp*$^{-/-}$ mice were born normally without apparent defect in growth, life span, and fertility. Taking advantage of *LacZ* activity we monitored the promoter activity of *Mt4-mmp* for the first time *in vivo*. Tissue distribution of *Mt4-mmp* mRNA was in good concordance with that of *LacZ*, and major tissues expressing these mRNAs were cerebrum, lung, spleen, intestine, and uterus. LacZ-positive cells were neurons in the cerebrum, smooth muscle cells in the intestine and uterus, and macrophages located in alveolar or intraperitoneal space. MT4-MMP is reported to act a TNF-α sheddase, and we re-evaluated this possibility exploiting our mutant macrophages. Differently from our expectation, the mutant macrophages released TNF-α as efficiently as the wild type cells did upon stimula-

tion with lipopolysaccharide. Expression of *Mt4-mmp* mRNA was rather repressed in the cells after the stimulation. Thus, our mutant mice are useful to analyze physiological functions of MT4 -MMP and also as a sensitive tracking system to monitor expression of MT4-MMP *in vivo*.

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

Isamu Gotoh, Takamasa Uekita and Motoharu Seiki

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. The 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 hADI 1. 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. U1-70 K over-expression induced nuclear accumulation of hADI1. Nuclear hADI1 expression significantly altered the splicing pattern of the adenovirus E1A mini-gene, which generates multiple alternatively spliced transcripts. Thus, hADI 1 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.

6. Application of systemic high-throughput proteomics to membrane protein complexes: Identification of the proteins associating with MT1-MMP/MMP-14

Taizo Tomari, Takashi Shinkawa, Nagayasu Egawa, Takayuki Uematsu, Naohiko Koshikawa, Toshiaki Isobe, Motoharu Seiki.

Membrane-type 1 matrix metalloproteinase (MT1-MMP) is a powerful modulator of the pericellular environment through its proteolytic activity. Expressed in tumor cells, MT1-MMP promotes cell migration, invasion, and proliferation. To carry out the functions properly, activity, localization, and turnover of MT1-MMP are regulated precisely through interaction with other proteins, although our knowledge is still very limited. In this study, we expressed MT1-MMP in human malignant melanoma A375 cells as a FLAG-tagged form and purified it together with associating proteins using an antibody-based affinity column. The purified complex contained a distinct set of membrane and cytoplasmic proteins in addition to MT1-MMP. The proteins were analyzed with an integrated system composed of nano-flow liquid chromatography and tandem mass spectrometry. We identified 158 proteins including ones reported to bind MT1-MMP such as αv and β1 integrins, CD63, and TIMP-3, although most are new candidates. To further evaluate the specificity of the result, eight membrane proteins were tested for their ability to form a complex with MT1-MMP. Seven of the eight test proteins were co-immunoprecipitated with MT1-MMP and four (5T4 antigen, IL13Rα2 chain, CD9, CD63) were found to co-localize with MT1-MMP at the ruffling membrane. Interestingly, the relevance of these proteins to malignant tumors has been proposed from independent studies. Thus, we believe our list of the proteins as a result of system-wide analysis of the MT1-MMP complex is a valuable source to shed light on the protein interaction networks regulating MT1-MMP on the cell surface and in the cytoplasm.

Publications

1. Gotoh, I., Uekita, T. and Seiki, M. Regulated nucleo-cytoplasmic shuttling of human aci-reductone dioxygenase (hADI1) and its potential role in mRNA processing. Genes Cells. 12: 105-117, 2007.
2. Rikimaru, A., Komori, K., Sakamoto, T., Ichise, H., Yoshida, N., Yana, I., and Seiki, M. Establishment of an MT4-MMP-deficient mouse strain representing an efficient tracking system for MT4-MMP/MMP-17 expression *in vivo* using beta-galactosidase. Genes Cells. 12: 1091-1100, 2007.
3. Taniwaki, K., Fukamachi, H., Komori, K., Nonaka, T., Sakamoto, T., Shiomi, T., Okada,

Y., Itoh, T., Itohara, S., Seiki, M., and Yana, I. Stroma-derived matrix metalloproteinase (MMP)-2 promotes membrane type 1-MMP-dependent tumor growth in mice. Cancer Res. 67: 4311-4319, 2007.

4. Yana, I., Sagara, H., Takaki, S., Takatsu, K., Nakamura, K., Nakao, K., Katsuki, M., Taniguchi, S., Aoki, T., Sato, H., Weiss, SJ and Seiki, M. Crosstalk between neovessels and mural cells directs the site-specific expression of MT1-MMP to endothelial tip cells. J Cell Sci. 120: 1607-1614, 2007.
5. Koshikawa, N., Minegishi, T., Nabeshima, K., and Seiki, M. Development of a new tracking tool for the human monomeric laminin γ2 chain *in vitro* and *in vivo*. Cancer Res. in press.

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Human cancers develop and progress toward malignancy through accumulation of multiple genetic alterations. Elucidation of these alterations is essential to provide molecular targets for prevention, diagnosis, and treatment of cancer. Our current interest is to understand the role of cell adhesion in cancer invasion and metastasis. To this end, a tumor suppressor protein, CADM1/TSLC1, and its cascade were identified and are being characterized. The genetic basis of the susceptibility of individuals to cancer is also being investigated.

1. The biological role of CADM1/TSLC1 protein in epithelial structure

Mika Sakurai-Yageta, Yumi Tsuboi, Miwako Iwai, Mari Masuda[1] and Yoshinori Murakami: [1]Tumor Suppression & Functional Genomics Project, National Cancer Center Research Institute

CADM1/TSLC1 is an immunoglobulin superfamily cell adhesion molecule and primarily involved in epithelial cell adhesion, while loss of its function could lead cancer cells to invasion and metastasis. We have previously shown that CADM1 associates with an actin-binding protein, 4.1B/DAL-1, and a scaffold protein, membrane protein palmitoylated 3 (MPP3). We have newly identified MPP1/p55 and MPP2/DLG2 as additional cytoplasmic proteins binding to CADM1 and investigated the roles of the CADM1 cascade in epithelial cell morphology and its malignant transformation. MPP1, MPP2, and MPP3 interact directly with 4.1B, forming a tripartite complex with CADM1. Whereas these complexes localize along the cell membranes in confluent HEK293 cells, only MPP2, but not MPP1 or MPP3, is recruited to the CADM1/4.1B complex in the early process of cell adhesion. When the CADM1 function is abrogated by RNAi, HEK293 loses epithelia-like structure and show flat morphology with immature cell adhesion. Furthermore, 4.1B and MPP2, as well as E-cadherin and ZO-1, are mislocalized from the membrane. Loss of CADM1 is also correlated with the transformed phenotype of lung cancer cells. These findings suggest that CADM1 is involved in the formation of epithelia-like cell structure with 4.1B and MPPs, while loss of its function could cause morphological transformation of cancer cells. Possible proteins functioning in the downstream cascade of CADM1 are being analyzed comprehensively by proteomics analysis in various tissues, including epithelia and ATL.

2. Patho-physiological function of the CADM 1 protein in animal models

Masayoshi Nagata, Takashi Obana, Hiromi Ichihara, Tomoko Masuda, Mari Masuda and Yoshinori Murakami

To understand the physiological role of the CADM1 protein in animal models, *Cadm1*-deficient mice were generated by disrupting exon 1 of the gene. *Cadm1*$^{-/-}$ mice were born

normally with the expected Mendelian ratio, but *Cadm1*$^{-/-}$ male mice were infertile due to disruption of cell adhesion between the spermatocytes and the Sertoli cells. Based on this finding, a possible involvement of CADM1 in human male infertility is being investigated in collaboration with other research groups. In addition, membrane proteins in the Sertoli cells that are interacting with CADM1 in spermatocytes were investigated in collaboration with others. We have also found that some *Cadm1*$^{-/-}$ mice spontaneously develop lung adenomas and adenocarcinomas at 15 months of age, indicating that *CADM1* is a potent tumor suppressor gene. The molecular mechanisms of lung tumorigenesis are being analyzed in *Cadm1*$^{-/-}$ mice.

3. Analyses of genetic abnormalities in human tumors

Masayoshi Nagata, Tomoko Masuda and Yoshinori Murakami

To understand the molecular features of multistage human carcinogenesis, promoter methylation of the *CADM1* and the *4.1B* genes was examined in various tumors, including lung cancers, renal cancers and nasopharyngeal cancers. Bisulfite SSCP analysis revealed that the promoters of the *CADM1* and the *4.1B* genes were methylated in 44% and 57% of NSCLC, respectively, where about 70% of tumors showed disruption of the CADM1-4.1B cascade. We also demonstrated that methylation of *CADM1 or 4.1 B* could provide novel indicators of poor prognosis in patients with NSCLC. Furthermore, we reported that CADM1 was inactivated in advanced stages of nasopharyngeal cancers, suggesting that aberration of CADM1 is involved in metastasis of nasopharyngeal cancer.

4. Analysis of cancer susceptibility by the quantitative detection of allele-specific expression

Shingo Usui, Tomoko Masuda and Yoshinori Murakami

A number of phenotypes in hereditary cancers and various common diseases are associated with a specific genotype. However, little is known about the molecular basis of phenotypic variations among individuals carrying the same mutation or polymorphism. To understand the biological significance of affected SNPs from the viewpoint of the amount of mRNA, we developed an RNA difference plot (RDP), a highly quantitative method for detecting a relative amount of mRNA from two polymorphic alleles with a coefficient of variation of less than 10%. Using RDP analysis, we found that the relative expression of a mutant gene showed a significant difference in the carriers harboring the identical mutation but varied phenotypes. This finding suggests that the diversity in the expression of a disease-associated allele is one of the mediators of phenotypic variation. RDP analysis was also applied in order to clarify the role of the polymorphisms associated with susceptibility to systemic lupus erythematosus.

Publications

Wakayama T., Sai Y., Ito A., Kato Y., Kurobo M., Murakami Y., Nakashima E., Tsuji A., Kitamura Y. and Iseki S. Heterophilic binding of the adhesion molecules poliovirus receptor and immunoglobulin superfamily 4A in the interaction between mouse spermatogenic and Sertoli cells. Biology Reprod. 76: 1081-1090, 2007.

Kawasaki A., Tsuchiya N., Ohashi J., Murakami Y., Fukazawa T., Kusaoi M., Morimoto S., Matsuda K., Hashimoto H., Takasaki Y. and Tokunaga K. Role of APRIL (TNSF13) polymorphisms in the susceptibility to systemic lupus erythematosus in Japanese. Rheumatology 46: 776-782, 2007.

Ito, A., Hagiyama, M., Oonuma, J., Murakami, Y., Yokozaki, H. and Takaki, M. Involvement of the SgIGSF/Necl-2 adhesion molecule in degradation of mesenteric mast cells. J. Neuro. Immunol. 184: 209-213, 2007.

Department of Cancer Biology

Division of Cellular and Molecular Biology
分子発癌分野

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Gene expression is largely regulated by signal transduction triggered by various stimulations. Several lines of evidence indicate that genetic defects of molecules involved in the signal transduction or the gene expression lead to abnormal cell differentiation or tumor formation. 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[1] 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) a 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. Molecular mechanism regulating thymic microenvironment to establish self-tolerance

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

Clonal deletion is one of the main mechanisms to maintain T-cell tolerance. T-cell clones that have high avidity for self-antigens are eliminated during their development in the thymus. Self-antigens are predominantly expressed and presented by epithelial cells (TECs) and dendritic cells. Medullary thymic epithelial cells (mTECs) have a unique property to promiscously express peripheral tissue specific self-antigens (TSAs). Therefore, it has been proposed that developing T-cells encounter TSAs in the thymic medullar for clonal deletion. This hypothesis is supported by the study of autoimmune regulator (Aire), inactivation or deficiency of which results in autoimmune polyendocrinopathy-candidiasis ectodermal dystrophy. Aire is preferentially expressed in mTECs and lack of Aire in mice results in the defective expression of some TSAs.

Even though an increasing body of evidence indicates crucial roles of mTECs on establishing self-tolerance in thymus, signaling pathways underlying the differentiation and proliferation of mTECs expressing Aire and TSAs are poorly understood. We previously reported that TNF receptor-associated factor 6 (TRAF6), a signal transducer activating the classical NF-κB pathway and MAPK pathway, plays a crucial role, suggesting that signal pathway dependent on TRAF6 are required for the development of mTECs expressing Aire and TSAs. However, the ligand and receptors upstream of TRAF6 remains to be determined. Analysis on the expression profile of TNF receptor superfamily in fetal thymic stroma revealed that RANK is a possible candidate of the upstream receptor of TRAF6.

3. Role of TRAF6 in development of lymphoid organs

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

Secondary lymphoid organs provide an environment that facilitates interactions among lymphocytes and between lymphocytes and antigen-presenting cells, interactions that are essential for initiation of an effective immune response. Although the structures and the development of the secondary lymphoid organs during ontogeny have been studied in detail, the molecular mechanisms involved in their development and organization remain unclear. We investigated the effect of deficiency of TNF-receptor associated protein 6 (TRAF6), which activates the classical NF-κB pathway, in splenic microenvironment formation. Two-week-old TRAF6-deficient mice showed defects in B-cell follicle and marginal zone formation, similar to mutant mice defective in lymphotoxin β-receptor (LtβR) signal. However, analysis revealed a TRAF6 role in architecture formation distinct from the early neonatal role played by LtβR signaling. LtβR signaling was essential for primary B-cell cluster formation and differentiation of follicular dendritic cells (FDCs) in neonatal mice. In contrast, TRAF6 was required only for converting B-cell clusters to B-cell follicles and maintaining FDCs through to later stages. Fetal liver transfer experiments suggested that the requirement for TRAF6 lies in the stromal cells. While FDC-specific surface marker expression gradually declines in neonatal TRAF6-deficient mice, CXCL 13 expression is lost before the FDCs disappear. These data suggest that developmentally regulated activation of TRAF6 in FDCs is required for inducing CXCL13 expression to maintain B-cell follicles.

4. Role of TRAF6 in osteoclastogenesis

Jin Gohda, Takayuki, Matsumura[1], Yuu Taguchi, Sayaka Yamane and Jun-ichiro Inoue

Osteoclasts are responsible for bone resorption and play a critical role in bone homeostasis. Excess formation or activity of osteoclasts pathological bone resorption, such as that in postmenopausal osteoporosis, rheumatoid arthritis, Paget's disease and bone tumor metastasis. Therefore, a precise understanding of the mechanisms that regulate osteoclast formation is required for understanding the onset of skeletal diseases and for developing drugs to treat such disease.

Osteoclast differentiation and activation requires Receptor activator of NFκB (RANK) and RANK ligand (RANKL)- mediated signaling. RANK is a member of the tumor necrosis factor receptor superfamily (TNFRSF), and its intracellular signaling pathways are mediated by TRAF family. We showed previously that TRAF6-

deficient mice exhibit severe osteopetrosis and are defective in osteoclast formation due to defective signaling from RANK. Furthermore, we demonstrated that overexpression of TRAF6 in osteoclast progenitor cells by a retrovirus vector system induces osteoclast formation. Thus, TRAF6 is not only required, but also sufficient for osteoclastogenesis.

On the other hand, CD40, another member of TNFRSF, is not able to promote osteoclastogenesis, although it activates NFκB and AP-1 in a TRAF6-dependent manner likely to RANK. RANK only can induce NFATc1 expression, a master gene in osteoclastogenesis, through sustained co-stimulatory signals mediated by immunoglobulin-like receptors/ITAM-containing adaptors complexes. Therefore, RANK, but not CD40, can transmit specific TRAF6-dependent signals in addition to those leading to NFκB and AP-1 activation. To elucidate the molecular mechanisms of this RANK-specific function in osteoclastogenesis, we searched for specific region in RANK critical for osteoclastogenesis. As a result, we identified such a functional region, HCR that is highly conserved in RANKs from various vertebrates and is essential for osteoclast formation. Addition of the HCR to the cytoplasmic tail of CD40 rendered CD40 capable of inducing sustained activation of co-stimulatory signals and subsequent activation of NFATc1. Furthermore, the HCR dramatically enhanced internalization of HCR-harboring receptors upon stimulation. These findings suggest that the HCR can continuously link the RANK-TRAF6 axis to sustained co-stimulatory signals through control of the membrane trafficking of receptors and that the HCR may be a novel therapeutic target for pathological bone resorption.

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 its activation plays a crucial role in T-cell transformation and survival of leukemic cells. Therefore, identification of the molecular mechanisms of constitutive NFκB activation may lead to development of drugs to treat ATL. In early stage of ATL development, constitutive NFκB activation is caused by HTLV-1-derived oncoprotein, Tax. Tax directly associates with NEMO, a noncatalytic subunit of IκB kinase (IKK) complex, and induces activation of IKK leading to NFκB activation. In contrast, ATL cells show constitutive NFκB activation despite the deficiency of *tax* gene or severe suppression of Tax expression. These facts suggest that NFκB activation in ATL is caused by Tax-dependent and Tax-independent mechanisms.

To clarify the molecular mechanism of Tax-induced IKK activation, we established a cell free system that activates IKK in response to Tax. Addition of recombinant Tax protein to cell extracts prepared from Jurkat cells led to the phosphorylation of IκBa. Depletion of IKK complex from the cell extracts and addition of IKK inhibitors to reaction mixtures prevented IκBa phosphorylation by Tax protein, indicating that the cell free system mimics the stringent requirement of IKK for Tax-induced IκBa phosphorylation. In contrast, Tax did not directly activate IKK complex purified from cell extracts. These results suggest the existence of intermediary factors required for IKK activation by Tax in the extracts. We are currently purifying such factors by fractionating the cell extracts. On the other hand, to elucidate the molecular mechanism of Tax-independent NFκB activation in ATL, we are identifying the NFκB inducers by RNA interference technique using a lentivirus vector system. To date, we have identified some candidates regulating NFκB activation in ATL leukemic cells.

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. Inability to proliferate HIV-1-specific memory CD4+ T cells is restored by suppressing HIV-1 reactivation in chronically HIV-1-infected individuals.

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

The loss of proliferative capacity by HIV-specific memory CD4+ T cells is a hallmark of the chronic progressive HIV-1 infection thus contributing to the lack of maintenance of a protective immunity against HIV-1. In order to address whether or not these cells have been eliminated or whether HIV-1 infection/replication in HIV-1-specific memory CD4+ T cells directly link to the functional defect of these memory T helper cells, we utilized a recombinant lentivirus expressing HIV-1-specific shRNA (Lenti shNef 366) for the lentivirus-mediated RNA interference. Lenti shNef366 efficiently inhibited the HIV-1 replication in CD4+ T cells. We demonstrated that by blocking ongoing HIV-1 replication, HIV-1-specific memory CD4+ T cells are able to restore their proliferative capacity in eight of twelve chronic HIV-1 patients, especially with a high viral load. Interestingly, the significant recovery of proliferative capacity was observed only cells at an intermediate stage of differentiation. Our results show that 1) HIV-specific CD4 T cells are not fully eliminated in chronic stages of the infection and remain present in an intermediate stage of differentiation 2) that HIV infection and replication strongly impairs their proliferative capacity for the immune system to work efficiently in chronic HIV-1 infection, it is absolutely important to restrict HIV-1 replication in HIV-1-specific memory CD4 + T cells during immune responses.

8. Characterization of breast cancer cells with constitutively activated NFκB

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

NFκB is usually maintained in an inactive form in the cytoplasm through its association with one of the specific inhibitory IκB proteins, whereas it is constitutively activated in many types of cancers and cancer cell lines. Overexpression of an IκBa mutant (IκB super repressor) which lacks phosphorylation sites by IKK revealed that NFκB activity contributed to the survival or growth of cancer cells, cancer invasion, and metastasis and angiogenesis. Cancer 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. However, it is not yet clear which types of cancer cells exhibit constitutive activation of NFκB.

To answer this question, we first examined basal NFκB activities of 125 human cancer cell lines from various tissues with electorophoretic mobility shift assays. We classified these cell lines on the basis of comparison of their NFκB activities with that of TNFa-stimulated Jurkat cells and revealed that 25 of 125 (20%) cell lines exhibit high basal NFκB activities (more than 20 % of that of TNFa-stimulated Jurkat cells). Next, we analyzed the gene expression profiles of breast cancer cell lines with high basal NFκB activity to identify discriminating genes that characterize these cells. Interestingly, it was found that an expression level of a mammary epithelial stem/progenitor cell marker gene has a sequential correlation with the basal NFκB activity. These results suggest that constitutively activated NFκB is involved in development of cancer cells derived from mammary epithelial stem /progenitor cells.

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

Takashi Miyasaka, Yuko Hata[2], Masaaki Oyama[2], Jun-ichiro Inoue, Kentaro Semba[1] and Tadashi Yamamoto[7]: [7]Division of Oncology, IMSUT

Lung cancer is the leading cause of cancer mortality all over the world including Japan. Recently accumulating evidence indicates that EGFR mutations in non-small cell lung cancer (NSCLC) have dramatic impact on lung cancer therapy with EGFR inhibitor, gefitinib. Mutant EGFR-mediated signal transduction pathways in NSCLC are, however, just beginning to be understood. Recent advanced 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 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 68 tyrosine phosphorylated proteins or their associated proteins and suggested novel signal pathways downstream from EGFR. We further confirmed that at least six proteins were tyrosine phosphorylated upon EGF stimulation. RNAi-mediated knockdown experiments suggested that two of them are required for cell survival or growth. Analysis of these proteins is in progress.

10. Identification of *FoxA1* as a lineage-specific oncogene in luminal type breast cancer

Noritaka Yamaguchi[1], Sakura Azuma, Junichiro Inoue, Kentaro Semba and Shinya Watanabe[5]

ErbB2/Her2/Neu is a receptor-type protein tyrosine kinase, whose amplification and increased expression is closely correlated with prognosis of breast cancer. Herceptin is a humanized monoclonal antibody that targets the extracellular domain of ErbB2 and has clinical activity against ErbB2-overexpressing breast tumors. However, the response rate to Herceptin as a single agent in first-line treatment of ErbB2-positive metastatic breast cancer is less than 40 % and the median duration of response is between 9 and 12 months. Thus, the efficacy of Herceptin is limited by intrinsic and acquired resistance, and it is hoped that an improved understanding of the molecular pathways involved in breast cancer development may facilitate development of novel targeted molecular therapies.

Forkhead box A1 (FoxA1) is a member of the Fox class of transcription factors that are characterized by an evolutionarily conserved 110 amino-acid DNA-binding domain known as the forkhead domain. High expression of FoxA1 has been reported in various tumors, including breast cancers. Thus, FoxA1 is thought to be involved in mammary tumorigenesis. However, the precise role of FoxA1 in breast cancer development is controversial. We examined expression of *FoxA1* in 35 human breast cancer cell lines and compared it with that of *ErbB2*. We found that *FoxA1* is expressed at high levels in all ErbB2-positive cell lines and a subset of ErbB 2-negative cell lines. Down-regulation of FoxA1 by RNA interference (RNAi) significantly suppressed proliferation of ErbB2-negative and FoxA1-positive breast cancer cell lines. Down-regulation of FoxA1 also enhanced the toxic effect of Herceptin on ErbB2-positive cell lines through induction of apoptosis. Taken together with previous data that FoxA1 is a marker of luminal cells in mammary gland, our present results suggest that FoxA1 plays an important role as a lineage-specific oncogene in proliferation of cancer cells derived from mammary luminal cells.

11. Identification of *NOTCH3* as a therapeutic target for ErbB2-negative breast cancer

Noritaka Yamaguchi[1], Sakura Azuma, Junichiro Inoue, Kentaro Semba[1] and Shinya Watanabe[5]

Gene amplification is one of the mechanisms of oncogene activation. Thus, amplified genes are candidates of molecular targets for cancer. One of the best-characterized genes that are amplified in breast cancer is ErbB2. Although treatment of ErbB2-amplified breast tumors with Herceptin is one of the few examples of a successful molecular-based therapy for breast cancer, its efficacy is limited because ErbB2-negative breast cancers do not respond to the medication.

Recently, we have established a new technique to identify amplified genes with microarray-based gene expression profiles. To identify novel amplified genes in ErbB2-negative breast cancer, we have explored amplified genes in 35 breast cancer cell lines with this technique and found that *NOTCH3* gene is amplified and overexpressed in an ErbB2-negative breast cancer cell line, HCC1143. Because NOTCH3 is one of the important receptor proteins that regulate cell proliferation and differentiation, we examined the functional importance of NOTCH3 in HCC1143 cells. RNAi-mediated knockdown experiments were performed, and it was found that down-regulation of NOTCH3 significantly suppressed proliferation of HCC1143 cells, suggesting that NOTCH3 is important for proliferation of these cells. We next examined the functional importance of NOTCH3 in NOTCH3-nonamplified and ErbB2-negative breast cancer cell lines. Interestingly, we found that down-regulation of NOTCH3 also significantly suppressed proliferation of NOTCH3-nonamplified and ERBB2-negative breast cancer cell lines. In contrast to ErbB2-negative cell lines, the sup-

pressive effects of NOTCH3-RNAi on proliferation of ErbB2-positive cell lines were less effective. Taken together, these data suggest that NOTCH3 plays an important role in proliferation of ErbB2-negative breast cancer cells and is a promising therapeutic target for ErbB2-negative breast cancer.

Publications

Inoue, J., Gohda, J., Akiyama, T., and Semba, K. NF-κB activation in development and progression of cancer. (review) *Cancer Sci*. 98, 268-274 (2007).

Gohda, J., Irisawa, M., Tanaka, Y., Sato, S., Ohtani, K., Fujisawa, J., and Inoue, J. HTLV-1 Tax-induced NFκB activation is independent of Lys-63-linked-type polyubiquitination., *Biochem. Biophys. Res. Commun*. 357, 225-30 (2007).

Ito, E., Honma, R., Yanagisawa, Y., Imai, J., Azuma, S., Oyama, T., Ohwada, S., Akiyama, T., Nomura, N., Inoue, J., Watanabe, S., and Semba, K. Novel clusters of highly expressed genes accompany genomic amplification in breast cancers. *FEBS Letter* 581, 3909-3914 (2007).

Qin, J., Konno, H., Ohshima, D., Yanai, H., Motegi, H., Hirota, F., Matsumoto, M., Takaki, S., Inoue, J., and Akiyama, T. Developmental stage-dependent collaboration between the TRAF6 and lymphotoxin pathways for B-cell follicle organization in secondary lymphoid organs. *J. Immunol*. 179, 6799-6807 (2007).

Saito, A., Nagasaki, M., Oyama, M., Kozuka-Hata, H., Semba, K., Sugano, S., Yamamoto, T., and Miyano, S. AYUMS: an algorithm for completely automatic quantitation based on LC-MS/MS proteome data and its application to the analysis of signal transduction. *BMC Bioinformatics* 8, 15 (2007).

Oyama, M., Kozuka-Hata, H., Suzuki, Y., Semba, K., Yamamoto, T., and Sugano, S. Diversity of translation start sites may define increased complexity of the human short ORFeome. *Mol Cell Proteomics 6*, 1000-1006 (2007).

Yamaguchi, N., Ito, E., Azuma, S., Honma, R., Yanagisawa, Y., Nishikawa, A., Kawamura, M., Imai, J., Tatsuta, K., Inoue, J., Semba, K., and Watanabe, S. FoxA1 as a lineage-specific oncogene in luminal type breast cancer. *Biochem. Biophys. Res. Commun* 365, 711-717 (2008).

Yamaguchi, N., Oyama T, Ito E, Satoh H, Azuma S, Hayashi M, Shimizu K, Honma R, Yanagisawa Y, Nishikawa A, Kawamura M, Imai J, Ohwada S, Tatsuta K, Inoue J, Semba K, & Watanabe S. NOTCH3 signaling pathway plays crucial roles in proliferation of ErbB2-negative human breast cancer cells. *Cancer Res*. in press (2008).

Miyasaka, T., Morita, M., Ito, K., Suzuki, T., Fukuda, H., Takeda, S., Inoue, J., Semba, K. and Yamamoto, T. Interaction of antiproliferative protein Tob with the CCR4-NOT deadenylase complex. *Cancer Sci*. in press (2008).

Haraguchi, K., Ohsugi, M., Abe, Y., Semba, K., Akiyama, T., and Yamamoto, T. Ajuba negatively regulates the Wnt signaling pathway by promoting GSK-3beta-mediated phosphorylation of beta-catenin. *Oncogene* 27, 274-284 (2008).

Department of Cancer Biology

Division of Biochemistry
腫瘍分子医学分野

Associate Professor Seiichi Takasaki, Ph.D.

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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

Interactions between tumor cells and the extracellular matrix (ECM) strongly influence tumor development, affecting cell proliferation and survival, as well as the ability to migrate beyond the original location into other tissues to form metastases. Many of these interactions are mediated by integrins, a ubiquitously expressed family of adhesion receptors. Integrins are essential for cell attachment and control cell migration, cell cycle progression, and programmed cell death, responses that they regulate in synergy with other signal transduction pathways. This large group of transmembrane proteins is formed from 18α and 8β subunits, which dimerize to yield at least 24 different integrin heterodimers, each with distinct ligand binding and signaling properties. With their extracellular domain, integrins can bind to different ECM molecules, such as collagens and laminins, or to cellular receptors, such as VCAM-1. Their intracellular domains connect directly or indirectly to the actin cytoskeleton, thus linking the cytoskeleton to the ECM. Integrins also serve as bidirectional signaling receptors, inducing changes in protein activities or gene expression in response to ligand binding, while also modulating adhesive affinity on the cell surface in response to changes in cellular physiology. Therefore, It is important to understand how integrin's function is regulated. We approach the issue from two different aspects. The first is to solve how function of integrins is affected by their transformation-associated glycosylation change. Based on the amino acid sequences, both integrin α3β1 and α5β1 contain fourteen and twelve potential asparagine-linked glycosylation sites on each of the α and β1 subunits, respectively. Our results using mouse melanoma cell lines with different metastatic potential indicate that expression of 2, 6-branched N-glycans increases in highly metastatic lines. The second is to identify proteins which work as cell surface ligands for integrins and to solve how integrin function is affected by the integrin-binding proteins. We are now focusing on ADAM (a disintegrin and metaloprotease) family proteins that are suggested to interact with integrins. When the expression of ADAMs of melanoma cell lines was examined, several ADAMs including ADAM 12 and 19 were shown to be up-regulated in highly metastasizing melanoma lines. Cell migration and invasion were induced

by transfection of the ADAM genes to the low-metastasizing line. Therefore, these ADAMs are involved in the expression of malignant phenotype, possibly by interacting with integrins on the cell surface, and analysis of the mechanism is now in progress.

ADAM was initially identified in sperm as a potential molecule involved in gamate fusion. It is known that six members of the ADAM family proteins (ADAM1 to 6) are expressed in mouse testis. Among then, function of ADAM4 to 6 is totally unknown. We produced their recombinant proteins in yeast, and their adhesive activities were examined. The result indicates that all three ADAMs are adhesive to several mammalian cells. Adhesion of the cells to ADAM-coated dish was inhibited by addition of fibronectin and laminin, suggesting that all three ADAMs have potential for binding to integrins (fibronectin receptors and laminin receptors). We also found that boar ADAM4 has an adhesive activity which is stronger that that of mouse ADAM4. These ADAMs might be involved in sperm-egg interaction and sperm-oviduct epithelial cell interaction. We are analyzing binding specificity of the ADAMs and their function in fertilization.

2. Analysis of cancer-related sialic acid and sialylated glycans

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Sialic acids usually occupy exposed terminal positions on the oligosaccharide chains of glycoconjugates and frequently serve as ligands for receptors such as selectins and siglecs, which mediate a variety of cell-cell adhesion processes in inflammation and the immune response. Owing to their negative charge, they may also influence the physicochemical properties of cell surfaces and individual glycoconjugates. Sialylated glycoconjugates also seem to be involved in tumour biology, since aberrant glycosylation patterns are very common in human and animal neoplasias. These tumor-associated antigens or tumor markers are of prognostic value, since their expression frequently correlates with invasiveness, metastasis and the tumor grade. The majority of sialylated tumour markers in man involve changes in the presentation of N-acetylneuraminic acid (NeuAc). However, evidence is accumulating to suggest that N-glycolylneuraminic acid (NeuGc) can appear in certain human cancers. Actually, we found that NeuGc NeuGc-containing glycoproteins are expressed in human hepatocarcinoma tissue and other tumor cell lines. We also found that sera from carcinoma patients contain antibodies recognizing NeuGc-containing glycoconjugates. Their content was much higher than that of normal humans. It is known that a gene encoding CMP-NeuAc hydroxylase that converts CMP-NeuAc to CMP-NeuGc has an exon deletion/frame shift mutation in humans. Therefore, the result that Neu5Gc occurs in cancerous human cells presents a paradox in glycobiology.

It is well known that sialic acid is usually linked to terminal galactose residues, internal N-acetylglucosamine, or N-acetylgalactosamine residues of glycans in α-configulation. Extensive studies so far done show that sialic acid residues of N-glycans are all α-linked. No β-linked sialic acids have been found in glycoproteins and glycolipids. However, it is possible that β-linked sialic acids are synthesized by the cells under pathological condition. To address this issue, an antibody raised against a synthetic β-linked sialic acid containing glycolipid was used. Interestingly, gangliosides from human cancer cell lines were stained with the antibody by the immunochemical method, but those from normal cells were not. The result suggests that β-linked sialic acids are expressed on the glycoconjugates of cancer cells. However, there is no chemical evidence that the epitose is really sialylated by β-linkage. Structural analysis of the epitope glycans is now in progress.

Selectin family interacting with its cognate carbohydrate ligands, sialyl Lewis related moieties, is well-studied molecules because of displaying multiple functions and important biological significances. Certain inflammatory mediators such as interleukin-1, tumor necrosis factor-α, or lipopolysaccharides induce vascular endothelia to express E-selectin which were shown to mediate adhesion of tumor cells. Interaction of selectin on endothelial cells and its carbohydrate ligands on cancer cells is deeply concerned with the arrest of metastatic cells in the first step of organ colonization. Accumulating data indicate that the adhesion of many kinds of carcinoma cells to E-selectin was greatly or totally affected by mucin-type glycoproteins carrying sialyl Lewis related moieties. In contrast to mucin-type glycans, only few reports of non-carcinoma cells indicated that N-glycans had main affinity binding for E-selectin. However, it is not clear whether human carcinoma cells express N-glycans with high affinity to E-selectin. Therefore, we examined several cancer cells and found that a human hepatoma cell line, HepG2, shows strong adhesion to E-selectin. The adhesion to an E-selectin/Fc chimera protein or IL-1 βinduced HUVEC were drastically reduced by treatment of the cells with 1-

deoxymannojirimycin, an inhibitor of N-glycan. processing. The result indicates that HepG2 express N-glycans working as ligands for E-selectin. We also analyzed cell surface N-glycans of human esophageal squamous carcinoma cell lines on the fine structural basis. The results indicate that the N-glycans have sialyl Lewis X structure and linear polyfucosylated N-acetyllactosamine outer chains called polyfucosylated sialyl Lewis X structures. Thus, it is obvious that some human carcinoma cells express E-selectin ligands on the N-glycans. Analysis of membrane glycoproteins expressed by human hepatoma and esophageal squamous carcinoma cells indicates that quite few glycoprotein species express N-glycans which serve as E-selectin ligands.