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癌細胞シグナル分野

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Structural and functional analysis of the protooncogenes reveals that a large majority of them encode proteins with protein-tyrosine kinase activity or transcription factors. The protooncogenes are important not only for 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 immune system, central nervous system, and cancer cells. We are also interested in the function of antioncogene products that are involved in regulation of cell cycle checkpoint.

1. Tyrosine phosphorylation and B cell signaling: analysis of BANK and Cbl

Kazumasa Yasuda, Tomoharu Yasuda, Tohru Tezuka, and Tadashi Yamamoto

B cell activation mediated through the antigen receptor is dependent on activation of protein tyrosine kinases (PTKs), such as Lyn and Syk, and subsequent phosphorylation of various signaling proteins. In search of substrates of tyrosine kinases, we identified a novel protein termed BANK (B cell scaffold protein with ankyrin repeats). BANK is expressed in B cells. BANK is tyrosine phosphorylated upon B cell antigen receptor (BCR) stimulation. BANK plays a role in BCR-induced calcium mobilization. Lyn and inositol 1,4,5-trisphosphate receptor (IP_3R) respectively associate with the distinct regions of BANK and that BANK promotes Lyn-mediated tyrosine phosphorylation of IP_3R. Our data suggest that BANK is a novel scaffold protein regulating BCR-induced calcium mobilization by. Because BANK expression is confined to functional BCR-expressing B cells, BANK-mediated calcium mobilization may be specific to a foreign antigen-induced immune response rather than to signaling required for B cell development.

Unlike Cbl-deficient B cells, Cbl-b-deficient DT40 B cells display reduced phospholipase C (PLC)-γ2 activation, Ca^{2+} mobilization, and c-Jun NH_2-terminal kinase activation upon B cell receptor (BCR) stimulation. Cbl-b helps association of PLC-γ2 with BTK as well as with BLNK and is indispensable for Btk-dependent sustained increase in intracellular Ca^{2+}. The tyrosine kinase-binding domain and C-terminal half region of Cbl-b are essential for its association with PLC-γ2 and for regulation of Ca^{2+} mobilization. Thus Cbl-b positively regulates BCR-mediated Ca^{2+} signaling by influencing Btk/BLNK/PLC-γ2 complex formation. We also find that Cbl-b translocates to lipid rafts upon BCR stimulation and Cbl-b acts as a scaffold protein of the Btk/BLNK/PLC-γ2 complex formation in the lipid rafts.

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

Tohru Tezuka, Takanobu Nakazawa, Kazumasa Yokoyama, Shin-ichiro Kina, Seiji Kawa, June Goto, Aya Sato, Sachiko Taniguchi, Miho Ohsugi, Jiro

Fujimoto and Tadashi Yamamoto

The Src-family protein-tyrosine kinases (PTKs) are implicated in various neural functions. For example, Src and Fyn phosphorylate glutamate receptors. Glutamate is a major excitatory neurotransmitter in the central nervous system. Two types of glutamate receptors, ionotropic and metabotropic receptors, have been described and are implicated in synaptic plasticity, synaptogenesis, and excitotoxity. Long-term potentiation (LTP) is one of the most striking examples of synaptic plasticity for supporting memory. Several lines of evidence suggest that protein-tyrosine kinases play regulatory roles in LTP induction. For example, there are data showing that 1) the N-methyl-D-aspartate (NMDA) receptor is highly tyrosine phosphorylated in neuronal cells; 2) stimulation of the NMDA receptor induces the intracellular protein-tyrosine phosphorylation; 3) Src interacts both physically and functionally with NMDA receptor (NMDAR); 4) specific inhibitors for protein-tyrosine kinases attenuate the induction of LTP. Moreover, LTP induction in hippocampus is also attenuated in *fyn*$^{-/-}$ mice. Our own studies have shown that the Src family kinase Fyn is involved in tyrosine phosphorylation of the NMDAR2A/2B subunits. Postsynaptic density protein PSD95 that directly interacts with NMDAR facilitates the tyrosine phosphorylation events. To analyze the biological significance of tyrosine phosphorylation of the NMDAR, we have determined tyrosine phosphorylation sites on MNDAR2A/B. Phosphorylation at Tyr-1472 on NR2B, which is a major tyrosine phosphorylation site, is significantly enhanced after induction of LTP in the hippocampal CA1 region, suggesting that Tyr1472 phosphorylation on NR2B is important fro synaptic plasticity. To further establish biological significance of Tyr1472 phosphorylation, mice with Tyr1472 to Phe mutation was generated. Analyses of the electrophysiological activities of the hippocampal neuron and the behavior of the knock-in mutant mice are underway. We also show that Tyr-1325 on NR2A is a major tyrosine phosphorylation site. Role of Tyr-1325 phosphorylation is going to be analyzed using the same strategy for Tyr-1472 of NR2B.

Accumulating evidence shows that not only tyrosine phosphorylation but also tyrosine dephosphorylation is involved in regulation of synaptic plasticity. We have shown that protein-tyrosine phosphatase PTPMEG is associated with glutamate receptors, NMDAR 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 brain. To know the physiological role of PTPMEG, especially in the tyrosine phosphorylation events of NMDA receptors, we generated PTPMEG$^{-/-}$ mice. We are currently analyzing their behavioral and elctrophysiological phenotypes.

Among the Src-family kinases, which are expressed in the brain, Lyn is specifically expressed in the granular layers of the cerebellum as well as in basal ganglia and cerebral cortex. Possible involvement of Lyn in LTP and LTD (long term depression) is also under investigation by using *lyn*$^{-/-}$ mice. To date, we have found that locomotors activity of *lyn*$^{-/-}$ mice is impaired due to the constitutive activation of NMDA receptor signaling. We have also shown that Lyn associates with AMPA receptor (AMPAR) and becomes activated upon strong stimulation of AMPAR. In parallel to this observation, we have preliminarily shown that AMPAR is tyrosine phosphorylated at least in the cultured cells.

To further study the role of protein-tyrosine kinases in neural function and development of CNS, we have been characterizing protein-tyrosine kinases, including ALK (see below) and AATYK1/2/3. AATYK1 is reportedly a tyrosine kinase, but the specificity of other two, AATYK2 and AATYK3, remains to be verified. All three members express specifically in the central nervous system.

In parallel of these studies, we have been trying to identify the targets of brain-specific protein-tyrosine kinases. We have already identified a number of novel proteins that could be phosphorylated by the Src family kinases. Characterization of these putative substrates is in progress. One of them is p250GAP, a novel GTPase-activating protein for Rho family proteins that interacts with the GluRε2 (NR2B) subunit of NMDA receptors *in vivo*. The p250GAP mRNA was enriched in brain, with high expression in cortex, corpus striatum, hippocampus, and thalamus. Within neurons, p250GAP was highly concentrated in the postsynaptic density and colocalized with the GluRε2 (NR2B) subunit of NMDA receptors and with PSD-95. p250GAP promoted GTP hydrolysis of Cdc42 and RhoA *in vitro* and *in vivo*. When overexpressed in neuroblastoma cells, p250GAP suppressed the activities of Rho family proteins, which resulted in alteration of neurite outgrowth. NMDA receptor stimulation led to dephosphorylation and redistribution of p250GAP in hippocampal slices. Taken together, p250GAP is likely to be involved in NMDA receptor activity-dependent actin reorganization in dendritic spines.

3. Elucidation of the biological role of Tob family proteins and Tob-mediated signaling pathway

Toru Suzuki, Junko Tsuzuku, Takahisa Nakamura, Rieko Ajima, Makoto Watanabe, Takashi Miyasaka, Mitsuhiro Yoneda, Amy Lai, Yutaka Yoshida, and Tadashi Yamamoto

By screening a cDNA expression library with autophosphorylated c-erbB-2 protein, we isolated cDNA clones coding for its possible substrates. One of the genes thus identified, termed *tob*, encodes a 45kDa protein with homology to the growth suppressing proteins Btg1 and PC3. We also cloned

novel genes homologous to *tob* that were termed *tob-2* and *ana*. Microinjection experiments showed that exogenously expressed Tob, Tob-2, and ANA proteins suppress growth of NIH3T3 cells. Thus, we propose a new family of antiproliferative genes that includes *tob*, *tob-2*, *btg1*, *pc3/tis21/btg2*, and *ana*. In our study to address the mechanisms by which Tob suppresses cell growth, we showed that overexpression of Tob family proteins resulted in hypophosphorylation of the Rb protein. Additionally, growth of Rb-deficient cells was not affected by Tob, suggesting that Tob inhibits cell growth in an Rb-dependent manner. We also found that Tob is rapidly phosphorylated at Ser152, Ser154, and Ser164 by Erk1 and Erk2 upon growth factor stimulation. Oncogenic Ras-induced transformation and growth factor-induced cell proliferation are efficiently suppressed by mutant Tob that carries alanines but not glutamates, mimicry of phospho-serines, at these sites. Wild-type Tob is little inhibitory to the cell growth when the three serine residues are phosphorylated but inhibitory when they are not phosphorylated. Intriguingly, cyclin D1 expression is elevated in serum-starved $tob^{-/-}$ cells. Reintroduction of wild-type Tob and mutant Tob with serine to alanine but not to glutamate mutations on the Erk phosphorylation sites in these cells restores the suppression of cyclin D1 expression. Thus, Tob inhibits cell growth by suppressing cyclin D1 expression, which is cancelled by Erk1- and Erk2-mediated Tob phoshorylation. We propose that Tob is critically involved in the control of early G1 progression.

To elucidate physiological function of Tob, we generated *tob*-deficient mice. *tob*-deficient mice had a greater bone mass resulting from increased number of osteoblasts. Orthotopic bone formation in response to BMP2 was elevated in *tob*-deficient mice. Overproduction of Tob repressed BMP2-induced, Smad-mediated transcriptional activation. Finally, Tob associated with receptor-regulated Smads (Smad1, 5, and 8), and colocalized with these Smads in the nuclear bodies upon BMP2 stimulation. The results indicate that Tob negatively regulates osteoblast proliferation and differentiation by suppressing the activity of the receptor-regulated Smad proteins.

We also found that aged mice lacking Tob expression develop a variety of tumors, such as hemangiosarcomas and hepatocarcinomas, at a very high frequency. Intraperitoneal injection, at two weeks after birth, of the carcinogen diethylnitrosamine (DEN) led to more frequent generation of liver tumors in *tob*-deficient mice than in wild-type mice. $tob^{-/-}p53^{-/-}$ mice show accelerated tumor formation in comparison with single null mice. Levels of tob mRNA are often decreased in human cancers, implying tob in cancer development. Furthermore, embryonic fibroblasts deficient for *tob* displayed a marked increase in chromosomal aberration, including breakage, translocation, and aneuploidy after the treatment of DEN. To circumvent functional redundancy between Tob and Tob-2, disruption of the *tob*-2 gene and subsequent generation of double knockouts ($tob^{-/-}tob\text{-}2^{-/-}$) were performed. Analysis of their phenotypes is underway.

Among the Tob family proteins, Tob and Tob2 proteins contain a putative nuclear localization signal (NLS) that is present near the amino-terminus. We showed that Tob is a nuclear protein by immunostaining. Nuclear localization of Tob is impaired by mutation of the putative NLS. Furthermore, Tob NLS sequence alone could drive nuclear import of the EGFP-LacZ fusion protein, indicating that the NLS of Tob is functional. Unlike wild-type Tob, Tob NLS mutant protein is poorly antiproliferative, suggesting that subcellular localization of Tob is related to the regulation of cell growth. Because the Tob family proteins interact with various transcription factors, such as Caf1, Tob is likely involved in transcription regulation. We assume that Tob acts as a transcriptional co-repressor. Indeed, expression of cyclin D1 mRNA is increased in the absence of Tob and Tob suppresses the cyclin D1 promoter activity through an interaction with histone deacetylase. There are data suggesting that Caf1 is associated with deadenylase activity, suggesting that Tob-Caf1 complex may be involved in translational regulation. Intriguingly, X ray analysis of the crystal structure reveals that three-dimensional structure of the Caf1-Tob complex is similar to that of E. coli Exonuclease I.

Other studies to establish biological significance of the *tob* family members are widely in progress. The studies include generation of *caf1*-deficient mice, search for the molecules that is relevant for regulation of the stability of Tob, purification and characterization of a large protein complex with Tob, and search for the genes whose expression are affected in the absence of Tob. Targeted inactivation of *caf1* leads to the male mice sterility, owing to oligo-astheno-teratozoospermia. Transplantation of germ cells from *caf1*-deficient male mice to germ cell-deficient male mice restored the structural abnormalities of the spermatozoa, suggesting that some of the spermatogonia in adult *caf1*-deficient males are potentially functional stem cells.

4. Role of kinesin-family protein Kid (kinesin-like DNA binding protein) in cell cycle regulation and chromosome segregation

Miho Ohsugi, Noriko Tokai-Nishizumi, Yasuomi Horiuchi, and Tadashi Yamamoto

Microtubule-associated motor proteins are involved in spindle formation and chromosome movements in mitosis and meiosis. We previously cloned cDNAs for a gene termed *kid* that encodes a novel member of the kinesin family of proteins. The *kid* gene product is a 73-kDa protein and related to

the Drosophila *nod* gene product, which is involved in chromosomal segregation during meiosis and mitosis. The microtubule-associated motor domain of Kid is present in its amino-terminal half and has the plus-end directed motor activity. Its carboxy-terminal half contains a helix-hairpin-helix DNA binding motif and is able to bind to specific DNA sequences similar to a part of α-satellite DNA. Overexpression of Kid, the motor domain, or the DNA binding domain abrogated chromosomal segregation. We also showed that Kida was phosphorylated at multiple sites during mitosis. We identified a Cdc2 kinase phosphorylation site on Kid and showed that Kid with a point mutation at the site failed to localize to chromosome, although the mutant Kid retains the ability to bind to mitotic chromosomes. These results suggest that Cdc2 kinase regulates the localization of Kid and the mutation on the phosphorylation site results in selective localization of Kid on microtubules. We have data suggesting that an electrostatic force is involved in the interaction. The force would prevent Kid from moving toward chromosome. Identification of other mitotic kinases that phosphorylate Kid and study of biological role of the phosphorylation are in progress. To further study the mechanisms for regulating localization, function and degradation of Kid, we are searching for Kid binding proteins by yeast two-hybrid system.

5. Characterization of LATS kinases, human homologs of a Drosophila tumor suppressor

Yoshinori Abe, Chenyu Bai, Jiro Fujimoto Miho Ohsugi and Tadashi Yamamoto

We have previously identified genes encoding human serine/threonine protein kinases with significant homology to a Drosophila tumor suppressor gene LATS. We named these two genes hLATS1 and hLATS2, and analyzed the function of the gene products. FISH analysis revealed that the hLATS1 and hLATS2 genes are localized to chromosome 6q and 13q, respectively. In these regions, loss of heterozygosity (LOH) is observed in various cancers including breast cancers, hepatocellular carcinomas, and renal carcinomas. We searched for mutations of the hLATS genes in various cancer cell lines and identified two cell lines that had alteration of the hLATS2 gene. One is a renal carcinoma cell line that had a large deletion in the hLATS2 gene resulting in suppression of the hLATS2 protein expression. The other is a colon cancer cell line and had a point mutation in the coding region of the hLATS2 gene. This mutation caused the substitution of a single amino acid that was highly conserved among the protein kinases. In these two cell lines, phosphorylation of the proteins such as Bcl-2 and Raf-1, which are reported to be involved in Taxol (a microtubule inhibitor)-induced apoptosis, as well as LATS1 was not observed in the presence of Taxol. Ectopic expression of wild-type LATS-2 protein in these cells caused the phosphorylation of these proteins and enhanced the apoptosis upon Taxol treatment. These results suggest that LATS-2 transmits an apoptotic signal caused by microtubule damages.

To further examine the role of LATS-2 in tumorigenesis and cell cycle regulation, we have searched for molecules that interact with LATS-2 and identified some centrosome-localized proteins such as Kendrin. Interestingly, our immunofluorescence study showed that LATS-2 was localized in centrosome area. Proper centrosome duplication is important for cell division and centrosomal abnormality is often observed in tumor cells. The role of LATS-2 in centrosome regulation is under investigation.

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Cell surface is an interphase for communication of the cells with extracellular environment and signals are transmitted in both directions through the cell membrane. Molecules on the surface and their ligands in the extracellular milieu are frequently regulated by proteolysis. In addition to degradation, such pericellular proteolysis includes processing that mediates activation, inactivation and functional conversion of the molecules there. Since the signals traverse the cell membrane regulate various cellular functions such as proliferation, apoptosis, differentiation, and invasion, abnormality in the pericellular proteolysis contributes to the behavior of cancer cells. The aim of our study is to shed light on the proteolytic events on cell surface that regulate the cell functions and their relevance to various diseases including cancer.

1. CD44 directs membrane-type 1 matrix metalloproteinase (MT1-MMP) to lamellipodia by associating with its hemopexin-like domain (PEX)

Hidetoshi Mori, Taizo Tomari, Naohiko, Koshikawa, Masahiro Kajita, Yoshifumi Itoh, Hiroshi Sato, Hideaki Tojyo, Ikuo Yana and Motoharu Seiki

Cell migration requires a continuous re-organization of the actin-based cytoskeleton within the cell and concerted action of adhesion molecules and proteases outside. Cell adhesion molecules play a particular role in gaining a foothold at the migration front and releasing it at the rear. At the same time, cells need to remove the extracellular matrix (ECM) to open the migration pathway. Matrix metalloproteinases (MMPs), also referred to as Matrixins, are responsible for the degradation of ECM components, and have therefore been implicated in this process. To eliminate the ECM barrier efficiently, MMP activity is required to work at the cell migration front, although the specific mechanism to regulate this polarized action is poorly understood. While the majority of MMPs are in a soluble form, six are membrane-type proteins (MT-MMPs) having devices that enable integration into the plasma membrane, such as transmembrane domains or glycosyl-phosphatidyl inositol (GPI) moieties. By having a potent pericellular proteolytic activity, MT-MMPs have a strong invasion-promoting activity compared to soluble MMPs. In particular, MT1-MMP is frequently expressed in invasive cancer cells and in endothelial cells during angiogenesis. The substrates of MT1-MMP include type I collagen, laminin and fibronectin. The enzyme also activates other proMMPs, including proMMP-2 and proMMP-13. Thus, MT1-MMP triggers MMP activation cascades on the cell surface.

Upon the stimulation of cells to migrate, MT1-MMP relocates to the lamellipodium and such a polarized localization seems particularly useful for the cells to invade tissue. The increased concentration of MT1-MMP at the lamellipodia also promotes the formation of homodimers via the hemopexin-like (PEX) domain. This dimer formation in turn facilitates proMMP-2 activation, since this process requires at least two adjacent MT1-MMP molecules; namely, one MT1-MMP molecule acts as a receptor for the proMMP-2/TIMP-2 complex while the other acts as an activator. In addition to its role as a protease in ECM degradation, MT1-MMP is a critical part of the mechanism of cellular locomotion as well. For example, cleavage of laminin 5 at the γ2 chain or

CD44H by MT1-MMP is linked to its migration-stimulating activity. Thus, it is particularly important to understand the mechanism by which the localization of MT1-MMP is regulated during cell migration.

Cell adhesion molecules should co-operate closely with ECM-degrading proteases during cell migration and recent findings support this idea. For example, MMP-1 associates with $\alpha_2\beta_1$ integrin during epithelial cell movement on type I collagen in the wound-healing process. MMP-2 binds $\alpha_v\beta_3$ integrin and promotes endothelial cell migration. We have also observed that MT1-MMP shed CD44H from the cell surface and stimulated cell migration in conjunction with the CD44 shedding. It also binds MMP-9 and promotes tumor invasion. CD44 is primarily a hyaluronan (hyaluronic acid, HA) receptor and binds collagen I, fibrin, and chondroitin sulfate proteoglycans. CD44 has multiple isoforms containing inserts derived from alternatively spliced exons at the variable region. Within the cytoplasm, CD44 interacts with ezrin/radixin/moesin (ERM) and/or ankyrin, both of which bind F-actin. CD44 is expressed in many types of migratory and metastatic tumor cells and promotes the migratory potential of these cells.

CD44 is spontaneously released from the cell surface by proteolytic processing. Interestingly, only CD44 shedding by metalloproteinases seems to play a role in cell migration because MMP inhibitors suppress the cell migration and shedding while serine proteinase inhibitors do not. Multiple metalloproteinases appear to have CD44 shedding activity, but only MT1-MMP has been clearly characterized as a shedding enzyme having migration-promoting activity. Both MT1-MMP and CD44H localize at the migration front and we speculate that the events occurring here are critical for cell migration. Thus, it is of particular interest how CD44H and MT1-MMP co-localize at the lamellipodia when the cells migrate.

This study aimed to elucidate how both MT1-MMP and CD44H are regulated to co-localize at the migration front. We found that MT1-MMP and CD44H form a complex mediated by the interaction between the PEX domain and the stem region of CD44H. Deletion of the PEX domain abolished the ability of the mutant MT1-MMP to localize at lamellipodia. Conversely, overexpression of the cytoplasmic-deletion mutant of CD44H, which shows no association with the actin cytoskeleton, prevented the localization of MT1-MMP to the lamellipodia. Thus, CD44H appears to link MT1-MMP to the actin cytoskeleton and regulate its localization. This interaction through the PEX domain is also critical for the shedding of CD44H and the cell migration-promoting activity of MT1-MMP. Our findings shed light on the close inter-relationship between CD44 and MT1-MMP, both of which are implicated in cell migration and invasion.

2. Roles of MT1-MMP in angiogenesis through type-I collagen

Ikuo Yana, Kaori Taniwaki, Kunika Nishibashi, Chieko Konishi, and Motoharu Seiki

It has been well documented that MT1-MMP plays a pivotal role in angiogenesis through type I collagen or fibrin. Two separate studies using the mice strain deficient in MT1-MMP gene have clearly shown a similar phenotype that skeletal development was severely impaired during postnatal period, probably caused by disorganized collagen turn-over, or malvascularization through collagenous tissue such as bone, and cornea. However, it has been remained to be precisely evaluated how MT1-MMP plays a role in the phenotypes. In order to understand expression profile on MT1-MMP in more detail, we have established MT1-MMP gene deficient strain in which exon 1-5 of MT1-MMP gene is recombined with the targeting fragment including lacZ gene, so that the galactosidase activity could mimic the expression profile on intrinsic MT1-MMP. Using the strain, an angiogenesis assay with murine muscle chunks has been performed to study the MT1-MMP expression in the time course of neovascularization. Our main goal is to understand the role in physiological expression of MT1-MMP during either the angiogenesis or vascular maturation, furthermore to compare the role to that during pathological angiogenesis.

3. MT1-MMP plays a role in in-vivo tumor invasion

Kaori Taniwaki, Ikuo Yana, Takahiro Nonaka, Kiyoshi Komori, Kunika Nishibashi, Hiroshi Fukamachi, Motoharu Seiki

MT1-MMP is thought to play an important role to facilitate cell invasion or migration through the extracellular matrix. MMP-2 is activated by MT1-MMP with a help of TIMP-2 on the cell membrane and degrades type IV collagen, a major and unique component of the basement membrane. In a specific condition, it is thought that MMP-2 also has a chance to activate MMP-9, another type IV colagen-degrading enzyme, and MMP-13 which degrades type I collagen in the tissue. Therefore, it has been thought that the combination of MT1-MMP and MMP-2 contributes to invasion and metastasis by tumor cells. However it is not sure enough if tumor cells utilize these enzymes to invade into extracelullar matrix under the physiological environment.

To assess the invasion profile through collagen or matrigel in the presence or absence of MT1-MMP. Cell lines were established from fibroblasts, and gastric mucosa of the mice having a defect in the MT1-MMP gene and they were transformed by onco-

genes. Pathological features of each tumor cells derived from wild type mice are compared with those from MT1-MMP knocked-out strain, respectively. We also evaluate the function of MMP-2 utilizing a MMP-2 gene deficient strain. One of the major issues in this study is to assess whether tumor cells utilize the MT1-MMP/MMP-2 system during transmigration through basement membrane or collagenouse tissue *in vivo*.

4. Proteolytic processing of laminin-5 by MT1-MMP in kidney epithelial tissues

Naohiko Koshikawa, Tomoko Minegishi, Vito Quaranta and Motoharu Seiki

Laminin-5 (Ln-5) is an extracellular matrix (ECM) ligand for integrins that supports epithelial cell adhesion, migration, as well as anchoring to basement membranes (BM) via hemidesmosomes. These Ln-5 functions are modulated by several proteolytic processing steps. The mechanisms whereby this processing occurs *in vivo* are incompletely understood. Here, we find that the membrane-type matrix metalloproteinase (MMP) MT1-MMP processes Ln-5 *in vivo*, affecting its functions and probably turnover. Thus, the amino-terminal truncated γ2', a well-known proteolytically processed form of the Ln-5 γ2 subunit, is absent or deficient in tissues of MT1-MMP knock-out (KO) mice. Absence of γ2' is associated with tubular epithelium alterations in KO kidney, including increased amounts of Ln-5 in the tubular BM and poorly differentiated epithelial cells, reminiscent of congenital mixed hypoplastic/dysplastic renal tubule disorders in humans. In parallel experiments, MT1-MMP expression was reconstituted by transfection in a Ln-5 positive, MT1-MMP deficient epithelial cell line. In three independent MT1-MMP transfectants, γ2 processing to γ2' became easily detectable and, at the same time, cells assumed a spread morphology on Ln-5, but not on fibronectin. We further determined that cleavage of γ2 reporter constructs by recombinant MT1-MMP occurs at a site that exactly matches the expected γ2' processing site. Taken together, these results strongly indicate that Ln-5 is a physiological substrate of MT1-MMP *in vivo*. Proteolytic processing of the γ2 subunit by MT1-MMP may influence Ln-5 turnover in epithelial BM and affect epithelial morphology, differentiation and organization.

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癌遺伝形質分野 (1)

Associate Professor Hiroaki Miki, Ph.D.
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Our aim is to elucidate the regulatory mechanism of cell morphology. Currently, we are focusing on the novel signaling pathway regulating the microtubule organization in response to Wnt-signal, which is known to play critical roles in morphogenesis and the development of human cancer. We are also trying to figure out an unexpected functional connection between the SF3a RNA splicing factor and cell morphology by employing biochemical and cell-biological approaches.

1. A novel Wnt-signaling pathway that regulates the dynamics of microtubules

Hideki Yamaguchi and Hiroaki Miki

It has been established that Wnts exert their effects through the activation (stabilization) of β-catenin, which forms a protein complex with transcription factors such as LEF/TCF and induces specific gene-expression. We have found that ectopic expression of Dishevelled, a critical Wnt-signal transducer, in neuroblastoma cells resulted not only in the accumulation of β-catenin in the nucleus but also in significant neurite formation. We then constructed various truncated fragments of Dishevelled and performed similar expression analyses in neuroblastoma cells. The results indicated that the neurite formation occurred in a manner independent of the accumulation of β-catenin. Since the morphology of cells are determined by the cytoskeleton, we then focused on possible effects of Dishevelled on the cytoskeleton and found that microtubules in the Dishevelled-expressing cells were stabilized, that is, they became resistant to microtubule-depolymerizing drug nocodazole. Interestingly, the partial fragment of Dishevelled that induced neurite-formation but lacked the ability to activate β-catenin also induced microtubule-stabilization. Dishevelled is therefore thought to regulate the cellular morphology of neuroblastoma cells through the stabilization of microtubules, which seems to be independent of the β-catenin function.

As for the effect of Wnt-signal on cell morphology, the group of Salinas has reported that Wnt-7a secreted from cerebellar granule cells induced the morphological change of the axonal structures of mossy fibers (Hall et al. Cell 100:525-535, 2000), but the details of the signaling mechanism still remains unknown. We tested various types of Wnts for their effect on β-catenin and microtubules, and found that some types of Wnts could induce the stabilization of microtubules, suggesting that this "Wnt/microtubule pathway" is a physiologically relevant one. We are now trying to elucidate the molecular mechanism of how the novel Wnt-signaling pathway leads to microtubule-stabilization.

2. A novel function of SF3a RNA splicing factor in the regulation of microtubules

Hiroaki Miki

I previously identified IRSp53 as a binding partner for WAVE2, a WASP-family actin-regulating protein

(Miki et al. Nature 408:732-735, 2000). IRSp53 contains an SH3 domain at its C-terminus and I performed a binding protein analysis for the IRSp53 SH3 domain. The results indicated that there existed several binding partners other than WAVE2. A mass spectrometric analysis revealed that the three major binding proteins (SF3a60, SF3a66, and SF3a120) were the components of the SF3a RNA splicing factor, which plays essential roles during pre-mRNA splicing by forming U2snRNP and is evolutionarily conserved from yeasts. Interestingly, the ectopic expression of SF3a66 in neuroblastoma cells specifically induced neurite formation as did IRSp53, whereas SF3a60 and SF3a120 did not show any significant effect. In addition, the co-expression of SF3a66 with IRSp53 resulted in the enhancement of neurite formation, but SF3a120 rather inhibited IRSp53-induced neurite formation. These results suggest the possibility that the SF3a complex cooperates with IRSp53 to induce neurite formation in neuroblastoma cells.

In order to investigate the mechanism of the neurite formation, I generated various truncated constructs of SF3a66 and subjected them to morphological analyses. It was found that the partial fragment of SF3a66 was sufficient for neurite formation and then I performed a binding protein analysis by affinity-chromatography with the GST-fusion proteins of the region. I observed two major binding protein bands that migrated at 55-kDa and 50-kDa regions, respectively, in SDS-PAGE. I am now trying to identify these proteins.

3. Complex formation between IRSp53 and Eps8, which regulates the actin cytoskeketon through the activation of Rac

Yohsuke Funato[1], Hideki Yamaguchi, Tadaomi Takenawa[1], and Hiroaki Miki : [1]Division of Biochemistry, IMSUT

As described above, we performed a binding protein analysis for IRSp53 and identified the SF3a complex as the major binding partner. In the analysis, we also identified Eps8, which had been characterized as a substrate for the EGF receptor tyrosine kinase. Indeed, we confirmed that Eps8 bound to IRSp53 very strongly and endogenous proteins of Eps8 and IRSp53 formed a tight protein complex *in vivo*. A report from the Di Fiore's laboratory indicated that Eps8 induces the activation of Rac, a Rho-family small G protein (Scita et al. Nature 401:290-293, 1999). We are now investigating the possible positive/negative effect of IRSp53 on the Eps8-induced activation of Rac.

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Department of Cancer Biology
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癌遺伝形質分野 (2)

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Our aim is to elucidate molecular mechanisms of infectious diseases caused by a variety of pathogens including tumor viruses. We are currently investigating the subject using a synthetic polynucleotide microarray system that we have independently devised and are ameliorating. Systematic transcriptome analysis with the microarray system can provide fundamental and comprehensive data on the genetic background of infectious diseases.

1. Comprehensive analysis of gene expression with synthetic DNA microarrays

Reiko Honma, Emi Ito, Jun-ichi Imai, and Shinya Watanabe

Toward the final goal of this project to print approximately 30,000 genes as a single microarray, we attempted to increase the number of genes printed within the same area that we previously used and to prepare a material that enables us to directly compare hybridization data independently obtained with a massive number of arrays in future. Eventually, we obtained comprehensive gene expression profiles for multiple human normal tissues and cell lines widely used in laboratories using the identical RNA sample as a reference throughout all hybridization as follows:

a. Microarray printing

We newly added 8,000 synthetic DNA representing individual genes to the previously prepared probes (14K arrays) and stably produced massive microarrays constituted of 22,000 different genes (designated 22K array).

b. Preparation of the common reference for hybridization

To obtain a material that enables to compare data derived from independent hybridization experiments one another, we prepared a mixture of RNA extracted from 22 immortalized cell lines that were established from a variety of tissues and subsequently divided into aliquots as a large identical batch. The RNA mixture should serve as an effective control in profile collection and subsequent analyses (designated common reference).

c. Profiling of gene expression for a variety of samples

We have obtained expression profiles for 29 normal adult human tissues, 8 normal fetal human tissues, and 35 immortalized cell lines widely used using the 14K arrays and the common reference. Finally, we compiled the data of the expression profiles and constructed an information complex that consists of over one million data elements. The information complex should enable to compare independent hybridization experiments for any pair of included samples by simulating in a computer. We believe that the information complex provides suit-

able raw material for data mining in bioinformatics.

2. Simultaneous and comprehensive monitoring for viral and host gene expression in human cytomegalovirus-infected cells

Emi Ito, Reiko Honma, Jun-ichi Imai, and Shinya Watanabe

Human cytomegalovirus (HCMV) is a ubiquitous pathogen associated with lethal opportunistic infections in immunocompromised individuals. To investigate interaction of HCMV and host cell at the transcript level, we generated microarrays containing 14,000 human genes and 173 HCMV genes within a single area and examined expression levels of the host and viral genes simultaneously using a common reference RNA mix consisting of uninfected and infected cells harvested at five time points. Of 173 viral genes printed on the host-virus array, 172 genes were detectable at any time point after infection. The detected HCMV genes were classified into the following four categories based on the expression kinetics: i) viral genes whose expression level was high at 4 hpi, decreased at 24 hpi, and increased again at later time points; ii) viral genes whose expression level was high at 4 hpi and subsequently decreased as progression of infection; iii) viral genes whose expression level was high at 4 hpi and increased successively as progression of infection; and iv) viral genes whose expression was still undetectable at 4 hpi but increased at 24 hpi and later time points. In other hand, cluster analysis of the expression profiles provided several criteria of host cell genes based on the differences in constituents of regulatory pathways for gene expression, including virion adsorption/penetration, newly synthesized viral gene products, and humoral factors excreted from cells after infection. Simultaneous transcriptome analysis for viral and host genes should provide a powerful tool to exploit a novel aspect of the host-parasite relationship.

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Division of Pathology has a unique research strategy to work directly on the pathogenic mechanisms and diagnosis of various human diseases through the analyses of pathological specimens. Elucidation of the primary events hidden in such specimens is the priority field of pathology. Our current targets include human lymphoid diseases and viral infections involving lymphoid tissues/cells.

I. Malignant lymphomas

1. Gene abnormalities and other pathogenetic abnormalities of human malignant lymphoma specimens

Shigeo Mori, Tomoko Nakajima, Hideaki Takahashi, Takamitsu Okamura, Naoto Aoki[1]: [1]Tokyo Metropolitan Laboratory of Hygienic Science

Chromosomal abnormalities and exogenous agents are the main primary causes of human malignant lymphomas. On specimens derived from lymphoma patients for diagnostic purpose, several surveys including abnormal human (patients') gene and exogenous genomes are under investigation. In those are included positional cloning of a B-cell lymphoma cell line carrying t(8;22) lacking c-myc gene rearrangement and subtraction analysis of a few lymphomas and a hepatoma in which viral infections are suspected.

2. Characterization of a novel sno-RNA isolated from a diffuse large B-cell lymphoma

Yuichi Soeno, Tsutomu Hatori, Ritsuko Tanaka, Shigeo Mori

The bcl-6 gene is known to be a promiscuous gene translocating to various different genes. Through the analysis of lymphoma cases bearing bcl-6 associated translocation, we have identified a novel partner gene, termed as H50HG, and found it to bear a new member of sno-RNA U50 that works on the regulation of ribosomal protein synthesis. Intending to clarify its pathologic role through animal model, we isolated murine host genes that harbor mouse U50. We found two novel host genes whose introns encode mU50 (Tanaka et al., in preparation). Our present aim is to clarify the function, especially in relation to lymphomagenesis, by the introduction of knock-out mice. In addition, we are now conducting a survey to identify new genes that are reversely encoded in U50HG site.

3. EBV infection status of Japanese lymphomas and normal individuals

Kengo Takeuchi[2], Shigeo Mori: [2]Department of Laboratory Medicine

One of the racial and geographical specificity of malignant lymphomas among Japanese resides in its high prevalence of EBV-associated cases. Hodgkin lymphoma is one of the lymphoma subtypes that as-

sociate with EBV. Hodgkin lymphoma is currently divided into five subtypes based on the morphology, and in four of those subtypes, EBV is suggested to take roles on its pathogenesis. Nodular sclerosis, one of those four, is known to occur much more frequently in young women, and the incidence is increasing rapidly in western countries. While studying the role of EBV on Japanese Hodgkin lymphomas, we recently noted that the incidence of EBV positivity is decreasing rapidly in those 50 years among Nodular Sclerosis subtype but not in other subtypes (Takeuchi et al., 2001). This data suggests the rapid change of EBV infectious status among Japanese in recent years. Thus, we conducted nation-wide epidemiological study to clarify the EBV infection status. As the result, we found that the incidence of EBV among childhood is decreasing dramatically in those 10 years from 90% to 50% (Takeuchi K et al., submitted).

4. The pathogenesis of so-called vascular neoplasia

Chihiro Kakiuchi[3], Kengo Takeuchi, Shigeo Mori: [3]Department of Pathology, Graduate School of Medicine, University of Tokyo

Vascular neoplasia is a rare neoplasia of parenchyma cells accompanied by the prominent proliferation of localized blood vessels and lymph node plasmacytosis. We experienced a case of vascular neoplasia and maintained the neoplastic cells in the form of primary culture. With intensive in vitro analysis we found the neoplastic cells to secrete high amount of IL-6 and vascular endothelial growth factor (VEGF). It is now evident, from this result that secretion of those cytokines are the cause of (at least a part of) so called vascular neoplasias (Kakiuchi, J Pathol 2002).

5. Hodgkin's lymphoma

Ryouichi Horie[4], Mariko Watanabe[4], Yasuyuki Morishita, Kinji Ito[5], Masae Nagai-Maruyama, Shigemi Aizawa, Takaomi Ishida, Shigeo Mori, Marshall E. Kadin[6], Lawrence J Abraham[7], Toshiki Watanabe: [4]Department of Hematology, Kitasato University, School of Medicine, [5]Department of Pathology, Toho University School of Medicine, [6]Beth-Israel Hospital, Harvard Medical School, [7]Biochemistry & Molecular Biology, School of Biomedical & Chemical Sciences, University of Western Australia

a. Ligand-independent signaling of overexpressed CD30 drives constitutive activation of NF-kB in Hodgkin/Reed-Sternberg cells

Hodgkin's lymphoma (HL) is a malignant lymphoma characterized by the presence of mononucleated Hodgkin cells and multinucleated Reed-Sternberg cells (H-RS cells) in a background of reactive cells comprising lymphocytes, eosinophils, plasma cells, histiocytic cells and fibroblasts. However, the biological mechanisms of its growth, regulation, and death remained unsettled. H-RS cells are characterized by overexpression of CD30, a member of tumor necrosis factor receptor (TNFR) superfamily. Recently, constitutively activated Nuclear factor-kB (NF-kB) (p50/p65) was reported to be a unique and common characteristics of H-RS cells, which prevent these cells from undergoing apoptosis and triggering proliferation. NF-kB is a pleiotropic transcription factor that interacts with the upstream regulatory regions of numerous genes. Ligation of CD30 by its ligand or an antibody triggers signals to activates NF-kB through recruitment of TNFR associated factor (TRAF) 2 and 5. We found that, through self-association, CD30 overexpression constitutively recruits TRAF2 and 5, and drives the NF-kB activation in H-RS cells. These findings linked two major characteristics of H-RS cells, CD30 overexpression and constitutive activation of NF-kB, and provided new insights into the mechanisms by which H-RS cells grow and express immuno-regulatory cytokines that characterize clinical features of Hodgkin's disease. To find out a cytospecific gene therapy based on the mechanisms of tumor cell growth, we examined adenovirus vector as a tool to efficiently transduce an effector gene into H-RS cells. We found that adenovirus vector can infect H-RS cell lines very efficiently, which contrasts to the relative resistance to adenovirus infection generally found in lymphoid and hematopoietic cells. We demonstrated that adenovirus-mediated transduction of a mutant CD30 that lacks the cytoplasmic region or dominant negative IkBa induced apoptosis of H-RS cell-derived cell lines. These findings provide a basis for a new strategy of cytospecific gene therapy of HD. (Horie et al., Oncogene, 2002)

Confocal immunofluorescence microscopy of cell lines derived from H-RS cells and HEK293 transformants highly expressing CD30 revealed aggregation of TRAF2 and TRAF5 in the cytoplasm as well as clustering near the cell membrane. In contrast, TRAF proteins were diffusely distributed in the cytoplasm in cell lines unrelated to HL and control HEK293 cells. Furthermore, the same intracellular distribution of TRAF proteins was demonstrated in H-RS cells of lymph nodes of HL, but not in lymphoma cells in lymph nodes of non-Hodgkin's lymphoma. Dominant negative TRAF2 and TRAF5 suppressed cytoplasmic aggregation along with constitutive NF-kB activation in H-RS cell lines. Confocal immunofluorescence microscopy also revealed co-localization of IKKa, NIK and IkBa with aggregated TRAF proteins in H-RS cell lines. These results suggest involvement of TRAF protein aggregation in the signaling process of highly expressed CD30 and suggest they function as scaffolding proteins. Thus, cytoplasmic aggregation of TRAF proteins appears to reflect constitutive CD30 signaling which is char-

acteristic of H-RS cells. (Horie et al., Am J Pathology, 2002)

b. AP-1 mediated relief of repressive activity of the CD30 promoter microsatellite in Hodgkin and Reed-Sternberg cells

Overexpression of CD30 is the hallmark of Hodgkin and Reed-Sternberg (H-RS) cells and drives constitutive NF-kB activation that is the molecular basis for the pathophysiology of Hodgkin lymphoma (HL). Transcription of the CD30 gene is controlled by the core promoter that is driven by Sp-1 and the microsatellite sequences (MS) that represses core promoter activity. To understand the mechanism(s) of CD30 overexpression in H-RS cells, we structurally and functionally characterized the CD30 MS. Although the CD30MS of H-RS cell lines was polymorphic, it was not truncated compared with that of control cells. A strong core promoter activity and constitutive Sp-1 binding were revealed in all cell lines examined irrespective of the levels of CD30 expression. In transient reporter gene assays, all MS clones derived from H-RS cell lines repressed the core promoter activity in unrelated cell lines, but not in the H-RS cell lines. An AP-1 binding site was found in the MS at nucleotide position of -377 to –371, the presence of which was found to relieve repression of the core promoter in H-RS cell lines but not in other tumor cell lines. H-RS cell lines showed constitutive and strong AP-1 binding activity, but other cell lines did not. The AP-1 complex contained JunB, whose overexpression activated reporter constructs driven by the CD30 promoter including the MS, and was dependent on the AP-1 site. JunB expression was detected in H-RS cells in vitro and in vivo, but not in reactive cells or tumor cells of non-Hodgkin's lymphoma of diffuse large B cell type. Taken together, overexpression and binding of JunB to the AP-1 site appear to relieve the repression of the core promoter by the CD30 MS in H-RS cells, which provide one basis for the constitutive overexpression of CD30 in HL (Watanabe M et al., Am J Pathol, in revision).

6. Molecular analysis of 1p36 chromosome translocation found in malignant lymphoma

Hitoshi Satoh

We have arrayed 21 cosmid and five P1 phage clones along with the short arm of chromosome 1 from telomere to the centromere direction by pairwise comparison experiment using multi-color FISH technique. The resulting order is as follows: 1pter-D1S1002 (cYS142)-D1S1053 (cYS1467)-**D1Z2**-D1S1085 (cYS1138)-D1S1013 (cYS1299)-D1S1032 (cYS1384)-D1S1010 (cYS1296) / D1S1047 (cYS1429)-**D1S96**-D1S989 (cYS1287) / D1S1131 (cYS1234)-**NPPA**-D1S975 (cYS1173)-D1S968 (cYS1121)-D1S1062 (cYS73)-D1S1092 (cYS1148)-D1S1130 (cYS1232) / D1S1028 (cYS1363)-D1S967 (cYS1120)-**PAX7**-D1S1111 (cYS1180)-D1S1073 (cYS191)-D1S1037 (cYS1406)-**D1S1112** (cYS1181)-D1S1040 (cYS144)-D1S112-cen. In reference to this FISH physical ordering map, we have determined the chromosomal breakpoints within the 1p36 region. Three of in vitro established and two of in vivo cell lines maintained in SCID mice were assessed for their breakpoints. As a result, all of the five cell lines had different breakpoints at cytogenetic level. However, the breakpoint of HMS24 cell line, an in vivo cell line maintained in SCID mouse, was mapped between DNA markers D1S96 and D1S989, showing the possibility that it corresponds to the distal breakpoint of rearranged chromosome 1 detected in BALL-1, a B-cell line derived from a patient with acute lymphoblastic leukemia. To make it sure whether these translocation events at 1p36 occurred at the same locus or not, we continued a detailed FISH mapping using YAC, BAC, and PAC clones. On the way to map the breakpoint, a YAC clone 762B5 was detected to contain the breakpoint of HMS24. Consecutive PFGE analysis and PCR screening of ESTs demonstrate that the breakpoint could be narrowed within the 260kb region between STS markers D1S1615 and SHGC-110595. Screening of the gene(s) locating on the breakpoint is still on going.

7. Expression of methionine aminopeptdase 2 in normal lymphoid tissues and malignant lymphomas

Takayuki Kanno, Kengo Takeuchi[2], Yasuyuki Morishita, Shigeo Mori

This topic will be described below.

II. Functional role of Lvcap·100A4, the tumor metastasis-associated protein

1. Association of s100A4 with methionine aminopeptidase 2

Hideya Endo, Takayuki Kanno, Masataka Asagiri

The mts1 gene (S100A4) is a mouse gene highly expressed in tumor with high metastatic potential. The molecular and cellular function of this gene is under investigation. With the introduction of GST fusion protein, co-immunoprecipitation and other in vitro and in vivo techniques, we found the S100A4 protein to associate with methionine aminopeptidase 2, the putative inhibitor of translation initiation, suggesting some specific role of S100A4 in such biochemical process (Endo et al., J Bio Chem 2002).

2. Expression of methionine aminopeptidase 2 (MetAP2) in normal lymphoid tissues and malignant lympho-

mas

Takayuki Kanno, Kengo Takeuchi[2], Yasuyuki Morishita, Shigeo Mori

By the introduction of two newly made antibodies reacting with different epitopes of MetAP2 protein, we incidentally found the MetAP2 to be highly expressed in human germinal center B cells and their neoplastic counterparts. This result suggests the presence in MetAP2 of hitherto-unclarified novel function(s) that may directly associate with the physiology of germinal center B cells (Kanno et al.,Lab. Invest. 2002).

III. Viral Pathogenesis of Human retroviruses, HTLV-1 and HIV

1. Multistep leukemogenesis of adult T-cell leukemia (ATL)-Possible involvement of PKCßII activation in the progression of HTLV-1 Tax-immortalized T-cells

Chieko Shionoiri[11], Risaku Fukumoto, Tsikasa Koiwa, Masao Matsuoka[12], Kazunari Yamaguchi[9], Toshiki Watanabe: [11]Department of Hematology, Graduate School of Medicine, University of Tokyo, [12]Institute for Virus Research, Kyoto University

Adult T cell leukemia (ATL) develops more than 50 years of the latency period. The age distribution of ATL onset statistically fits in with the Weibull's multistep carcinogenesis model and five independent leukemogenic events are involved in ATL development. HTLV-1 infection of T cells immortalizes the infected cells by the action of viral transcriptional regulator Tax. However, HTLV-1 Tax-immortalized T cells differ significantly from *in vivo* transformed ATL cells in terms of biochemical and biological phenotypes, suggesting that more steps are involved in progression to ATL cells. Characterization of the gene expression profiles of ATL cells by the differential display analysis (DDA) resulted in identification of PKCβII overexpression as one of the characteristics that distinguishes *in vivo* transformed cells from *in vitro* immortalized ones. In addition to overexpression of the mRNA and protein, constitutive activation of PKCβII was demonstrated in cell lines derived from ATL clones but not in those immortalized by *in vitro* infection or introduction of Tax. Transduction of a constitutive active form of PKCβII by a retrovirus vector into Tax1A cells resulted in a growth advantage and resistance to apoptosis induced by IL-2 withdrawal (R Fukumoto et al., in preparation). Since GSK-3β-β-catenin/TCF pathway is reported to be a target of PKCbII in colon carcinogenesis, we examined whether GSK-3β-β-catenin/TCF pathway is also activated by PKCβII in ATL leukemogenesis. We found accumulation of b-catenin in ATL cells, ATL derived cell lines and Tax-immortalized cell lines transduced with a constitutively active PKCβII and phosphorylation of GSK-3β in some of these cells. Thus, it was suggested that the GSK-3β-β-catenin/TCF pathway is involved in ATL leukemogenesis.

2. Involvement of the IL-2/IL-2R system activation by the parasite antigen in the polyclonal expansion of CD4+25+ HTLV-1-infected T-cells in dual carriers of HTLV-1 and *S. stercoralis*

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The intermediate state of HTLV-1 infection, often found in individuals dually infected with *Strongyloides stercoralis* (*S. stercoralis*) and HTLV-1, is assumed to be a preleukemic state of adult T-cell leukemia (ATL). To investigate the effects of *S. stercoralis* superinfection on the natural history of HTLV-1 infection, we characterized peripheral blood samples of these individuals in Okinawa, Japan, an endemic area for both HTLV-1 and *S. stercoralis* and we studied effects of the parasite antigen on T-cells. The dually infected individuals showed a significantly higher provirus load and an increase in CD4+25+ T cell population, with a significant, positive correlation. This increase was attributable to polyclonal expansion of HTLV-1-infected cells, as demonstrated by inverse-long PCR analysis of the integration sites. *S. stercoralis* antigen activated the IL-2 promoter in reporter gene assays, induced production of IL-2 by PBMC *in vitro*, and supported growth of IL-2 dependent cell lines immortalized by HTLV-1 infection or the transduction of Tax. Taken collectively, these results indicate that *S. stercoralis* infection induces polyclonal expansion of HTLV-1-infected cells by activating the IL-2/ IL-2R system in dually infected carriers, an event which may be a precipitating factor for ATL and inflammatory diseases. (Sato et al., Oncogene, 2002)

3. CpG methylation of LTR as a regulatory mechanism of latency of human retroviruses

Transcriptional repression of heterologous genetic elements such as proviruses is often observed concomitantly with their integration into chromatin in the host cell genome. Deacetylation of nucleosomal histones and methylation of cytosines, particularly in the dinucleotide 5' CpG, negatively influence transcriptional activity in cis. Therefore, we have been investigating the regulatory roles of CpG methylation and chromatin structures in latency induction and reactivation of human retroviruses, HIV and HTLV-1.

a. CpG methylation-dependent and -independent regulation of HIV latency

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(1) Reactivation of latent HIV is associated with CpG demethylation

The incidence of AIDS and AIDS-related mortality has decreased with availability of highly active antiretroviral therapy. This has led to considerable optimism, however, it became evident that complete eradication of latent HIV in the reservoir pool is very difficult. Thus, invention of a novel therapy to overcome treatment resistance of latent HIV and to achieve containment or purging of HIV depends on a better understanding of mechanisms involved in latency and reactivation of HIV. DNA methylation has been implicated in HIV latency. Cytokines such as TNF-a can induce HIV gene expression in HIV-infected T cell lines as well as in latently infected lymphocytes *in vivo*. Using HIV-infected T-cell lines and HIV transgenic mice, we obtained evidence that LPS-induced reactivation of heavily methylated provirus is cell cycle-dependent and is associated with demethylation of specific CpG sites located in the binding sites for CREB/ATF family transcription factors. Our evidence shows binding of a common factor (s) (other than known CREB/ATF factors) to sequences around these CpG sites, irrespective of the methylation status. Since protein binding can specify sites of DNA demethylation, our results suggest a mechanism for reactivation where extracellular signal-induced DNA replication results in demethylation of CpG sites that are protected from maintenance methylation by binding of CREB/ATF-like factors. The present study provides novel clues to delineation of the mechanism for signal-mediated demethylation and reactivation of HIV, and to design of a treatment strategy to contain or purge HIV. (Tanaka et al., AIDS, 2002, Ishida et al., submitted to J Virol)

(2) Repression of HIV gene expression by the "repressive histone code"

Analysis of CpG methylation of integrated provirus in autopsied samples showed almost total absence of CpG methylation of the LTR. The results suggested other mechanisms than CpG methylation to stably repress viral gene expression. We have found a latently infected cell line, OM10.1, where the provirus LTR is totally unmethylated. Chromatin immunoprecipitation analysis (ChIP) revealed histone modification at the nucleosome B region that is compatible with the repressive histone code (inclusion of H1, absence of H3 acetylation), which changed into a permissive one after TNF-a stimulation and induction of viral gene expression (absence of H1, hyperacetylation of H3 and H4). These results suggest presence of CpG methylation independent mechanisms for viral latency that is controlled by chromatin structure controlled by histone modification (Ishida et al., manuscript in preparation).

b. Involvement of CpG methylation in the latency of HTLV-1

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(1) 5'-LTR-selective CpG methylation of latently integrated provirus in vivo and in vitro

HTLV-1 causes leukemia and inflammatory diseases after several decades of latency period. *In vivo*, viral gene expression is suppressed in HTLV-1-infected T cells whether they are transformed or not. CpG methylation of LTR has been implicated in the HTLV-1 latency, however, little information is available as to the methylation of the integrated provirus LTR. To gain insights into the mechanisms of HTLV-1 latency, we studied methylation of individual CpG sites in the U3-R region of the integrated provirus LTR by the bisulfite genomic sequencing method. In addition to non-selective analysis with LTR primers, 5' and 3' LTRs were selectively studied with a sense primer located in the 5'-flanking sequence of the integrated provirus and that in the pX region, respectively. Results revealed selective hypermethylation of 5'-LTR and demethylation of 3' LTR in the latently infected cell lines and an ATL sample having a complete provirus. Almost complete demethylation was demonstrated in the LTR of 5'-defective proviruses of ATL samples, which is in line with selective demethylation of 3' LTR. Non-selective analysis of PBMC samples of asymptomatic carriers showed uniformly methylated CpG sites in about half of the copies sequenced, whereas 3'-LTR specific analysis revealed almost complete demethylation. Thus, the integrated HTLV-1 provirus in these carriers appears hypermethylated in 5'-LTR and demethylated in 3'-LTR. These observations, together with reactivation of provirus expression by 5'-azacytidine in latently infected cell lines, indicate that selective hypermethylation of 5'-LTR appear to be the norm both in vivo and in vitro, by which gene expression of HTLV-1 is repressed. (Koiwa et al., J Virol, 2002)

(2) Demethylation of 5'-LTR of the integrated HTLV-1 provirus in carriers with increased levels of provirus load

Since polyclonal expansion of HTLV-1-infected T cells has been well documented in patients with TSP/HAM or HU and dual carriers of HTLV-1 and S. stercoralis, we next investigated methylation status of 5′-LTR of provirus in the PBMC of these individuals. Results showed demethylation of the 5′-LTR which is correlated with the levels of provirus load in the PBMC which was determined by quantitative real time PCR method. These results suggested demethylation induced by signaling of some extracellular stimuli, since CpG methylation of the provirus LTR in MT-1 cells showed demethylation following reactivation of virus gene expression by TPA treatment. As expected, stimulation by a combination of anti-CD3 antibody and IL-2 resulted in demethylation of CpG sites in the 5′-LTR in TL-om1 cells. Thus, polyclonal expansion of HTLV-1-infected T cells in these individuals appear to results from TCR and IL-2 signal-mediated growth of HTLV-1-infected cells that is associated with demethylation of 5′-LTR, where reactivation of virus expression and production of Tax protein may have conferred growth advantage on these cells (Koiwa et al., submitted to J Virol).

IV. Cytogenetic Studies

1. Molecular analysis in the domestic cat

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The detection of integrated feline leukemia viruses (FeLVs) in cat lymphoid tumor cell lines is on going. FeLV is an oncogenic type-C retrovirus associated with lymphoid and hematopoietic malignancies in cats. The FeLV-induced tumors are thought to be caused, at least in part, by somatically acquired insertional mutagenesis in which the integrated provirus activates a proto-oncogene or disrupts a tumor suppressor gene. To enumerate and map somatically acquired FeLV proviral insertions in FeLV-associated tumor cells, fluorescence in situ hybridization using an 8.4kb FeLV-A/Glasgow-1 proviral genome was done on the metaphases from a feline thymic lymphoma cell line (FT-1). At least six loci of chromosomal regions, A2p22, B2p14, B4p15, D4q23, E1p13, and E2p13, were suspected to be positive for FeLV integration. Simultaneously, southern blot hybridization using a probe specific for the long terminal repeat U3 region of exogenous FeLV revealed multiple copies of integrated FeLV proviral genome in FT-1 cells.

2. Chromosomal assignment of novel genes in human, mouse, rat, and the domestic cat

Hitoshi Satoh, Masahiko Fujiwara[19]: [19]Pathology Division, KOTOBIKEN Medical Laboratory Inc.

The following nine novel genes were mapped this year: DKK4 (dickkopf (Xenopus) homolog 4) to 8p11.2-p11.1, CKTSF1B1 (cysteine knot superfamily, BMP antagonist 1; Gremlin) to 15q13, and ARPP/ANKRD2 (ankyrin repeat domain 2(stretch responsive muscle)) to 10q23.1-q25.1 in the human genome. Murine Msi1 and Msi2 (Musashi (Drosophila) homolog 1 and 2) to mouse chromosomes 5qE3-F and 11qB5-C, respectively. Arpp/Ankrd2 (a murine homolog of ARPP) to mouse chromosome 19C3-D1 and likely to rat chromosome 1q53-q55 (Fujiwara et al., submitted). TNFRSF6 (tumor necrosis factor receptor superfamily, number 6; Fas) to D2p13-p12.2 and TNFSF6 (tumor necrosis factor (ligand) superfamily, number 6; FasL) to F1q12-q13 in the cat genome.

V. Service activities of the Histopathology and Cytogenetics Service Laboratory

Tomoko Nakajima, Hitoshi Satoh, Shigeo Mori

Following services were conducted during April – December, 2002. Paraffin blocks: 520 specimens; Thin section cutting: 850 sections; Hematoxylin and Eosin staining: 205 slides; Immunostaining 24 specimens, chromosome analysis, 2 cases, and immunostaining as a cooperative study: 1 case. Those were requested from 8 divisions.

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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 three such proteins including Tumor necrosis factor receptor-associated factor (TRAF), E coli. Ras-like protein (ERA), and WT-1. Our current studies indicate that these three proteins play critical roles in cell proliferation and differentiation. Therefore, elucidation of how these proteins work is definitely required for understanding onset of diseases.

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

Kazuyuki Meguro, Kohei Kojima, Ayaka Moriya, Shigenori Yagi, Jin Gohda, Taishin Akiyama and Junichiro Inoue

Transcription factor Rel/NFκB binds specifically to a decameric motif of nucleotide, κB site, and activates transcription. The κB sites are present in the enhancers of many genes including the immunoglobulin κ light chain gene, Interleukin-2 receptor α-chain gene, β-interferon gene and LTR of human immunodeficiency virus, which are involved in the regulation of the immune system. Recent studies showed that the activation of Rel/NFκB links to the anti-apoptotic signal. Thus, it is essential to understand the molecular mechanisms by which Rel/NFκB transcription factor is regulated. 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 Lys 48-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. Last year, we demonstrated 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. TRAF6 becomes ubiquitinated upon IL-1 stimulation. We also found that TRAF6 becomes conjugated by a Lys48 (K48) polyubiquitin chain and degraded upon stimulation. Therefore, it is possible that poly ubiquitin chain regulates TRAF6 both posi-

tively and negatively. To elucidate mechanisms of the ubiquitination-mediated regulation of TRAF6, we are trying to identify ubiquitination sites of TRAF6. Several lines of evidence suggest that TRAF6 is able to activate some kinase other than TAK1. To identify such kinases, we have set up cell-free system in which IKK becomes activated upon addition of recombinant TRAF6 protein. By using this system, we are trying to identify the molecule(s) that function between TRAF6 and the IKK complex by biochemical protein purification. To elucidate how TRAF6 transduces signals, crystallographic studies are required. To do this end, we have prepared large amount of the purified recombinant TRAF6 protein produced by baculovirus expression system. We are trying to set up experimental condition for crystallization.

2. Role of TRAF6 in osteoclastogenesis

Jin Gohda, Kazuyuki Meguro, Yasuko Kuzuwa, Asuka Naito, Taishin Akiyama and Jun-ichiro Inoue

We have previously identified TRAF6 as a putative signal transducer of CD40 by yeast two-hybrid system. However, it is not known whether TRAF6 plays pivotal roles in immune system in vivo, and whether it has additional functions yet to be identified. Thus, we have generated TRAF6-deficient mice by homologous recombination-based gene knockout. TRAF6-/- mice exhibit severe osteopetrosis due to the lack of osteoclasts in bone. Ex vivo co-culture experiments revealed the inability of osteoclast precursor cells derived from TRAF6-/- mice to differentiate to functional osteoclasts in response to osteoclast differentiation factor (ODF), indicating that RANK, a receptor of ODF, utilizes TRAF6 for signal transduction. The number of sIgM+B220+ B cells is markedly reduced in both spleen and bone marrow, whereas T cell development is normal in the absence of TRAF6. However, thymocytes from TRAF6 deficient mice do not proliferate in response to IL-1. IL-1 does not activate JNK and NFκB of TRAF6 deficient embryonic fibroblast, while TNFα does. These results indicate that TRAF6 is essential for IL-1 signaling. Furthermore, TRAF6-/- mice are defective in lymph-node organogenesis. Thus, TRAF6 is an essential component of ODF signaling pathway, and also show that TRAF6 plays pivotal roles in immune and inflammatory systems *in vivo*. To understand how TRAF6 activates downstream pathways that lead to osteoclastogenesis, we tried to identify functional domains of TRAF6 under physiological conditions established by appropriate expression of TRAF6 mutants in TRAF6-deficient cells. In IL-1 and LPS signaling pathways, the RING finger and first zinc finger domains are not required for NFκB activation but required for full activation of MAPK. However, IL-1 and LPS signals utilize distinct regions within the zinc finger domains of TRAF6 to activate NFκB. By expressing various TRAF6 mutants in TRAF6-deficient progenitors of osteoclasts, we found that the RING finger domain is not required for differentiation of splenocytes to multinuclear osteoclasts but essential for osteoclast maturation. Thus, TRAF6 plays essential roles in both differentiation and maturation of osteoclasts by activating various kinases via its multiple domains. Although we learned that RANK-TRAF6 linkage is essential for osteoclastogenesis, why CD40 and IL-1 signals, which activate TRAF6, are unable to induce formation of osteoclasts remains to be elucidated. To address this question, we have generated various RANK mutants to find out the structural characteristics that are specifically required for osteoclastogenesis.

3. Role of TRAF6 in ectodermal differentiation

Asuka Naito, Jin Gohda, Taishin Akiyama and Jun-ichiro Inoue

Recently, we report that *TRAF6* deficiency results in defective development of epidermal appendices, including guard hair follicles, sweat glands, sebaceous glands of back skin, and modified sebaceous glands such as meibomian glands, anal glands, and preputial glands. Except the sebaceous gland impairment, these abnormal phenotypes are identical to those observed in *Tabby* (*Ta*), *downless* (*dl*) and *crinkled* (*cr*) mice, which are models of hypohidrotic (anhidrotic) ectodermal dysplasia (HED) in human. β-catenin and Mucosal addressin cell adhesion molecule-1 (MAdCAM-1), an early marker of developing guard hair follicles is absent in the skin of *TRAF6*-deficient embryos. Thus, TRAF6 is essential for development of epidermal appendices. TRAF6 does not associate with the cytoplasmic tail of DL/EDAR, which, when mutated, results in HED. However, TRAF6 associates with XEDAR and TROY/TAJ, which are EDAR-related members of the TNFR superfamily that are expressed at high level in epidermal appendices. Furthermore, TRAF6 is essential for the XEDAR-mediated NFκB activation. Thus, our results suggest that TRAF6 may transduce signals emanating from XEDAR or TROY/TAJ that are associated with development of epidermal appendices.

4. Regulation of TRAF6 by TRAF6-binding protein

Hiroshi Takatsuna, Shigenori Yagi, Hiroki Kato, Ayaka Moriya, Jin Gohda, Taishin Akiyama, Kentaro Semba and Jun-ichiro Inoue

TRAF6 transduces signals from members of the Toll/interleukin-1 (IL-1) receptor family by interacting with IL-1 receptor-associated kinase-1 (IRAK-1) after IRAK-1 is released from the receptor/MyD88 complex upon IL-1 stimulation. However, the molecular mechanisms underlying regulation of the

IRAK-1/TRAF6 interaction are largely unknown. We have identified TIFA, a TRAF-interacting protein with a forkhead-associated (FHA) domain. The FHA domain is a motif known to bind directly to phosphothreonine and phosphoserine. In transient transfection assays, TIFA activates NFκB and JNK. However, TIFA carrying a mutation that abolishes TRAF6 binding or mutations in the FHA domain that are known to abolish FHA domain binding to phospho-peptide fails to activate NFκB and JNK. TIFA, when overexpressed, binds both TRAF6 and IRAK-1 and significantly enhances the IRAK-1/TRAF6 interaction. Furthermore, analysis of endogenous proteins indicates that TIFA associates with TRAF6 constitutively, whereas it associates with IRAK-1 in an IL-1 stimulation-dependent manner *in vivo*. Thus, TIFA is likely to mediate IRAK-1/TRAF6 interaction upon IL-1 stimulation.

5. Elucidation of CD40 signaling in lymphocyte development

Yasuko Kuzuwa, Kazuyuki Meguro, Jin Ghoda, Sakura Azuma, Taishin Akiyama and Jun-ichiro Inoue

CD40 is expressed in late B cells in bone marrow, mature B cells and certain accessory cells, including bone marrow-derived dendritic cells and follicular dendritic cells and is a receptor for CD40 ligand (CD40L) present in activated CD4+ T cells. Signals through CD40 play crucial roles in B cell differentiation including blocking apoptosis of germinal center B cells. We have previously demonstrated that CD40 signalings induce Bcl-xL, Cdk4&6 expression and suppress p27kip-1 expression to block apoptosis induced by sIgM crosslinking. We have also demonstrated that sIgM signaling stabilized p27Kip-1 and destabilized Cdk4&6, whereas CD40 signaling maintained their stability at the level of unstimulated cells. CD40 signaling modulates the immune response at least in part by activation of nuclear factor κB (NFκB). We have previously shown that two distinct domains in the CD40 cytoplasmic tail, namely cyt-N and cyt-C, independently activate NFκB. We have reported that TRAF2, TRAF3 and TRAF5 bind cyt-C, whereas TRAF6 binds cyt-N. We have demonstrated that cyt-C is sufficient for blocking apoptosis induced by sIgM crosslinking. The cyt-C signal was shown to suppress p27kip-1 induction and Cdk4&6 reduction induced by sIgM signaling. Furthermore, These activities of cyt-C were abolished by substitution of threonine-254 with alanine (T254A). Since this mutation also abolished the binding of cyt-C to TRAF2, TRAF3 and TRAF5, this result strongly suggests that these TRAF proteins were involved in blocking apoptosis induced by sIgM crosslinking. However, full length CD40 carrying T254A mutation has partial activity to block apoptosis, suggesting that cyt-N could play a role in blocking apoptosis. To prove that TRAF6 is involved in CD40 signaling, CD40 was ectopically expressed in TRAF6-deficient cells. CD40-mediated NFκB activation was largely impaired in the absence of TRAF6, indicating that TRAF6 mediates CD40 signaling in vivo.

6. Identification and functional characterization of vertebrate homologues of *E. coli* Ras-like protein (ERA) and Developmentally Regulated GTP-binding protein (DRG)

Taishin Akiyama, Saiko Shibata, Taizo Ishikawa, Kosuke Ishikawa, Sakura Azuma, Jin Gohda, Kentaro Semba and Jun-ichiro Inoue

We have obtained a novel gene encoding a putative GTP binding protein from human placenta cDNA library. Sequence homology analysis indicated that this novel GTP binding protein appeared to be a mammalian homologue of ERA (*E. coli* Ras-like protein), which was previously shown to be essential for cell cycle control in bacteria from the results of the experiments using the temperature-sensitive lethal mutant. Further analyses of the DNA sequence data base and our cDNA screening suggested that the homologues of this GTP binding protein were found to exist in mouse, avian, Drosophila, and plant as well as human and bacteria. All of these homologues possess a common unknown domain at carboxyl terminal of the protein in addition to the typical GTPase domain at amino terminal, suggesting that these GTP binding proteins are a novel GTP binding family, which might play a pivotal role on cellular function of not only prokaryote but also eukaryote. As corollary of above consideration, we decided to explore the biological function of this novel GTP binding protein. To address physiological role of ERA, ERA was overexpressed in HeLa cells. Interestingly, overexpression of the GTPase mutants resulted in an apoptosis of HeLa cells. Since the apoptosis was efficiently inhibited by the co-expression with Bcl-xL or Bcl-2, the apoptosis induced by the GTPase mutants are most likely to correlate to the well-known apoptosis pathway mediated through mitochondria. In addition, recent several reports indicated the ERA might directly bind to single strand RNA. In vitro RNA binding experiments revealed that the C-terminal half of ERA, which has a KH domain, directly binds to RNA, suggesting that RNA is a possible candidate of the effector or regulator of this GTP binding protein. We have recently performed a genetic analysis of chicken ERA (GdERA) in DT40 cells. Depletion of GdERA diminished the growth rate of the cells, accompanied by an accumulation of apoptotic cells. The analysis of cell cycle indicates that the elimination of GdERA caused arrest at G1 phase, but not at M phase, which highlights the distinct role of vertebrate ERA in the cell cycle progression compared to prokaryotic ERA.

Furthermore, human ERA (HsERA) rescued the phenotype of GdERA-deficient cells, whereas a mutant of HsERA deprived of RNA binding activity did not. These data suggest that vertebrate ERA regulates the G1 phase progression via an as yet unknown molecular mechanism, which involves RNA recognition by ERA. Developmentally Regulated GTP binding protein (DRG) is conserved in archaebacteria and eukaryotes. We are now trying to identify physiological roles of the DRG protein in mouse and Xenopus.

7. Synthesis of compounds which specifically regulates the activity of Rel/NFκB transcription factors

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Chemical compounds are designed and synthesized to test their ability to modulate the signal transduction pathways linked to the physiological response of cells. One of the research targets is the signaling linked to Rel/NFκB activation. We have previously synthesized various compounds comprising dimethylaminopyridine and histidine. One of the compounds inhibits the activation of Rel/NFκB by TNF-α only when the compound is within the cell. This year we have synthesized oligonucleotides 26-mers including single 5-formyl-2′-deoxy-uridine or 5-formyl-2′-*O*-methyluridine in place of thymidine at the κB site. One of the 26-mer with 5-formyl-2′-deoxy-uridine was critically discriminated by the NFκB p50 homodimer. These findings should provide a better understanding of the NFκB-DNA interaction and give us basic information for drug design. Our another trial is to synthesize a number of derivatives of bleomycin conjugated with oligonucleotides that preferentially associate with κB motif present in the enhancer of several genes to form a triple helix structure and cut out double strand DNA. Our goal of this project is to use this compound for the inactivation of human immunodeficiency virus (HIV), since HIV has tandem copies of κB sites in its own LTR. We have already succeeded in cutting out double strand DNA by forming triple helix structure using the oligonucleotides derived from the regulatory element of interleukin-2 receptor α-chain. We are trying to apply this system to HIV.

8. Function of a chimeric EWS-WT1 oncoprotein

Kentaro Semba, Shinya Watanabe[4], Sakura Azuma and Jun-ichiro Inoue: [4]Divison of Cancer Genomics, IMSUT

WT1 gene was originally identified as a tumor suppressor gene for Wilms' tumor, a pediatric kidney tumor. Several lines of evidence show that WT1 is required for development of various organs including kidney, gonad, spleen, heart and retina. WT1 is a transcription factor, which binds to the specific DNA sequence via its zinc finger domain and then regulates transcription of target genes. WT1 is also involved in DSRCT, desmoplastic small round cell tumor. In this tumor, the WT1 gene is fused to the EWS gene as a result of chromosomal translocation between chromosome 11 and 22. This fusion gene product consists of three of the four zinc fingers of WT1 and transcriptional activation domain of EWS, and thus it acts as a transcriptional activator. We are searching for target genes of EWS-WT1 using synthetic polynucleotide microarray, which covers more than 20,000 human genes. Recently, we found that two genes encoding membrane proteins, TALLA1 and BAI1 were highly expressed in EWS-WT1-expressing cell lines and in DSRCT tumor specimens. BAI1 was originally identified as a target of p53. This seven-transmembrane protein is thought to inhibit angiogenesis mediated by the extracellular thrombospondin domain. The reason why the oncogenic EWS-WT1 protein induces anti-oncogenic BAI1 is currently under investigation. Another candidate is TALLA1, a member of tetraspanin family. This family member usually forms a complex with integrin and modulates integrin-mediated signals. Since TALLA1 also binds to PI4K, TALLA1 itself may directly mediate signals. We are interested in the function of TALLA1 in movement and attachment of cancer cells.

9. Function of WT1 in sex determination

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In humans, as in other mammals, sex determination is controlled by a dominant switch termed TDF for Testis Determining Factor. The SRY gene is thought to be the TDF, which encodes a transcription factor with one HMG box as a DNA binding domain. Mutations in the SRY gene have been identified in 15% cases of XY sex reversal in humans. Introduction of mouse SRY gene (Sry) into XX female mice induced testis differentiation and subsequent male development. However, little is known about mechanism of transcriptional regulation by SRY. WT1 mutations have frequently been observed in Denys-Drash syndrome (DDS) patients with urogenital malformation. During analysis of WT1-associated proteins, we found that WT1 bound to several types of transcription factors. One of them was Sox30, which encodes a novel transcription factor with one HMG box. Further analysis showed that WT1 bound to its HMG box.

This observation prompted us to analyze interaction between WT1 and SRY. To date, we showed i) WT1 binds to SRY *in vitro* and in cultured cells, ii) this binding is mediated by the zinc finger domain of WT1 and the HMG box of SRY, iii) WT1 and SRY synergistically activates transcription from a promoter which contains SRY binding sequence, while WT1 mutants found in DDS did not show this activity, iv) the SRY gene itself is a candidate for target genes which are regulated by WT1 and SRY synergy, v) WT1 is recruited on SRY-binding sequence in a SRY-dependent manner, while recruitment of DDS mutants is significantly reduced, vi) one SRY mutant (L163ter) found in a Japanese family with complete gonadal dysgenesis had reduced affinity with WT1. These observations suggest that WT1 and SRY interaction plays an important role in early gonadal development and its disease. We are currently searching for the target genes which are induced by coexpression of WT1 and SRY.

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Our major projects are to clarify (1) signal transduction systems in cell growth, reorganization of cytoskeleton and morphogenesis (2) roles of inositolphospholipids as biomodulators. Currently we are focusing on the clarification of mechanisms in cortical actin reorganization and cell movement, and the roles of inositolphospholipid-mediated signallings in trafficking and a variety of physiological functions using knockout mice.

1. WICH, a novel verprolin homology domain-containing protein that functions cooperatively with N-WASP in actin-microspike formation

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We describe a novel protein that contains a verprolin-homology (V) region, through which several actin-regulating proteins, including Wiskott-Aldrich syndrome protein (WASP) family members, bind directly to actin. The amino acid sequence is homologous to the sequences of WASP-interacting protein (WIP) and CR16, both of which associate with WASP and/or N-WASP, and thus these three proteins constitute a new protein family. We named the protein WICH (WIP- and CR16-homologous protein). WICH associates strongly with N-WASP but only weakly with WASP via its C-terminal WASP-interacting (W) region. Ectopic expression of WICH induces actin-microspike formation through cooperation with N-WASP. In addition, expression of the W fragment of WICH suppresses microspike formation induced by N-WASP, indicating an essential role for WICH in N-WASP-induced microspike formation.

2. Sustained Activation of N-WASP through Phosphorylation is Essential for Neurite Extension.

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Neurite extension is a key process for constructing neuronal circuits during development and remodeling of the nervous system. Here we show that Src family tyrosine kinases and proteasome degradation signals synergistically regulate N-WASP in neurite extension. Src family kinases activate N-WASP through tyrosine phosphorylation, which induces Arp2/3 complex-mediated actin polymerization. Tyrosine phosphorylation of N-WASP also initiates its degradation through ubiquitination. When neurite growth is stimulated in culture, degradation of N-WASP is markedly inhibited, leading to accumulation of the phosphorylated N-WASP. On the

other hand, under culture conditions that inhibit neurite extension, but favor proliferation, the phosphorylated N-WASP is degraded rapidly. Collectively, neurite extension is regulated by the balance of N-WASP phosphorylation (activation) and degradation (inactivation), which are induced by tyrosine phosphorylation.

3. N-WASP is recruited to rafts and associates with endophilin A in response to EGF.

Makiko Otsuki, Toshiki Itoh, and Tadaomi Takenawa

Neural Wiskott-Aldrich syndrome protein (N-WASP) has been implicated in endocytosis; however, little is known about how it interacts functionally with the endocytic machinery. Sucrose gradient fractionation experiments and immunofluorescence studies with anti-N-WASP antibody revealed that N-WASP is recruited together with clathrin and dynamin, which play essential roles in clathrin-mediated endocytosis, to lipid rafts in an epidermal growth factor (EGF) dependent manner.

Endophilin A (EA) binds to dynamin and plays an essential role in the fission step of clathrin-mediated endocytosis. In the present study, we show that the Src homology 3 (SH3) domain of EA associates with the proline-rich domain of N-WASP and dynamin *in vitro*. Co-immunoprecipitation assays with anti-N-WASP antibody revealed that EGF induces association of N-WASP with EA. In addition, EA enhances N-WASP-induced actin-related protein 2/3 (Arp2/3) complex activation *in vitro*. Immunofluorescence studies revealed that actin accumulates at sites where N-WASP and EA are co-localized after EGF stimulation. Furthermore, studies of overexpression of the SH3 domain of EA indicate that EA may regulate EGF-induced recruitment of N-WASP to lipid rafts. These results suggest that, upon EGF stimulation, N-WASP interacts with EA through its proline-rich domain to induce the fission step of clathrin-mediated endocytosis.

4. SKIP negatively regulates insulin-induced GLUT4 translocation and membrane ruffling formation

Takeshi Ijuin and Tadaomi Takenawa

Skeletal muscle and kidney enriched inositol phosphatase (SKIP) is an inositol polyphosphate 5-phosphatase that hydrolyzes phosphatidylinositol 3,4,5-trisphosphate ($PI(3,4,5)P_3$) to downregulate intracellular levels. In this study, we show that SKIP inhibits phosphoinositide 3-kinase (PI 3-kinase) signaling in insulin-stimulated CHO cells. Ectopic expression of SKIP did not inhibit insulin-induced $PI(3,4,5)P_3$ generation but did rapidly decrease insulin-induced intracellular $PI(3,4,5)P_3$ levels compared to control cells. Further, insulin-induced phosphorylation of some downstream targets such as Akt and p70 S6 kinase was markedly inhibited by the ectopic expression of SKIP, whereas phosphorylation of MAP-kinase was not. In contrast, downregulation of intracellular SKIP levels by antisense oligonucleotides dramatically enhanced Akt (PKB) phosphorylation in response to insulin, suggesting that endogenous SKIP downregulates insulin signaling. SKIP also markedly inhibited GLUT4 translocation and membrane ruffle formation. We conclude that SKIP preferentially regulates glucose transport and actin cytoskeletal re-arrangement among a variety of $PI(3,4,5)P_3$ downstream events.

5. Phospholipase Cδ1 is required for skin stem cell lineage commitment

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Phosphoinositide-specific phospholipase C (PLC) is a key enzyme in phosphoinositide turnover and is involved in a variety of physiological functions. Here we report that PLCδ1-deficient mice undergo progressive hair loss in the first postnatal hair cycle. Epidermal hyperplasia was observed, and many hairs in the skin of PLCδ1-deficient mice failed to penetrate the epidermis and became zig-zagged owing to occlusion of the hair canal. Subsequently, many cysts that developed as a result of transdifferentiation of the hair follicles into interfollicular epidermis as well as hyperplasia of sebaceous glands were observed. Molecular markers of interfollicular epidermis were expressed in these cysts. From these results, we conclude that PLCδ1 is required for skin stem cell lineage commitment.

6. Phospholipase C-L2, a novel PLC like protein, negatively regulates B cell activation

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Several Phospholipase C (PLC) isoforms have been found in immune cells and are known to play important functions dowmstream of immunogloblin (Ig) superfamily receptors by regulating the calci-

um/protein kinase C signaling pathway. PLC-L2 is a novel PLC like protein, which does not have PLC activity, though it is very homologous to PLC δ type. PLC-L2 is highly expressed in hematopoetic cells. However, to date, its physiological roles in the immune system have not been clarified yet. To elucidate the function of PLC-L2 in the immune system, we generated the *PLC-L2* gene deficient mice. PLC-L2 deficient splenic mature B cells are hyper proliferative and show enhanced calcium influx in response to B cell receptor (BCR) cross-linking. PLC-L2 deficient splenice B cells also have enhanced Ig secretion *in vitro*. PLC-L2 deficient mice have stronger T-independent II antigen response compared to wild type mice in vivo. Higher titer of anti-nuclear antigen IgM is observed in aged PLC-L2 deficient mice, although they had showed no apparent autoimmune disease. Surprisingly, in contrast to conventional B cells, B1-a cells in the peritoneal cavity is significantly reduced. These results indicate that PLC-L2 negatively regulates B cell activation, suggesting that PLC-L2 is involved in B cell homeostasis and tolerance.

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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. Establishment of rapid and sensitive methods to identify carbohydrate binding proteins and its application to analysis of sperm proteins which might be involved in fertilization

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A hemagglutination assay has been widely used to detect carbohydrate recognition molecules (lectins) because of its simplicity, but their identification has to be done by other methods. Affinity chromatography using glycoprotein-coupled beads is used for purification of lectins. However, the column work is time-consuming, and usually requires a large amounts of samples. It is also required to consider effects of the peptide moieties of glycoproteins coupled to gels in most cases. Carbohydrate-coupled affinity gels, some of which are commercially available, only possess simple saccharides such as monosaccharides and small oligosaccharides. Carbohydrate binding proteins in vivo recognize fine structures of carbohydrate ligands such as glycosyl linkages and antennary structures. Thus, it is better to use multivalent probes carrying native oligosaccharides in our assay. Taking these problems into consideration, we developed a new method to detect and identify carbohydrate binding proteins. A unique point in the established method is to use a 96-well plastic plate, wells in which were coated with various dextran-based oligosaccharide probes carrying approximately 130 natural oligosacharide chains in a molecule. Multivalency of oligosaccharides in the probe supports enhanced lectin binding and therefore increase possibility to find out novel lectins that have not been identified by conventional approaches. Crude samples including lectins such as membrane proteins solubilized by detergent are added to the coated wells. After washing, proteins bound to the wells are biotin-labeled. The labeled proteins are eluted from the wells with the sampling buffer followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After Western blotting, the proteins are detected by the chemiluminescence method using horseradish peroxidase-labeled avidin. When RCA-I lectin was applied to the detection system as a model, it was easily detected on the blot even if a solution (50μl) of 4 ng/ml was used. The method was also successfully applied to detection of asialoglycoprotein receptors of mouse liver. The amount of the oligosaccharide probes coated to the wells was roughly equal in spite of oligosaccharide structures of the probes. Utilization of a 96-well plas-

tic dish enables us to examine many samples from various cells and tissues comparatively and simultaneously.

Mammalian eggs are surrounded by an extracellular matrix called the zona pellucida (ZP) which consists of a few glycoproteins. The ZP plays important roles in sperm-egg binding, induction of sperm acrosome reaction, and block to polyspermy. It has so far been suggested that multiple mechanisms recognizing glycans on the ZP are working in the process of sperm-egg interaction, those are still unveiled. On the basis of oligosaccharide structures of porcine zona pellucida glycoproteins so far elucidated, we prepared various multivalent oligosaccharide probes, and applied them to detection of the putative sperm carbohydrate recognition molecules. The results indicated that two kinds of molecules, one recognizing the N-acetyllactosamine structure and the other recognizing the Le^x structure, are expressed on boar sperm heads. These carbohydrate recognition molecules are detected more strongly on capacitated sperm than on uncapacitaed sperm, but not detected on acrosome-reacted sperm, indicating that their expression depends on the physiological state of sperm. The biotinylated oligosaccharide probes were also successfully used to detect carbohydrate recognition molecules on the boar plasma membrane which were isolated and coated on a plastic dish. The inhibition study using this assay system confirmed that there are at least two types of carbohydrate recognition molecules on boar sperm plasma membrane. To identify the recognition molecules, plasma membranes of boar sperm were solubilized with Triton X-100, and then applied to plastic wells coated with the oligosaccharide probes containing N-acetyllactosamine structure. Analysis of proteins bound to and eluted from the wells suggested that two proteins with apparent molecular masses of 70 kDa and 40 kD are candidates for sperm carbohydrate binding proteins recognizing the N-acetyllactosamine structure. Both proteins were also detected in the retarded or bound fraction when the detergent solubilized plasma membrane fraction of boar sperm was applied to an affinity column of asialoorosomucoid coupled beads. Partial amino acid sequences of proteolytic peptide fragments of the 70 kD protein protein were determined by ESI-Q/TOF-MS/MS. On the basis of the sequence, cDNA encoding a large part of the protein could be cloned from cDNA library of boar testis and its nucleotide sequence was determined. We are currently producing the recombinant proteins and analyzing their carbohydrate binding activity.

2. Role of O-glycosylation in stable expression of dysadherin, a carcinoma-associated antigen and in adhesion of carcinoma cells

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The cadherin-catenin cell-cell adhesion system is known to play a crucial role in tumor development. Loss of E-cadherin function in tumors results in progression of relatively benign tumors to invasive, metastatic carcinomas. Inactivation of E-cadherin-mediated cell-cell adhesion in tumors has been reported to be caused by its genetic alteration, promoter methylation, and tyrosine phosphorylation of β-catein that is associated with cytoplasmic domain of E-cadherin. Quite recently, it has been shown by Dr. Hirohashi's group that transfection of dysadherin, a tumor-associated antigen in many cancer tissues, causes E-cadherin down-regulation and disturbs homotypic cell adhesion in a human hepatocellular carcinoma cell line. The amino acid sequence of dysadherin predicted from cDNA sequence indicated that it is a type 1 transmembrane protein with features typical of membrane-associated mucins including serine-, threonine-, and proline-rich ectodomains. There was no tripeptide sequence, Asn-X-Ser/Thr, known as a potential *N*-glycosylation site in the predicted amino acid sequence of dysadherin. Thus, it has been suggested that dysadherin expresses only O-glycans.

We showed evidence that dysadherin is actually O-glycosylated. This was based on a direct carbohydrate composition analysis of a chimera protein of an extracellular domain of dysadherin fused to an Fc fragment of immunoglobulin. To assess the importance of O-glycosylation in dysadherin function, dysadherin-transfected hepatocarcinoma cells were cultured in a medium containing benzyl-α-GalNAc, a modulator of O-glycosylation. This treatment facilitated homotypic cell adhesion among dysadherin transfectants accompanied with morphological changes, indicating that the anti-adhesive effect of dysadherin was weakened. Modification of O-glycan synthesis also resulted in down-regulation of dysadherin expression and up-regulation of E-cadherin expression in dysadherin transfectants, but did not affect E-cadherin expression in mock transfectants. Structural analysis of O-glycans released from the dysadherin chimera proteins indicated that a series of O-glycans with core 1 and 2 structures are attached to dysadherin, and their sialylation is remarkably inhibited by benzyl-α-GalNAc treatment. However, neuraminidase treatment of the cells did not affect calcium-dependent cell aggregation, which excluded the possibility that sialic acid itself is directly involved in cell-cell adhesion. Taken together, it is suggested that aberrant O-glycosylation in carcinoma cells inhibits stable expression of dysadherin and leads to the up-regulation of E-cadherin expression by an unknown mechanism, resulting in increased cell-cell adhesion. The carbohydrate-directed approach to the regulation of dysadherin expression will be a new strategy for cancer therapy.

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腫瘍抑制分野

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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. During several years we have been mainly focusing on the functions of tyrosine kinases [VEGF receptors-1 and -2 (Flt-1/KDR), FGF receptor and Bcr-Abl] and adaptor proteins, Shc, FRS2 and Vav. This year we examined the role of VEGFR-1 in tumor metastasis, and found that VEGFR-1 is deeply involved in lung-specific metastasis via upregulation of MMP9 in the lung tissue. We are also examining the biological role of FRS2 in embryogenesis, and our data suggest that this adaptor molecule is essential for FGF-receptor signaling in the anterior-posterior axis formation in early embryogenesis.

1. Involvement of MMP9 induction by Vascular Endothelial Growth Factor Receptor-1 in lung specific metastasis

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Vascular endothelial growth factor (VEGF) and its receptor system has been shown to be involved in most of the pathological angiogenesis such as tumor angiogenesis, diabetic retinopathy and rheumatoid arthritis. However, the molecular mechanism of tissue-specific metastasis in tumors endogenously expressing members of the VEGF family is not yet clear. In this study using tumor transplantation mouse model, we clearly demonstrated that MMP9 is specifically induced in pre-metastatic lung endothelial cells and macrophages by distant primary tumors via VEGFR-1/Flt-1 tyrosine kinase (TK), and that it significantly promotes lung metastasis. In a genetic approach using gene-targeted mice, we found that deletion of either TK domain of VEGFR-1 or MMP9 gene markedly suppressed MMP9 induction as well as lung metastasis. Furthermore, the MMP9 levels in endothelial cells of normal lung lobes from patients carrying distant tumors were significantly elevated as compared with those from patients without tumors. These results strongly suggest that a block of MMP9 induction via VEGFR-1 inhibition is useful for the prevention of tumor metastasis in lung.

2. VEGFR-2-specific ligand VEGF-E induces non-edematous hyper-vascularization in mice

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VEGF family members play important roles in angiogenesis and vascular permeability. VEGF-A is expected to be a good candidate for proangiogenic molecule in clinical field. However, VEGF-A transgenic mice showed an increased vascularization with edema due to hypervascular permeability and subcutaneous hemorrhage as side effects. VEGF-A binds and activates two receptors, VEGFR-1 (Flt-1) and

VEGFR-2 (KDR/Flk-1). To dissect the signals of these two receptors, we generated transgenic mice overexpressing either the VEGFR-2-specific ligand VEGF-E_{NZ-7} or VEGFR-1-specific ligand PlGF-II under the control of the Keratin-14 promoter. VEGF-E-mice showed a significant increase in vascularization (about 10-fold compared to control mice) in subcutaneous tissues, whereas PlGF-mice showed only a 2-3 fold increase. Interestingly, VEGF-E-mice did not show any clear edematous lesions or hemorrhagic spots on the skin. Microscopically, VEGF-E-induced capillary networks have a well organized structure with the recruitment of pericytes. These results indicate that VEGF-E is a new angiogenic agent with less side effects for clinical usage.

3. A set of loop-1 and -3 structures in the novel VEGF family member, VEGF-E_{NZ-7}, is essential for the activation of VEGFR-2 signaling

Atsushi Kiba, Naoyuki Yabana, and Masabumi Shibuya

Recently we have shown that novel members of VEGF family encoded in Orf virus genome, VEGF-E, function as potent angiogenic factor by specifically binding and activating one of the VEGF receptors namely VEGFR-2 (KDR). VEGF-E is about 45%-homologous to VEGF-A at amino acid levels, however, to our surprise the amino acid residues in VEGF-A crucial for the VEGFR-2-binding are not conserved in VEGF-E protein. To understand the molecular basis of the biological activity of VEGF-E, we have constructed a series of chimeric molecules by exchanging the domains between VEGF-E_{NZ-7} and PlGF which binds only to VEGFR-1 (Flt-1), and functionally mapped residues important for interaction of VEGF-E with VEGFR-2. Exchange on the amino- and carboxyl-terminal regions had no suppressive effect on biological activity. However, exchange on either the loop-1 or loop-3 region of VEGF-E_{NZ-7} significantly reduced activities. On the other hand, introduction of the loop-1 and -3 of VEGF-E_{NZ-7} to PlGF rescued the biological activities. In addition to the chimera between PlGF and VEGF-E_{NZ-7}, the chimera between VEGF-A and VEGF-E_{NZ-7} gave essentially the same results. These findings strongly suggest that a common rule exists for VEGFR-2 ligands (VEGF-E_{NZ-7} and VEGF-A) that they build up the binding structure for VEGFR-2 through the appropriate interaction between loop-1 and loop-3 regions.

4. Analysis of VEGFR-2 signaling toward blood vessel formation using point mutation-carrying "knock-in" mice

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VEGFR-2 (KDR in human/Flk-1 in mouse) generates a major positive signal for VEGF-dependent endothelial cell growth. We have recently demonstrated that 1175 Tyrosine residue on VEGFR-2 is the autophosphorylation site critical for the activation of unique signaling pathway to cell proliferation: PLCgamma-PKC-MAP kinase-DNA synthesis pathway. In addition to 1175Tyr, we found that 1214Tyr is another major autophosphorylation site on VEGFR-2 in the presence of VEGF-A. To elucidate the biological roles of these two tyrosine residues *in vivo*, we made two knock-in mice which carry Tyr to Phe-point mutation at one of these Tyr residues in the Flk-1 genome. Our results suggest that 1173Tyr to Phe (corresponding to 1175 in human) but not 1212 Tyr to Phe (corresponding to 1214 in human) mutation showed a severe vascular defect. We are currently further analyzing at which step in the vascular development the 1173Phe-VEGFR-2-carrying mice have a lethal dysfunction.

5. Signaling through FRS2 proteins

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a. Docking proteins, FRS2α and FRS2β, activators of MAP kinase signaling, show distinct spatial and temporal expression patterns during mouse development

FRS2 family docking proteins, FRS2 α and FRS2 β, become tyrosine phosphorylated in response to stimulation with fibroblast growth factor (FGF) or nerve growth factor (NGF). Tyrosine phosphorylated FRS2 α serves as a platform for recruitment of multiple signaling proteins including Shp2, Grb2 and Cbl, induces sustained activation of mitogen-activated protein (MAP) kinase, and plays a critical role in FGF-signaling. However, signaling through FRS2 β and the biological roles of the FRS2 proteins *in vivo* are unclear. Here, we show that exogenous expression of FRS2 β in FRS2 α -deficient mouse embryonic fibroblasts (MEFs) restores activation of MAP kinase to a level comparable to that of FRS2α in response to FGF, and this is associated with formation of an FRS2 β /Shp2 complex. *In situ* hybridization of mouse embryos revealed that FRS2 α is expressed ubiquitously during early development and organogenesis and that FRS2 β is expressed specifically in the nervous system. In the nervous system, FRS2 α and FRS2 β show distinct spatial-temporal expression patterns. Thus, we conclude that the distinct expression patterns of FRS2 α and FRS2 β reflect the physiological roles of these proteins.

b. A critical role for docking-protein mediated FGF-receptor signaling in the anterior-posterior axis formation

In order to understand the biological role of FRS2 α *in vivo*, we introduced a targeted mutation into the murine *Frs2* α gene. Although mesodermal cells seem to appear in these mutant embryos, a distinct primitive streak is not formed and the embryos die by E8. The primitive streak markers and anterior visceral endoderm (AVE) markers were not expressed in the appropriate regions, indicating that the mutant embryo fails to establish an anterior-posterior axis. We found reduced expression of *Eomesodermin* and *Bmp4* as well as decreased activation of MAP kinase in the extraembryonic ectoderm of mutant embryos during E6.0-E6.5, suggesting that the FGF4-dependent trophoblast stem (TS) cells are not appropriately maintained. We propose that FGF4-stimulated FRS2 α signaling in the extraembryonic ectoderm plays a critical role in anterior-posterior axis formation. Moreover, chimera study showed that FRS2 α -/- ES cells accumulated at the primitive streak, revealing that FRS2 a plays an important role in cell migration during gastrulation.

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