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Oncogenes and protooncogenes play important roles in the genesis and/or development of cancer. 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, which is mediated predominantly by Syk. Overexpression of BANK in B cells leads to enhancement of BCR-induced calcium mobilization. We found that both Lyn and inositol 1,4,5-trisphosphate receptor (IP_3R) associate with the distinct regions of BANK and that BANK promotes Lyn-mediated tyrosine phosphorylation of IP_3R. Given IP_3R channel activity is upregulated by its tyrosine phosphorylation, BANK appears to be a novel scaffold protein regulating BCR-induced calcium mobilization by connecting PTKs to IP_3R. 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.

Genetic studies have revealed that Cbl-b plays a negative role in antigen receptor-mediated proliferation of lymphocytes. However, we show that Cbl-b-deficient DT40 B cells display reduced phospholipase C (PLC)-γ2 activation, Ca^{2+} mobilization, and c-Jun NH2-terminal kinase activation upon B cell receptor (BCR) stimulation. In addition, overexpression of Cbl-b in WEHI-231 mouse B cells resulted in augmentation of BCR-induced Ca^{2+} mobilization. Cbl-b helped association of PLC-γ2 with BTK as well as BLNK and was indispensable for Btk-dependent sustained increase in intracellular Ca^{2+}. The tyrosine kinase-binding domain and C-terminal half region of Cbl-b were essential for its association with PLC-γ2 and for regulation of Ca^{2+} mobilization. These results demonstrate that Cbl-b positively regulates BCR-mediated Ca^{2+} signaling likely 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

Thoru Tezuka, Takanobu Nakazawa, Kazumasa Yokoyama, Shin-ichiro Kina, Seiji Kawa, June Goto, Aya Sato, Miho Ohsugi, Jiro Fujimoto and Tadashi Yamamoto

The Src-family protein-tyrosine kinases (PTKs) are implicated in various neural functions. For example, 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 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 facilitated the tyrosine phosphorylation events. To analyze the biological significance of tyrosine phosphorylation of the NMDAR, we have determined major tyrosine phosphorylation sites on MNDAR2A/B. Tyrosine phosphorylation at Tyr-1472 on NR2B, which is a major tyrosine phosphorylation site, was significantly enhanced after induction of LTP in the hippocampal CA1 region, suggesting that NR2B Tyr1472 phosphorylation is important for 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 have also 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 are generating PTPMEG$^{-/-}$ mice.

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. We have 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 known to be a tyrosine kinase, but the specificity of other two, AATYK2 and AATYK3, remained to be verified. All three members express specifically in the central nervous system.

Another line of study is to identify the targets of brain-specific protein-tyrosine kinases. We have already identified a number of proteins that could be phosphorylated by the Src family kinases. Characterization of these putative substrates is in progress.

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

Yutaka Yoshida, Toru Suzuki, Junko Tsuzuku, Takahisa Nakamura, Rieko Ajima, Makoto Watanabe, Takashi Miyasaka, Eri Hosoda, Hisashi Umemori and Tadashi Yamamoto

By screening a cDNA expression library with autopohosporylated 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 protein Btg1 and PC3. Elevated expression of the Tob protein suppressed growth of the NIH3T3 cells. We assume that antiproliferative property of Tob might be canceled by the signaling through membrane tyrosine kinases. We also cloned novel genes homologous to *tob* that were termed *tob-2* and *ana*. Microinjection experiments showed that both exogenously expressed 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, suggesting that activity of cyclin-dependent kinases (CDKs) were affected. We have also found that Tob is rapidly phosphorylated at multiple sites upon growth factor stimulation, and that Ser152, Ser154, and Ser164 are phosphorylated by Erk1 and Erk2 MAPKs. Tob mutant with Ser152, 154, 164 to alanine substitution largely suppressed Ras/MAPK dependent cell proliferation and transformation, whereas wild-type and the mutant with glutamates at those three positions, which are negatively charged amino acids mimicking phospho-serines, had no significant effect. These results suggest that the antiproliferative activity of Tob is controlled by MAPK-mediated phosphorylation and Tob is involved in regulation of growth factor-induced cell cycle entry. 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 have preliminary data showing suppression of cyclin D1 gene by Tob. There are data suggesting that Caf1 is associated with deadenylase activity, suggesting that Tob-Caf1 complex may be involved in translational regulation.

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. Furthermore, aged mice lacking Tob expression were shown to 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. 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. 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 on presence of Tob.

4. Characterization of ALK (Anaplastic Lymphoma Kinase) signaling in lymphoma malignancy and neural function

Jiro Fujimoto, Akira Motegi, Minoru Chikamori, and Tadashi Yamamoto

Ki-1 lymphoma is a subtype of human malignant lymphomas characterized by the expression of CD30 (Ki-1 antigen) and an anaplastic large cell morphology. It is occasionally accompanied by a unique reciprocal chromosome translocation t(2;5)(p23;q35). We previously showed that a gene encoding the Ltk-like kinase, termed ALK, is located at the breakpoint and is responsible for the tumor formation as fusion protein p80(NPM-ALK). We also have cloned full-length proto-*alk* cDNA and analyzed its expression. Alk is expressed in the brain and spinal cord of embryonic and neonatal mice, especially in specific regions of the nervous system such as the thalamus, mid brain, olfactory bulb, and ganglia. Expression of ALK in adult mice is low, suggesting its role in the development of the brain.

To further characterize ALK-mediated signaling, we isolated monoclonal antibodies against the extracellular domain of ALK. Some of the antibodies could stimulate the kinase activity of ALK and induce neurite outgrowth of ALK-expressing neuroblastoma cell line and suppress apoptosis upon serum withdrawal. This result suggested that ALK could mediate neurotrophic signals. Though natural ligand of ALK receptor is still not identified, this antibody-mediated approach could help to investigate the biological and biochemical activities of ALK.

We previously reported that IRS-1 and SHC adaptor proteins associate with phosphorylated ALK and identified the binding site as an NPXY-like phosphotyrosine-containing sequence. However, the oncogenic activity of p80(NPM-ALK) was not eliminated when the association of these two adaptor molecules was suppressed by a point mutation of the binding sites. This result suggested that there is another downstream factor, which is essential for the oncogenicity of activated ALK kinase. We have been searching for its novel interacting molecules by yeast two-hybrid screening and identified SNT2(FRS2-beta) as the possible downstream factor of ALK-mediated signaling. SNT2 interacted with c-terminal tail of ALK and was strongly phosphorylated by p80(NPM-ALK). Furthermore, we showed that interaction of ALK with SNT2 as well as SNT1 largely contributed to the transforming activity of p80(NPM-ALK). To elucidate the function of SNT2/SNT1 in the ALK-signaling, we have been identifying the precise binding properties of SNT2/SNT1 with ALK.

5. 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, Hirohisa Umemoto, 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 Kid 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 between Kid and microtubules. 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.

6. Identification and analysis of LATS kinases, human homologs of a Drosophila tumor suppressor

Jiro Fujimoto, Yoshinori Abe, 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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Cells are held in tissue through interaction with extracellular matrices (ECM). Such interaction is important not only to maintain tissue architecture but also in regulating various cell functions such as cell growth, differentiation, apoptosis, migration and morphology. Malignant cancer cells are defective in interacting with ECM and destructive against surrounding tissue. We are interested in understanding how matrix-degrading enzymes such as matrix metalloproteinases (MMPs) are used during cancer cell invasion and trying to apply the knowledge to develop therapeutic way to treat cancer.

1. Membrane-type 5 matrix metalloproteinase is expressed in differentiated neurons and regulates axonal growth

Hiromi Hayashita-Kinoh, Hiroaki Kinoh, Akiko Okada, Kiyoshi Komori, Yoshifumi Itoh, Tadashige Chiba, Masahiro Kajita, Ikuo Yana, and Motoharu Seiki

Expression of membrane-type 5 matrix metalloproteinase (MT5-MMP) in the mouse brain was examined. MT5-MMP was expressed in the cerebrum in embryos, but it declined after birth. In contrast, expression in the cerebellum started to increase postnatally and continued thereafter. The cells expressing MT5-MMP were post-mitotic neurons that showed gelatinolytic activities. Specific expression of MT5-MMP was observed in the neurons but not in the glial cells when embryonal mouse carcinoma P19 cells were differentiated *in vitro* by retinoic acid treatment. Neurons isolated from dorsal root ganglia (DRG) also expressed MT5-MMP and it was localized at the edge of growth cone. Proteoglycans are inhibitory against neurite extension and regulate synaptogenesis. The inhibitory effect of the proteoglycans against neurite extension of DRG neurons was effectively eliminated by recombinant MT5-MMP. Thus, MT5-MMP expressed in neurons may play a role in axonal growth that contributes to the regulation of neural network formation.

2. Membrane-type 1 matrix metalloproteinase cleaves CD44 and promotes cell migration

Masahiro Kajita, Yoshifumi Itoh, Tadashige Chiba, Hidetoshi Mori, Akiko Okada, Hiroaki Kinoh and Motoharu Seiki

Migratory cells including invasive tumor cells frequently express CD44, a major receptor for hyaluronan, and membrane-type 1 matrix metalloproteinase (MT1-MMP) that degrades extracellular matrix (ECM) at pericellular region. In this study, we demonstrate that MT1-MMP acts as a processing enzyme for CD44H, releasing it into the medium as a soluble 70-kDa fragment. Further more, this processing event stimulates cell motility although expression of either CD44H or MT1-MMP alone did not stimulate cell motility. Co-expression of MT1-MMP and mutant CD44H lacking the MT1-MMP-processing site did not result in shedding and did not promote cell migration, suggesting that the processing of CD44H by MT1-MMP is critical in the migratory stimulation. Moreover, expression of the mutant CD44H inhibited the cell migration promoted by CD44H and MT1-MMP in a dominant negative manner. The pancreatic tumor cell line, MIA PaCa-2, was found to shed the 70-kDa CD44H fragment in a MT1-MMP dependent manner. Expression of the mutant CD44H in the cells as well as MMP inhibitor treatment effectively inhibited the

migration, suggesting that MIA PaCa-2 cells indeed use the CD44H and MT1-MMP as migratory devices. These findings revealed a novel interaction of the two molecules that have each been implicated in tumor cell migration and invasion.

3. Homophilic complex formation of MT1-MMP facilitates proMMP-2 activation on the cell surface and promotes tumor cell invasion

Yoshifumi Itoh, Akiko Takamura, Noriko Ito, Yoshiro Maru, Hiroshi Sato, Naoko Suenaga, Takanori Aoki and Motoharu Seiki

Activation of proMMP-2 by MT1-MMP is considered to be a critical event in cancer cell invasion. In the activation step, TIMP-2 bound to MT1-MMP on the cell surface acts as a receptor for proMMP-2. Subsequently, adjacent TIMP-2-free MT1-MMP activates the proMMP-2 in the ternary complex. In this study, we demonstrate that MT1-MMP forms a homophilic complex through the hemopexin-like (PEX) domain that act as a mechanism to keep MT1-MMP molecules close together to facilitate proMMP-2 activation. Deletion of the PEX-domain in MT1-MMP, or swapping the domain with the one derived from MT4-MMP abolished the ability to activate proMMP-2 on the cell surface without affecting the proteolytic activities. In addition, expression of the mutant MT1-MMP lacking the catalytic domain (MT1PEX-F) efficiently inhibited the complex formation of the full-length enzymes and the activation of proMMP-2. Furthermore, expression of MT1PEX-F inhibited proMMP-2 activation and Matrigel invasion activity of invasive human fibrosarcoma HT1080 cells. These findings elucidate a new function of PEX domain regulating MT1-MMP activity on the cell surface that accelerates cellular invasiveness in the tissue.

4. Cytoplasmic tail dependent internalization of membrane-type 1 matrix metalloproteinase (MT1-MMP) is important for its invasion-promoting activity

Takamasa Uekita, Yoshifumi Itoh, Ikuo Yana, Hiroshi Ohno and Motoharu Seiki

Membrane-type 1 matrix metallorproteinase (MT1-MMP) is an integral membrane proteinase that degrades the pericellular extracellular matrix (ECM) and is expressed in many migratory cells including invasive cancer cells. MT1-MMP has been shown to localize at the migration edge and to promote cell migration, however, it is not clear how the enzyme is regulated during the migration process. Here we report that MT1-MMP is internalized from the surface and that this event depends on the sequences of its cytoplasmic tail. Di-leucine ($Leu^{571\cdot572}$ and $Leu^{578\cdot579}$) and tyrosine^{573} residues are important for the internalization, and the μ2 subunit of adaptor protein 2 (AP2), a component of clathrin-coated pits for membrane protein internalization, bound to the LLY^{573} sequence. MT1-MMP was internalized predominantly at the adherent edge and was found to co-localize with clathrin-coated vesicles. The mutations that disturb internalization caused accumulation of the enzyme at adherent edge, though the net proteolytic activity was not affected much. Interestingly, while expression of MT1-MMP enhances cell migration and invasion, the internalization-defective mutants failed to promote either activity. These data indicate that dynamic turnover of MT1-MMP at the migration edge by internalization is important for the enzyme to function properly during cell migration and invasion.

5. Identification of *cis*-acting promoter elements that support expression of membrane-type 1 matrix metalloproteinase (MT1-MMP) in v-*src* transformed Madin-Darby Canine Kidney Cells

Hee-Jae Cha, Akiko Okada, Kyu-Won Kim, Hiroshi Sato, Motoharu Seiki

Membrane-type 1 matrix metalloproteinase (MT1-MMP) expressed in tumor cells is believed to be important for the pericellular degradation of extracellular matrices during invasion and metastasis. To analyze the mechanism by which MT1-MMP becomes expressed in cancer cells, we assessed the MT1-MMP promoter region for the presence of *cis*-acting promoter elements that support transcription in transformed cells. Our tumor model consisted of Madin-Darby canine kidney (MDCK) cells transformed by v-*src* (src4 cells). MT1-MMP mRNA was only faintly detected in parental cells but was strongly expressed in the src4 cells. In parallel, src4 cells invaded into collagen gels, whereas MDCK cells did not. When MDCK and src4 cells were transiently transfected with a plasmid containing of -3000 to -99 nt from the upstream region of the MT1-MMP gene, the promoter activity was 2.6 fold higher in src4 cells than in MDCK cells. Furthermore, the region between -399 and -356 nt was found to contain the src4-specific enhancer element(s). Tandem Sp1 binding sites were also found to be essential in promoting transcription. An Egr-1 site that partially overlaps with the Sp1 sites was found to cooperate with the src4-specific enhancer and to also contribute weakly to the basal promoter activity. The presence of transcription factors that bind to the src4-specific enhancer site was detected by mobility-shift assays in src4 cell nuclear extracts but only weakly in MDCK extracts. Thus, we have identified a novel enhancer element that acts specifically in the transformed cells to enhance MT1-MMP expression.

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Department of Cancer Biology
Division of Cancer Genomics

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. A novel microarray system with synthetic polynucleotides

Shinya Watanabe

Viruses require host factors to proliferate in cells. As the host factors may restrict viral replication, identification and characterization of the host factors and their genes should play an important role in investigation of viral pathogenesis and eventual control of viral infection. In addition, to study the host factors involved in viral life cycles as a tool may contribute to inquire about complex biological phenomena in the cell. We have obtained a novel methodology to study the host factors involved in viral pathogenesis from a view point different from the conventional paradigm in biology, which utilizes DNA microarrays and leads us to survey alteration of gene expression simultaneously and comprehensively.

We have independently ameliorated the cDNA microarray system originally established in Stanford, using 80mer of synthetic polynucleotides and commercially available slide glasses. When compared the Stanford cDNA microarray, our synthetic polynucleotide microarrays showed equivalent qualities on the signal-noise ratio and the detection sensitivity of low-abundant transcripts. The synthetic polynucleotide array system have dramatically reduced the total cost of making arrays and operational labor and opened a way to utilize thousands of arrays for collaborators who are interested in transcriptome analyses in a variety of biological phenomena.

By the end of 2001, we have successfully printed 12,000 human RefSeq genes within a 4 cm^2-area on a slide glass and are struggling to increase the number of arrayed genes to 14,400 within the fiscal year of 2001. In addition, we have already prepared 3,800 rat gene microarrays that are useful for investigation with animals. These microarrays are widely available for those who have enthusiasm for conducting comprehensive analysis of gene expression in every biological field as collaborative studies with us.

2. Transcriptome analysis of cellular responses against HCMV infection in permissive fibroblasts

Shinya Watanabe

Human cytomegalovirus (HCMV) replicates efficiently in normal human fibroblasts but restrictedly or scarcely in most immortalized cell lines. To investigate a molecular strategy under which HCMV conquests and governs the permissive fibroblasts toward succeeding virus propagation, we analyzed transcriptomes for HCMV-infected cells using a microarray system with synthetic polynucleotides representing 9,600 of human named genes in the RefSeq database of NCBI. We compiled expression profiles obtained from wild-type HCMV-infected and UV-inactivated HCMV-infected fibroblasts as well as fibroblasts inoculated with culture media of the infected cells and found that HCMV elicits a wide range of cellular responses via adsorption and/or penetration of viral particles and subsequently via factors exrtracellularly secreted after the initial events. Moreover, comparison of the transcriptomes between wild type and UV-inactivated virus-infect-

ed cells revealed that HCMV eventually suppresses expression of the cellular genes which are responsible against exogenous stimuli by means of viral gene products synthesized *de novo* during the viral replication cycle. These results suggest that suppression of the cellular responsible genes mostly involved in inflammation may indicate the overall submission to of the host cell to the virus.

In this year, we have submitted the following patent that can facilitate development of the microarray technology and that allows to provide an optimal hybridization condition for synthetic DNA microarrays with a simple device: 特願 2001-323412, スライドガラスハイブリダイゼーションチャンバ (title of invention: slide glass hybridization chamber).

3. Patent

Shinya Watanabe

Publications

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Department of Cancer Biology
Division of Pathology

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.
Sponsored by this division, a new laboratory for histopathologic and cytogenetic services started in September 2001 under the doorplate of "Histopathology and Cytogenetics Service Laboratory".

I. Malignant lymphomas

1. Maintenance of human malignant lymphoma specimens, establishment of new lymphoma lines and their characterization

Izuru Maekawa, Takamitsu Okamura, Naoto Aoki[1], Shigeo Mori:[1]Tokyo Metropolitan Laboratory of Hygienic Science

Fresh pathology specimens are prerequisite for the molecular analysis of various human diseases. We are continuing to maintain resected human lymphoma tissues in these 10 years and could maintain 376 cases successively. A T-cell lymphoma cell line expressing the Epstein-Barr virus (EBV) latent gene products has been investigated on those days for its genomic abnormalities (Maekawa et al. 2001). The positional cloning of genes locating at t(1;18) (q32;q21) and t(6;12) (p12;q24) are now on-going. Another positional cloning is now under investigation on another B-cell lymphoma cell line carrying t(8;22) lacking c-myc gene rearrangement in its ordinal manner.

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

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).

3. EBV infection status of Japanese Hodgkin lymphoma

Kengo Takeuchi[2], Shigeo Mori:[2]Department of Pathology, Graduate School of Medicine, University of Tokyo

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 associate 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 realized 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., 2000). This data suggests the rapid change of EBV infectious status among Japanese in recent years. We are now conducting nation-wide epidemiological study to clarify the EBV infection status.

4. The pathogenesis of so-called vascular neoplasia

Chihiro Kakiuchi[3], Kengo Takeuchi, Yasuyuki Morishita, 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.

5. Hodgkin's lymphoma: molecular mechanisms of constitutive activation of NF-κB in Hodgkin/ Reed-Sternberg cells

Ryouichi Horie[4], Yasuyuki Morishita, Kinji Ito[5], Masae Nagai-Maruyama, Shigemi Aizawa, Takaomi Ishida, Shigeo Mori, Marshall E. Kadin[6], 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

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-κB (NF-κB) (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-κB 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-κB 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-κB activation in H-RS cells. These findings linked two major characteristics of H-RS cells, CD30 overexpression and constitutive activation of NF-κB, 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 IκBα 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, in press).

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-κB activation in H-RS cell lines. Confocal immunofluorescence microscopy also revealed colocalization of IKKα, NIK and IκBα 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 characteristic of H-RS cells. (Horie et al., Am J Pathol, in press)

6. Development of adenovirus mediated gene therapy for lymphoid malignancies

Takurou Watanabe[7], Horie R[4], Shin Koga[8], Takaomi Ishida, Kazunari Yamaguchi[9], Toshiki Watanabe:[7]Department of Hematology, Graduate School of Medicine, The University of Tokyo, [8]Central Amakusa General Hospital, [9]Blood Transfusion Service, School of Medicine, Kumamoto

University

Although adenovirus vectors has many advantages in efficient gene transfer, they have not been used for hematopoietic and lymphoid cells because of low transduction efficiency. As described above, we found that adenovirus vectors infect some of the neoplastic cells of Hodgkin/Reed Sternberg cells, and also HTLV-1-infected T cell lines as well as ATL cells. Furthermore, transduction of a dominant negative form of IκBα into these cells induced apoptosis. To examine the feasibility to apply adenovirus vectors to clinical situation, characterization of adenovirus infection to *ex vivo* tumor cells and screening of effector molecules are now underway.

7. 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 600kb region between STS markers D1S1615 and D1S274E. Screening of the gene(s) locating on the breakpoint is still on going.

8. 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. Human herpes virus type 8 (HHV8)

1. Transmission mode of HHV-8 from HHV-8 positive lymphoma cells to endothelial cells

Shinsaku Sakurada, Harutaka Katano[10], Shigeo Mori:[10]Department of Pathology, National Institute of Health, Japan

To know the transmission mode of HHV8 onto blood vessels, we conducted in vitro study with the use of HHV-8 free human umbilical endothelial cells and HHV8-infected lymphoma cell line that is established in this laboratory. We found the direct contact of those cells facilitates viral transmission. It is speculated from this result that same process can happen on HHV-8 infected patients and this process may relate to the occurrence of the Kaposi sarcoma (Sakurada et al., 2001).

2. HHV-8 infection status of Japanese Castleman's disease

Tetsuji Suda, Harutaka Katano[10] and Shigeo Mori

Multicentric Castleman's disease (MCD) is a rare systemic disease characterized by the prolonged polyclonal hyperimmunoglobulinemia complicated with generalized symptoms such as fever, anemia, hepatosplenomegaly and lymphadenopathy. High incidence of HHV8 carrier are reported from mid-east European and Mediterranean countries. In our systemic study of MCDs occurring among Japanese, it was clarified that the incidence of HHV8-carrier is low, less than 2% and that such HHV-8 positive MCD cases are restricted to AIDS (Suda et al. 2000). This result, together with our epidemiological studies suggest that the incidence of HHV-8 associated disease remain lower, if not zero, among Japanese.

III. Functional role of S100A4, the tumor metastasis-associated protein

1. Association of s100A4 with methionine aminopeptidase 2

Hideya Endo, Takayuki Kanno

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., submitted).

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

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., submitted).

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

1. Multistep leukemogenesis of adult T-cell leukemia (ATL) -Possible involvement of PKCβII 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 PKCβII in colon carcinogenesis, we examined whether GSK-3β-β-catenin/TCF pathway is also activated by PKCβII in ATL leukemogenesis. We found accumulation of β-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*

Masao Satoh[13], Shimeru Kamihira[14], Masao Matsuoka[12], Kazunari Yamaguchi[9], Eitaro Hori[15], Toshiki Watanabe:[13,15]Department of Immunology, Saitama Medical School, [14]Department of Laboratory Medicine, School of Medicine, Nagasaki University

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, in press)

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. Reactivation of latent HIV is associated with CpG demethylation

Takaomi Ishida, Jun Tanaka[16], Tsukasa Koiwa, Masae Nagai-Maruyama, Youichirou Iwakura[16], Toshiki Watanabe:[16]Center for Experimental Medicine, Division of Cell Biology

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-α 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., submitted, Ishida et al., in preparation)

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

Tsukasa Koiwa, Akiko Hamano-Usami, Takaomi Ishida, Akihiko Okayama[17], Kazunari Yamaguchi[9], Shimeru Kamihira[14], Toshiki Watanabe: [17]Department of Internal Medicine, Miyazaki Medical College

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., submitted)

V. Cytogenetic Studies

1. Molecular analysis in the domestic cat

Hitoshi Satoh, Yasuhito Fujino[18], Hajime Tsujimoto[19]: [19]Dept. Veterinary Internal Med., Graduate School of Agricultural and Life Sciences, The University of Tokyo.

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 inser-

tional 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[20]:[20]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.

VI. Service activities of the Histopathology and Cytogenetics Service Laboratory

Tomoko Nakajima, Hitoshi Sato, Shigeo Mori

Following services were conducted during September 2001 - March 2002. Paraffin blocks: 613 specimens; Thin section cutting: 1329 sections; Hematoxylin and Eosin staining: 266 slides; Pathology diagnosis service: 1 cases. Those were requested from 6 divisions.

Publications

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Department of Cancer Biology
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Our interest is to elucidate the regulation of gene expression in relation to development and tumor formation. We are currently focusing on the function of a tumor suppressor gene, WT1 which is involved in Wilms' tumor, leukemia and sex determination.
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 kidney and reproductive organs. Surprisingly, recent progress demonstrated that WT1 is overexpressed in leukemic cells and thought to function as an oncogene in leukemogenesis. However, molecular mechanism of WT1 remains to be elucidated. We are trying to answer the questions, why WT1 exerts such two distinct functions.

1. Analysis of "oncogenic" function of WT1 in leukemia

Kentaro Semba and Yoshimasa Sakamoto

In contrast to the previous reports which showed that overexpression of WT1 inhibited growth of a variety of cultured cells, WT1 is required for growth of leukemic cells. To analyze the "oncogenic" function of WT1 in those cells, we isolated cDNAs encoding WT1-associated proteins from human erythroleukemia cell line, K562. 1.6 x 10^7 clones were screened and 467 clones have been shown to bind to WT1 specifically. 263 of 467 clones are human ubiquitin conjugating enzyme 9 as previously reported. In addition, four small nuclear ribonucleoprotein particles genes have been identified. Furthermore, eight clones were likely to encode transcription factors. Among them, we focused on GATA family proteins. GATA family plays a critical role in differentiation of hematopoietic cells. We showed i) WT1 binds to GATA-1 and GATA-2 in vitro and in cultured cells, ii) WT1 and GATA-1 synergistically activates transcription from a promoter that contains GATA binding sequence, iii) DNA pull down assay showed that WT1 was recruited on GATA-binding sequence in a GATA-dependent manner, iv) WT1 competed with FOG-1, a cofactor of GATA-1 in luciferase reporter assay. In this assay, FOG-1 repressed GATA-mediated transcription, whereas, WT1 activated GATA-mediated transcription in a dose-dependent manner even in the presence of FOG-1, v) WT1 formed ternary complex with GATA-1 and FOG-1 in a GATA-dependent manner.

Our current hypothesis is that WT1 may perturb the differentiation process of hematopoietic cells by inhibition of FOG-1 and subsequently constitutive activation of GATA-mediated transcription. We are currently testing this hypothesis.

2. Analysis of WT1 function in sex determination

Kentaro Semba and Yumiko Matsuzawa-Watanabe

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. Recently we have established cell lines which express both WT1 and SRY. We are currently analyzing the expression profile of this cell line, which will reveal target genes regulated by WT1 and SRY.

3. Characterization of a novel Sox family gene, Sox30

Kentaro Semba, Gensuke Takayama, Emiko Osaki, Yukio Nishina[1], Johji Inazawa[2], Jun-ichi Hata[3], Iwao Fukui[4] and Haruo Sugano4:[1]Department of Biology, Graduate School of Integrated Science and Faculty of Science, Yokohama City University, [2]Department of Molecular Cytogenetics, Division of Genetics, Medical Research Institute, Tokyo Medical and Dental University, [3]Keio Univ., School of Medicine, [4]Japanese Foundation for Cancer Research

During searching for WT1-associated proteins, we cloned Sox30 cDNA which encodes a novel transcription factor with a SRY-like HMG box (Sox). Sequence analysis of Sox30 identified a novel subclass H of Sox family transcription factors. Sox30 protein showed sequence-specific binding to ACAAT motif and activated transcription from synthetic promoter containing this binding motif. Immunohistochemical and northern blot analyses revealed that Sox30 was highly expressed in germ cells. Interestingly, RT-PCR analysis showed that aberrant Sox30 transcripts were observed in two human germ cell tumor cell lines and 17 out of 18 tumor specimens. Sequence analysis of those transcripts and corresponding genomic clones revealed that aberrant splicing occurred in tumor cells but not in normal testis, which resulted in missing the DNA binding domain of Sox30. Furthermore aberrant splicing was observed not only in primary lesion but also in metastatic lymphnodes. Those mutants did not show any activation of transcription. It still remains to be tested whether reduced expression of Sox30 is involved in development of germ cell tumors, nevertheless, our finding may be served as molecular marker of male germ cell tumors.

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Department of Cancer Biology
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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 with knockout mice.

1. Identification of another actin-related protein (Arp) 2/3 complex binding site in neural Wiskott-Aldrich syndrome protein (N-WASP) that complements actin polymerization induced by the Arp2/3 complex activating (VCA) domain of N-WASP

Shiro Suetsugu, Hiroaki Miki, and Tadaomi Takenawa

Neural Wiskott-Aldrich syndrome protein (N-WASP) is an essential regulator of actin cytoskeleton formation via its association with the actin-related protein (Arp) 2/3 complex. It is believed that the C-terminal Arp2/3 complex-activating domain (verprolin homology, cofilin homology, and acidic (VCA) or C-terminal region of WASP family proteins domain) of N-WASP is usually kept masked (autoinhibition) but is opened upon cooperative binding of upstream regulators such as Cdc42 and phosphatidylinositol 4,5-bisphosphate (PIP2). However, the mechanisms of autoinhibition and association with Arp2/3 complex are still unclear. We focused on the acidic region of N-WASP because it is thought to interact with Arp2/3 complex and may be involved in autoinhibition. Partial deletion of acidic residues from the VCA portion alone greatly reduced actin polymerization activity, demonstrating that the acidic region contributes to Arp2/3 complex-mediated actin polymerization. Surprisingly, the same partial deletion of the acidic region in full-length N-WASP led to constitutive activity comparable with the activity seen with the VCA portion. Therefore, the acidic region in full-length N-WASP plays an indispensable role in the formation of the autoinhibited structure. This mutant contains WASP-homology (WH) 1 domain with weak affinity to the Arp2/3 complex, leading to activity in the absence of part of the acidic region. Furthermore, the actin comet formed by the DeltaWH1 mutant of N-WASP was much smaller than that of wild-type N-WASP. Partial deletion of acidic residues did not affect actin comet size, indicating the importance of the WH1 domain in actin structure formation. Collectively, the acidic region of N-WASP plays an essential role in Arp2/3 complex activation as well as in the formation of the autoinhibited structure, whereas the WH1 domain complements the activation of the Arp2/3 complex achieved through the VCA portion.

2. Requirement of the basic region of N-WASP/WAVE2 for actin-based motility

Shiro Suetsugu, Hiroaki Miki, Hideki Yamaguchi, and Tadaomi Takenawa

WASP family proteins activate nucleation by the Arp2/3 complex, inducing rapid actin polymerization in vitro. Although the C-terminal portion of WASP family proteins (VCA) activates nucleation by the Arp2/3 complex in pure systems, we find that this fragment lacks activity in cell extracts. Thus, polystyrene beads coated with VCA did not move in brain cytosol, while beads coated with N-WASP or WAVE2 did move. The basic clusters between the WH1 domain and the CRIB domain of N-WASP were critical for movement since beads coated with N-WASP or WAVE2 constructs missing the basic clusters (Delta basic) also did not move. Furthermore, VCA and N-WASP/WAVE2 Delta basic

constructs were much less able than wild-type N-WASP and WAVE2 to induce actin polymerization in cytosol. All of the proteins, with or without the basic domain, were potent activators of nucleation by purified Arp2/3 complex.

3. Essential role of N-WASP in podosome formation and degradation of extracellular matrix in *src*-transformed fibroblasts

Kiyohito Mizutani[1], Hiroaki Miki[1], Hong He[2], Hiroshi Maruta[2], and Tadaomi Takenawa[1]:[2]Ludwig Institute for Cancer Research, PO Royal Melbourne Hospital

Transformation of cells by the *src* oncogene causes dramatic changes in adhesive structures. In *v-src* transformed 3Y1 rat fibroblasts (3Y1-src), there are actin-rich protrusive structures called podosomes, by which attachment to the extracellular matrix is thought to occur. In this study, we found that neural Wiskott-Aldrich syndrome protein (N-WASP) colocalizes with F-actin in podosomes. Expression of dominant-negative mutants of N-WASP, Δcof N-WASP and ΔVPH N-WASP, both of which are incapable of activating the Arp2/3 complex, suppressed podosome formation, suggesting that N-WASP is essential in this process. Localization of N-WASP in podosomes appears to be due to interaction between N-WASP and the SH3 domain of cortactin. Indeed, microinjection of the cortactin SH3 domain suppressed podosome formation. We also observed that 3Y1-src cells cultured on fibronectin degrade the fibronectin primarily at the podosomes and that the inhibition of podosome formation by Δcof N-WASP abolishes the fibronectin degradation. These results suggest the importance of N-WASP in podosome formation and extracellular matrix degradation, which are processes thought to underlie the invasive phenotype of 3Y1-src cells.

4. Role of the ENTH-domain is phosphatidylinositol 4,5-bisphosphate binding and endocytosis

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Endocytic proteins such as epsin, AP180, and Hip1R (Sla2p) share a conserved modular region termed the epsin N-terminal homology (ENTH) domain, which plays a crucial role in clathrin-mediated endocytosis through an unknown target. Here we demonstrate a strong affinity of the ENTH domain for phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2). With nuclear magnetic resonance analysis of the epsin ENTH domain, we determined that a cleft formed with positively-charged residues contributed to phosphoinositide binding. Overexpression of a mutant, epsin K76A, with an ENTH domain defective in phosphoinositide binding, blocked epidermal growth factor internalization in COS-7 cells. Thus interaction between the ENTH domain and PtdIns(4,5)P_2 is essential for endocytosis mediated by clathrin-coated pits.

5. Identification and characterization of a sac domain-containing phosphoinositide 5-phosphatase

Tetsuya Minagawa, Takeshi Ijuin, Yasuhiko Mochizuki, Tadaomi Takenawa

We have characterized a novel Sac domain-containing inositol phosphatase, hSac2. It was ubiquitously expressed but especially abundant in the brain, heart, skeletal muscle, and kidney. Unlike other Sac domain-containing proteins, hSac2 protein exhibited 5-phosphatase activity specific for phosphatidylinositol 4,5-bisphosphate and phosphatidylinositol 3,4,5-trisphosphate. This is the first time that the Sac domain has been reported to possess 5-phosphatase activity. Its 5-phosphatase activity for phosphatidylinositol 4,5-bisphosphate (K(m) = 14.3μ) was comparable with those of Type II 5-phosphatases. These results imply that hSac2 functions as an inositol polyphosphate 5-phosphatase.

6. Requirement of phospholipase Cδ4 for the zona pellucida-induced acrosome reaction

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Several Phospholipase C (PLC) isoforms have been found in male and female mammalian gametes and splicing isoforms of PLCδ4 are predominantly expressed in testis. Here we report that PLCδ4 gene-disrupted male mice either produced few small litters or were sterile. *In vitro* fertilization studies showed that insemination with PLCδ4$^{-/-}$ sperm resulted in significantly fewer eggs becoming activated and that the calcium transients associated with fertil-

ization were absent or delayed. PLCδ4$^{-/-}$ sperm were unable to initiate the acrosome reaction, an exocytotic event required for fertilization and induced by interaction with the egg coat, the zona pellucida. These data demonstrate that PLCδ4 functions in the zona pellucida-induced acrosome reaction during mammalian fertilization.

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Fukami, K. (Review): Structure, regulation, and function of phospholipase C isozymes. J. Biochem. in press

Department of Cancer Biology
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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. Development of new methods and tools for analysis of protein-carbohydrate interaction

Naoei Yoshitani and Seiichi Takasaki

Carbohydrate binding proteins *in vivo* recognize native structures of carbohydrate ligands. Fine structures of oligosaccharides such as glycosyl linkages and antennary structures are quite important to be recognized as ligands. Thus, it is better to use multivalent probes carrying native oligosaccharides in your assay in order not to miss the novel carbohydrate-mediated interactions. These kinds of probes are expected to have much potential than conventional neoglycoconjugates that carry monosaccharides or small synthetic oligosaccharides, but are not commercially available. Therefore, we have developed a simple and efficient method for the synthesis of dextran-based multivalent oligosaccharide probes. Strategy of the synthesis is as follow. First, oligosaccharides derived from glycoproteins or isolated from milk are derivatized with succinic dihydrazide. Secondly, thus prepared oligosaccharide-hydrazides are conjugated to oxidized dextran (500 kDa). Subsequent labeling by hydrazide possessing fluorescence or biotin group can be performed. Finally, to the reaction mixture is added dimetylamine borane, stabilizing a hydrazone bond by reduction. If the labeling reagent is not added in the final step, non-labeled multivalent oligosaccharide polymer can be prepared. It should be particularly noted that the multivalent probes can be synthesized using the nanomole ranges of oligosaccharides by the method. This is because more than 50% of oligosaccharides added to the reaction mixture can be utilized for conjugation. The high efficiency of the reaction is beneficial to our use of limited amounts of native oligosaccharides. To be noted also is that the oligosaccharide density of the probe can be easily controlled by changing the amounts of oligosaccharide hydrazides added to the reaction mixture. The oxidized dextran used as a carrier molecule of oligosaccharides is also convenient, because it is easily prepared from commercial sources of dextran with various molecular weights and has a good solubility in water and a biological adaptability. Densities of fluorescent reagent or biotin incorporated to the oligosaccharide probes were also nearly equal, indicating that difference in the structures of oligosaccharides does not affect subsequent labeling under the conditions used. Further, the non-labeled probes were easily immobilized to gels having hydrazino groups, suggesting that the gels thus prepared can be used as a tool to purify carbohydrate binding proteins. To be particularly advantageous is that the method does not require any special instruments and skilled techniques. Affinity to various lectins of the dextran-based oligosaccharide polymers thus prepared was more than 1,000 times as strong as that of free oligosaccharides, indicating that the polymers are powerful tools for studying carbohydrate binding proteins.

2. Analysis of sperm carbohydrate binding pro-

teins which might be involved in fertilization

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Sperm capacitated in the female tract penetrate the cumulus oophorus and then meet the zona pellucida (ZP) on eggs, an extracellular matrix which is composed of several glycoproteins synthesized during oogenesis. The sperm loosely adhere and then tightly bind to the ZP. Bound sperm then undergo induction of acrosome reaction that involves fusion and vesiculation of sperm plasma membrane and outer acrosomal membrane at many sites, release of the vesicles and acrosomal contents, and exposure of the inner acrosomal membrane. Then, the acrosome-reacted sperm penetrate the ZP, and finally fuse with the egg. The initial sperm-egg interaction has been thought to occur between ZP glycoproteins and carbohydrate binding proteins on the sperm head. We have so far revealed that glycans of porcine ZP glycoproteins have the following non-reducing terminal structures: Siaα2-3(6)Galβ1-4GlcNAc—, Galβ1-4GlcNAc—, GlcNAc—, Fucα1-2Galβ1-4GlcNAc—, Galβ1-4(Fucα1-3)GlcNAc—, and Fucα1-3GlcNAc—. On the basis of structural information of the glycans, we prepared various dextran-based multivalent oligosaccharide polymers by our established method and examined their activities to inhibit sperm-oocyte binding. The result indicated that Fet-Dex and As-Fet-Dex, the polymers composed of fetuin N-glycans (containing Siaα2-3(6)Galβ1-4GlcNAc as the terminal structure) and its asialo-N-glycans (containing terminal GlcNAc residues), respectively or LNFP III-Dex (containing the Lewis X structure) cause a remarkable inhibition at the oligosaccharide-based concentration of 50μ M. Thus, boar sperm are suggested to recognize the sialyl and non-sialyl N-acetyllactosamine structures, +/-Sia-Galβ1-4GlcNAc, or the Lewis X structure, Galβ1-4 (Fucα1-3) GlcNAc that physiologically occur in the outer chain moieties of N-glycans of ZP glycoproteins.

Then, we applied the dextran-based oligosaccharde probes to detection of the putative carbohydrate binding molecules on the cell surface of boar sperm. When Lucifer yellow-labeled Fet-Dex, AsFet-Dex and LNFP III-Dex probes were used, strong fluorescence staining of capacitated boar sperm were obtained. As compared with capacitated sperm, uncapacitated sperm were stained very weakly by Fet-Dex and AsFet-Dex, and moderately with LNFP III-Dex, respectively. On the other hand, almost no fluorescence signal was observed using Ova-Dex (containing terminal mannose residues) from both uncapacitated and capacitated sperm. AgFet-Dex exhibited no fluorescence signal, either. These results indicate that the molecule(s) recognizing the sialyl and non-sialyl N-acetyllactosamine structures is expressed on the sperm head more prominently after capacitation. It is also evident that the molecule recognizing LNFP III (Lewis X structure) is expressed at a moderate level on the head of uncapacitated sperm and its expression further increases after capacitation. In addition, it was found that both molecules disappear from the sperm head after induction of acrosome reaction. Thus, the two kinds of carbohydrate molecules are expressed on the plasma membrane of boar sperm depending on their physiological state. The biotinylated probes were also able to detect the binding proteins on the isolated plasma membrane coated on a plastic dish. Therefore, the inhibition study was performed by this assay system. Binding of both biotinylated Fet-oligo-Dex and As-Fet-oligo-Dex to the sperm plasma membranes on the dish was remarkably inhibited by N-glycans prepared from fetuin and from asialofetuin at approximately 10μ M, but not significantly by LNFP III. By contrast, biotinylated LNFP III-Dex binding to the membrane was almost completely inhibited by LNFP III at 150μ M, but not by N-glycans of fetuin nor asialofetuin. Thus, it is evident that there are at least two types of carbohydrate recognition molecules on boar sperm plasma membrane. Quite recently, we could detect several proteins in a Triton X-100 extract of boar sperm that bound to the oligosaccharide probes coated on plastic plates, and are currently analyzing their protein and gene structures.

3. Carbohydrate analysis of functional glycoproteins

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The members of the G-protein coupled receptor exhibit considerable sequence similarity and form a common topological structure consisting of seven α-helical segments spanning the lipid bilayer. In addition, many G-protein coupled receptors share common post-translational modifications such as fatty acid acylation, phosphorylation and glycosylation. O-glycosylation of G-protein coupling receptor has been unnoticed. Quite recently, the existence of O-glycans in two G-protein coupled receptors, V2 vasopressin receptor and δ-opioid receptor has been demonstrated. However, the conclusion of the site and structure of the O-glycans by this approach was based upon indirect evidence. The lack of certainty in the analysis prompted us to perform a substantial analysis of the O-glycans of octopus rhodopsin, a member of the G-protein coupled receptors. In collaboration with Drs. Tsuda and Takao, the structure and sites of O-glycan in octopus rhodopsin were de-

termined using a combination of enzymatic and manual Edman degradation in conjunction with FAB mass spectorometry. The result indicated that two N-acetylgalactosamine residues are O-linked to Thr^4 and Thr^5 in the N-terminus of octopus rhodopsin. In addition, we found chicken iodopsin, but not bovine rhodopsin, contains N-acetylgalactosamine. This is the first time to determine the structure and sites of O-glycan in G-protein coupled receptors. The function of O-glycans of octopus rhodopsin is not elucidated at present. Further efforts are required to understand the function of O-glycans of a large family of G-protein coupled receptors which might contribute to maturation, transport and cell surface expression.

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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), EGF receptor, FGF receptor and Bcr-Abl] and adaptor proteins, Shc and Vav. This year we examined a hierarchy among angiogenic factors including VEGF and Ang1, and found that basal level of VEGF is required for stimulatory effect of several angiogenic factors such as Ang1, FGF and HGF. We are also examining molecules which could explain genomic instability in chronic myeloid leukemia (CML). For this purpose we have been working on XBP which associates with Bcr protein.

1. Angiopoietin1-Tie2 stimulates tube-like structures via PI3K-Akt pathway in VEGF dependent manner

Momomi Saito, Maho Hamazaki, and Masabumi Shibuya

Angiopoietin-1-Tie2 system is known to be important for the process of angiogenesis and vascular remodeling. However, its intracellular signaling and its effects on the morphogenesis of endothelial cells compared with other angiogenic factors are still not well characterized. In this study we used two experimental systems (1) *in vitro* HUVEC culture for cell growth and survival, and (2) HUVEC-fibroblast coculture for tube-like structure formation. In the first system we found that, compared with VEGF-induced VEGFR2 (KDR) signaling, Ang1-Tie2 signaling showed much weaker autophosphorylation, a clear activation of PI3K-Akt pathway for survival but very small activation of MAP-kinase with no growth stimulation. On the other hand, VEGFR2 signaling strongly stimulated MAP kinase pathway and cell growth, but very low in the activation of PI3K-Akt pathway. These results indicate that two major endothelial cell-specific tyrosine kinase receptors are different qualitatively and quantitatively, that is VEGF for MAP kinase activation, whereas Ang1 for PI3K-Akt activation.

In the second system which mimics *in vivo* angiogenesis (tube formation), we found that Ang1 stimulates the numbers and the length of tube-like structure, however, these effects are highly dependent on the endogenous VEGF possibly secreted from the co-cultured fibroblasts. Blocking of VEGF by either neutralizing antibody or soluble VEGFR1 strongly suppressed the whole morphogenesis of tube-like structure. Furthermore, other angiogenic factors such as FGF and HGF showed essentially the same phenotype as Ang1 i.e. a stimulatory effect only in the presence of VEGF. Ang1 stimulated tube-like structures via a suppression of apoptotic pathway. These results suggest that a hierarchy exists among angiogenic factors, and the basal level of VEGF signaling is essential for the stimulatory effects of Ang1 on tubular formation.

2. Soluble Flt-1 (soluble VEGFR-1), a potent natural anti-angiogenic molecule in mammals, is phylogenetically conserved in avians

Sachiko Yamaguchi, Kumi Iwata, and Masabumi Shibuya

The *flt-1* gene encodes for both full length receptor Flt-1 (VEGFR-1) and a soluble form designated sFlt-

1. sFlt-1 carries the VEGF-binding domain of Flt-1 as well as a 31-amino-acid stretch derived from an intron, and tightly binds VEGF, suppressing its angiogenic activity. The *flt-1* gene has so far been identified only in mammals, and is highly expressed in placenta as well as in vascular endothelial cells. In placenta, sFlt-1 is abundant in trophoblast layer during the pregnancy, suggesting that sFlt-1 is a negative regulator against an excess angiogenesis and vascular permeability in feto-maternal border in mammals. However, we show here at the first time that the *flt-1* gene exists and is highly conserved in chickens. Surprisingly, chicken *flt-1* gene also encodes for sFlt-1 in addition to the full length receptor. Similar to the mammalian sFlt-1, chicken sFlt-1 carries the VEGF-binding domain and 31-amino-acid carboxyl region derived from an intron, which was significantly homologous to that in mammals. Chicken sFlt-1 is expressed in early embryogenesis. These findings strongly suggest that natural anti-angiogenic molecule sFlt-1 is widely conserved in vertebrates and regulates angiogenic process.

3. Differential display analysis of endothelial cells that show invading tubulogenesis

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BCR-FLTm1 is a constitutively activated form of the Flt-1 kinase which induces invading endothelial tubulogenesis. By differential display analysis, the expression level of CD2-associated protein (CD2AP) was found to be slightly up-regulated when BCR-FLTm1 is expressed. Knock-out mice of CD2AP have been reported to display nephrotic syndrome. We challenged to maintain liquid cultures of cells derived from glomerulus. Among three types of cells with distinctive morphologies, mesangial cells as well as cells positive in acetylated LDL uptake were found to express CD2AP. Primary sinusoidal endothelial cells which grow in a VEGF/KDR-dependent manner were also found to express CD2AP. RT-PCR analysis showed that the expression level of CD2AP appeared to decrease after starting liquid cultures with VEGF. Those informations suggest that expression of CD2AP may be regulated by VEGF via two specific receptors and play certain roles in endothelial cells.

4. A search for molecules by which to explain genomic instability in chronic myeloid leukemia (CML)

Guang Li, Masabumi Shibuya, and Yoshiro Maru

Yeast two-hybrid screening gave the XPB gene whose product binds specifically to the DH domain of BCR but not of other Dbl family members. Coexpression of P210BCR-ABL and XPB in 27-1 cells (27-1/XPB/P210) counteracted against the repair-correcting ability of XPB. Although the helicase activity of P210-bound XPB was inhibited at least *in vitro*, a low stoichiometry of P210 binding to XPB *in vivo*, a high anti-repair activity of P210 in 27-1 cell system, and a change of *in vitro* transcription activity of TFIIH purified from 27-1/XPB/P210 cells prompted us to perform differential display analysis. PO, a ribosomal protein with AP-endonuclease activity, was found to be one of the genes whose expression level correlated with sensitivity to genotoxins. PO was detected in anti-XPB immune complex, and its expression in 27-1 cells enhanced the repair activity against methylmethane sulfonate and hydrogen peroxide.

5. Critical role for docking-protein mediated FGF-receptor signaling in vertebrate anterior-posterior axis formation

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Receptor tyrosine kinases (RTK) play an important role in the control of a variety of cellular processes including regulation of the cell cycle, cell metabolism and migration as well as cell proliferation and differentiaion. Upon ligand induced activation, RTKs undergo rapid autophosphorylation on numerous tyrosine residues. The tyrosine autophosphorylated RTKs function as platforms for the assembly of a variety of signaling proteins. Fibroblast growth factor (FGF) or nerve growth factor (NGF) stimulation leads to tyrosine phosphoryaltion of the docking proteins FRS2α and β enabling recruitment of multiple Grb2/Sos complexes leading to activation of the Ras/MAP kinase signaling pathway. FRS2 proteins contain myristyl anchors and PTB domains in their N-termini and large regions with multiple tyrosine phosphorylation sites at their C-termini. FRS2 α contains four binding sites for the adaptor protein Grb2 and two binding sites for the protein tyrosine phosphatase Shp2.

In order to understand the biological role of FRS2 α *in vivo*, we introduced a targeted mutation into the murine *Frs2 α* gene. We demonstrated that FRS2 α plays a critical role in the determination of anterior-posterior identity and the formation of the primitive streak, revealing a crucial role for the docking protein in embryonic development. Similar defects were found in mice deficient in *Cripto* suggesting a potential connection between FRS2 α and signaling via Cripto. We have analysed mouse embryonic fibroblast deficient in FRS2 α to demonstrate that FRS2 α plays a critical role in signaling via FGF receptors. Deficency in FRS2 α results in impairment in cell mi-

gration, MAP kinase stimulation, PI-3 kinase activity and cell proliferation.

6. Adaptor protein APS directly binds the autoinhibitory domain of guanine nucleotide exchange factor Vav3 and augments its activity

Naoyuki Yabana, Katsunori Shibata, and Masabumi Shibuya

Although guanine nucleotide exchange factors (GEFs) for Rho-family small G-proteins have been characterized oncogenically, the regulatory mechanisms of the GEF activities in signal transduction remain to be addressed. The N-terminal calponin homology (CH) domain of Vav3, a GEF for RhoA and RhoG, is thought to serve an autoinhibitory role, however its precise function is not entirely clear. We therefore attempted to identify proteins binding to the CH domain of Vav3, and as a result adaptor protein APS was isolated.

The binding of Vav3 and APS was apparently stabilized by the tyrosine phosphorylation of Vav3 by Lck, and the association of APS with Vav3 in turn enhanced the Lck-mediated phosphorylation of Vav3. Focus formation assays demonstrated that APS could increase the transforming activity of proto-Vav3. Further analyses revealed that the Vav3 CH domain could bind the pleckstrin homology domain of APS and that this binding was indispensable to enhance the transforming activity of Vav3. Finally co-expression of APS increased the degree of Vav3 associating with the membrane- and cytoskeleton-containing particulate fraction.

These observations suggest that APS directly relieves the autoinhibitory CH domain to recruit Vav3 to the membrane, thus allowing efficient association with its activator Lck and substrates Rho proteins. Furthermore our results identified Vav3 as a novel target of APS and it is intriguing to analyze the biological role of this signaling pathway.

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