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Contents

Division of Cell Biology

General Summary of Division of Cell Biology

In the past three years the division has been dedicated to basic researches on new RNA functions, biogenesis of liver-specific microRNA, miR-122 and function of hemocytes in ascidians.

A) Biogenesis of liver-specific microRNA, miR-122.

miR-122 is a liver-specific and highly abundant miRNA implicated in cholesterol and lipid metabolism and in replication of HCV. Interestingly miR-122 is down-regulated in HCCs as well as hepatoma cell lines. It suggests that the downregulation of miR-122 is associated with hepatocarcinogenesis. Therefore we have studied about the miR-122 gene and its expression.

Human miR-122 gene is about 5kb and transcribed at least 4 alternatively spliced forms as non-coding and exonic pri-miR-122 RNA. Transcription of miR-122 gene is stimulated by three liver abundant transcriptional factors HNF3b, C/EBPβ and unknown factor that interact at 200base upstream from transcription initiation site. Expression of miR-122 gene was strongly stimulated by promoter-proxymal splicing event.

B) Functional analysis of a non-coding small RNA involved in acetylation of 18S ribosomal RNA.

Many non-coding RNA species are involved in transcriptional and post-transcriptional regulation of gene expression. Among hundreds of known non-coding RNAs localized in nucleoli, U13 snoRNA is the first RNA involved in 4-acetylcytidine modification in the 3’-end region of 18S ribosomal RNA. We are currently interested in the structure required for the biogenesis and target recognition of U13 snoRNA. Various mutant RNAs have been expressed in a U13 gene-knockout DT40 cell line and their acetylation activities have been assayed.

C) Function of hemocytes in ascidians

Ascidians have no adaptive immunity but have innate immunity that allows elimination and degradation of potentially pathogenic foreign microorganisms. Phagocytosis by hemocytes is a major effector system of the innate immunity. Our knowledge on the ascidian phagocytes is still fragmentary and sometimes conflicting. Therefore the hemocytes with phagocytic capacity was studied in the ascidians. It was firstly shown that four types of hemocytes have phagocytic capacity in *Ciona intestinalis*. The high phagocytosis rate possibly assists to investigate the function of ascidian hemocytes in the future.

Biogenesis of liver-specific microRNA, miR-122.

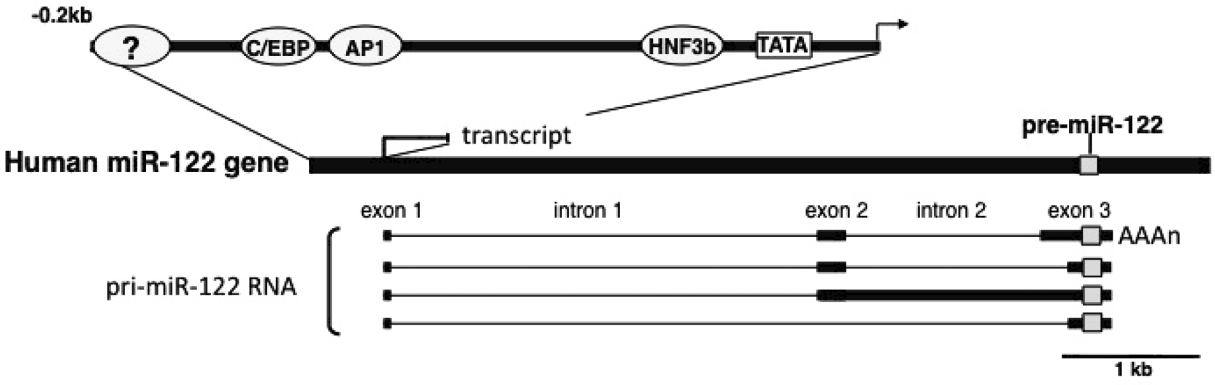
K. Kuroki, Y. Usami, Y. Fukushima*, T. Ishikawa*, F. Harada
(*Dept. of Internal Medicine, University of Tokyo)

MicroRNAs (miRNAs) are small non-coding RNAs (18～24 nucleotides) that negatively regulate gene expression through interactions with 3' untranslated regions of their target mRNAs, which leads to mRNA cleavage or translational repression.

miR-122 is a liver-specific and highly abundant miRNA, constitutes 70% of the total hepatic miRNA, implicated in cholesterol and lipid metabolism and in replication of HCV RNA. Interestingly miR-122 is down-regulated in primary HCCs as well as hepatoma cell lines. It suggests that the downregulation of miR-122 is associated with hepatocarcinogenesis. Human miR-122 sequence is mapped in chromosome 18q21. But the structure of the gene is not understood until now. Therefore we study about the miR-122 gene and its expression.

We have determined the structure of human miR-122 gene by the sequence analysis of pri-miR-122 cDNAs, mapping of the transcription initiation site using 5' RACE method, and by the reporter assay for the promoter analysis. Human miR-122 gene is about 5kb and transcribed at least 4 alternatively spliced forms as non-coding and exonic pri-miR-122 RNA. Transcription of miR-122 gene is stimulated by three liver abundant transcriptional factors HNF3b, C/EBPβ and unknown factor that interact at 200base upstream from transcription initiation site.

Expression of miR-122 gene was strongly stimulated by promoter-proxymal splicing event like many protein-coding genes. Transcription of miR-122 gene was also regulated epigenetically by the modification of histone3.



Mutational analyses of U13 small nucleolar RNA : a guide RNA involved in the acetylation of 18S ribosomal RNA.

Y. Kido, T. Suzuki and F. Harada

U13 small nucleolar RNA (U13 snoRNA) is essential for 4-acetylcytidine modification in the 3'-end region of 18S ribosomal RNA (Fig. 1). According to oligo RNA-ligation and RT-PCR on U13 snoRNA in human, mouse and chicken cells, we demonstrated that there are only two nucleotides between box D and the 3'-end of U13 snoRNA. This suggests that the terminal stem of the k-turn structure in U13 snoRNA is truncated (Fig. 2). The k-turn in snoRNAs is known as the target motif for binding of 15.5kd protein, one of the snoRNA core proteins. A recombinant 15.5kd protein bound to the truncated k-turn *in vitro* and its binding was disrupted when the box D sequence was mutated. A single base substitution in box D also decreased the expression of U13 snoRNA *in vivo*. These observation s suggest that the box C/D sequence of U13 snoRNA, a truncated k-turn, make a functional binding site for core proteins and is important for the assembly of U13 snoRNP complex.

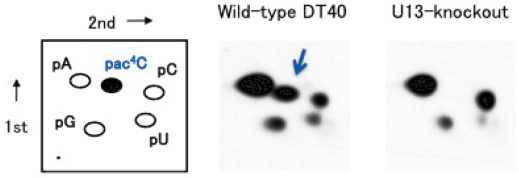


Figure 1

U13 RNA has two regions complementary to 3'-end of 18S rRNA. To identify sequences essential for recognition of the acetylation-target site, we introduced base-substitution mutations in the rRNA-complementary region on U13 RNA gene. There are some primary sequences essential for expression of the RNA, however, we could not find any mutation that lost target-recognition. These observation suggest that U13 snoRNA recognizes the target site not by a short and rigid complementarity but by a long and loose complementarity.

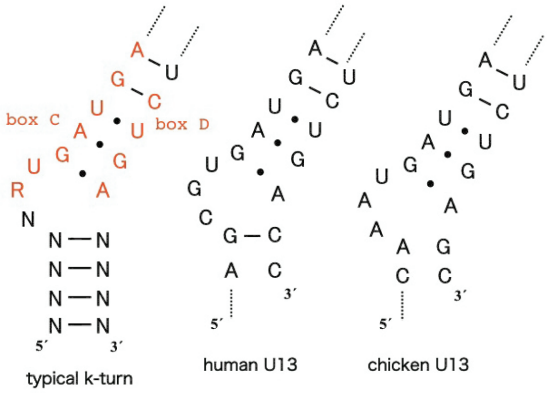


Figure 2

Expression of human or mouse U13 snoRNA in a U13-knockout DT40 cell partially recover the acetylation activity. This implies that the protein machineries recognize common structures on these U13 RNAs and "acetylation-guidance" by U13 snoRNA is conserved, therefore, important function in higher eukaryotes.

Hemocytes with phagocytic capacity in the ascidian *Ciona intestinalis*

Shigetoyo Amano

Phagocytosis is an innate immune response shared by all metazoans allowing elimination and degradation of potentially pathogenic foreign microorganisms. In ascidians a number of studies have shown that hemocytes phagocytose foreign particles, however, our knowledge on the ascidian phagocytes is still fragmentary and sometimes conflicting. Therefore the phagocytic capacity was studied qualitatively and quantitatively in the ascidian *Ciona intestinalis.*

Yeast cells were applied on the monolayer of hemocytes *in vitro*. Using a peptide antibody that reacts with ascidian hemocytes, it was shown immunohistochemically that hemocytes with phagocytic capacity are hyaline amoebocytes, granular amoebocytes, morula cells, and small amoebocytes. The serial tomograms of those hemocytes proved that the yeast cells were really engulfed by those hemocytes. It was firstly shown in this study that the morula cells and small amoebocytes have phagocytic capacity.

The phagocytosis rates of four types of phagocytic hemocytes (Table) evidently show that hyaline amoebocytes are most active phagocytes because nearly 100% of them ingested yeast cells. There is a consensus among the researchers of ascidian hemocytes that hyaline amoebocytes are professional phagocytes. Granular amoebocytes are also active phagocytes because more than 90% of them ingested yeast cells. The other two types of phagocytic hemocytes are considerably less active phagocytes; the phagocytosis rate of morula cells is approximately 33% and that of small amoebocytes is approximately 27%. These results strongly suggest that ascidians have multiple types of phagocytic hemocytes with different roles in innate immunity like in vertebrates.

Table. Phagocytosis rates of the hemocytes of the ascidian *Ciona intestinalis*

Hemocytes	Phagocytic	Non-phagocytic	Phagocytosis rate (%)
Hyaline amoebocyte	1112	13	98.9±0.9
Granular amoebocyte	558	35	93.8±3.9
Morula cell	233	452	33.0±12.8
Small amoebocyte	39	105	27.4±7.9

The number of yeast cells ingested in a hemocyte was counted. Hyaline amoebocytesingested up to six yeast cells, and the average is 2.62 yeast cells. Granular amoebocytesingested up to five yeast cells, and the average is 2.38 yeast cells. Morula cells ingested one or two yeast cells, and the average is 1.17 yeast cells. Small amoebocytes were able to ingest only one yeast cell.

Phagocytosis requires combinational receptor-mediated recognition of foreign particles, however,

in ascidians we have scanty knowledge about the ligands and receptors that trigger phagocytosis. In mammals Toll-like receptors, an important family of the receptors, activate phagocytosis when the pathogen-associated molecular patterns of foreign particles attach to them. The high phagocytosis rate (nearly 100%) of hyaline amoebocytes shown in this study possibly assist to investigate these problems about ascidian phagocytes in the future.

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Division of Molecular Pathology

General Summary of Division of Molecular Pathology

Our main research interest is to clarify how cells recognize DNA damage and transduce signals to cell cycle control, DNA repair and apoptotic machineries. To achieve this goal, we are currently using a gene knockout approach in chicken DT40 cell lines.

A) Direct ATM activation by toxic metabolites

ATM (ataxia-telangiectasia mutated) is activated by a variety of noxious agent, including oxidative stress, and ATM deficiency results in an anomalous cellular response to oxidative stress. However, the mechanisms for ATM activation by oxidative stress remain to be established. We provide evidence that ATM is activated through the direct modification of its SH groups, independent of DNA damage, and this activation leads, downstream, to apoptosis.

B) c-ABL tyrosine kinase stabilizes RAD51 chromatin association

It is unclear how ABL tyrosine kinase mechanistically regulates Rad51 functions during homologous recombination repair (HRR). We show that phosphorylation on Tyr-315 by c-ABL is required for chromatin association of oligomerization-defective RAD51 mutants, but is insufficient to restore oligomerization, suggesting a new model for the regulation of early steps of HRR.

C) NBS1 is directly involved in ATR activation

Many recent studies have provided evidence for a role for Nbs1 as a damage sensor and activator acting upstream of ATM in cellular response to DSB. We provides evidence that Nbs1 but not the Mre11/Rad50/Nbs1 complex, plays unique role in ATR-mediated Chk1 phosphorylation and FANCD2 ubiquitination, induced by various DNA replication-stalling agents, such as cisplatin, pierisin, UV and hydroxyurea.

D) Cell cycle and chromatin control by nuclear small G protein Ran

The Ran GTPase system regulates the direction and timing of several cellular events, such as nuclear-cytosolic transport, centrosome formation, and nuclear envelope assembly in telophase. Most nuclear Ran localizes to chromatin, by both RCC1-dependent and independent mechanisms. Our results suggest that active Ran GTPase opens the heterochromatin structure at the telomere in yeast.

Due to the retirement of Prof. S. Murakami, Assistant Prof. N. Hayashi moved to our laboratory in April 1, 2006. We are very grateful to investigators outside of this institute for their contributions to our projects; Kenshi Komatsu, Radiation Biology Center, Kyoto University; Koji Uchida, Graduate School of Bioagricultural Science, Nagoya University; Hiroshi Tauchi, Department of

Environmental Science, Faculty of Science, Ibaraki University; Masayuki Takahashi, UMR 6204 Centre National de la Recherche Scientifique & Universite de Nantes; Hitoshi Kurumisaka, Graduate School of Science and Engineering, Waseda University; Shunichi Takeda, Graduate School of Medicine, Kyoto University; Minoru Takata, Radiation Biology Center, Kyoto University; Keiji Wakabayashi, National Cancer Center Research Institute; Ashok R. Venkitaraman, Cancer Research UK Department of Oncology, University of Cambridge; Yosef Shiloh, Sackler School of Medicine, Tel Aviv University; Steve Jackson, The Wellcom Trust and Cancer Research UK Gurdon Institute, Cambridge University; Larry M. Karnitz, Department of Molecular Pharmacology and Experimental Therapeutics, Mayo Clinic College of Medicine.

NBS1 is directly involved in ATR activation

Masahiko Kobayashi, Kousuke Maeda, Makiko Ikei, and Ken-ichi Yamamoto

Many recent studies have provided evidence for a role for Nbs1 as a damage sensor and activator acting upstream of ATM in cellular response to DSB. There is also evidence for a direct physical interaction between ATM and Nbs1. In addition, both of Nbs1 and ATM are involved in DSB repair by the homologous recombinational DNA repair system. We found that Chk1 phosphorylation (Ser-345) and FancD2 ubiquitination induced by various DNA replication-stalling agents, such as cisplatin, pierisin, UV and hydroxy urea, were severely diminished in Nbs1-/-/- DT40 cells. However, Chk1 phosphorylation and FancD2 ubiquitination induced by these agents were not significantly compromised in conditional Mre11-knockout cells. Furthermore we found that Nbs1 interacts with TopBP1 and ATR in 293T cells. These results indicate that Nbs1 but not the Mre11/Rad50/Nbs1 complex plays unique role in ATR-mediated Chk1 phosphorylation and FancD2 ubiquitination. This functional relationship between Nbs1 and ATR may explain the embryonic lethality of *NBS1* knockout in mice, which is distinct from the non-essential feature of ATM in mice and human. Furthermore, other clinical features of NBS (Nijmegen breakage syndrome caused by a hypomorphism in *NBS1*), such as microcephaly, developmental delay, and characteristic facial features, which are also seen in ATR-Seckel patients, are not seen in A-T patients, who display progressive cerebellar ataxia. These shared clinical features in ATR-Seckel and NBS patients further support the functional relationship between Nbs1 and ATR.

Direct ATM activation by toxic metabolites

Masahiko Kobayashi, Hiroto Ono, Keiko Mihara, and Ken-ichi Yamamoto

ATM (ataxia-telangiectasia mutated) is essential for cellular response to double strand breaks in vertebrate cells. However, ATM is activated by a variety of noxious agent, including oxidative stress, and ATM deficiency results in an anomalous cellular response to oxidative stress. While this defective response to oxidative stress may underlie the pathogenesis of cerebellar ataxia, premature aging and cancer predisposition in ataxia-telangiectasia, mechanisms for ATM activation by oxidative stress remain to be established. Furthermore, it is not clear whether ATM responds to oxidative DNA damage or to a change in the intracellular redox state, independent of DNA damage. To address these questions, we studied ATM activation by protein sulfhydril (SH)-group-modifying agents, N-methyl-N'-nitro-nitrosoguanidine (MNNG) and 15-deoxy- Δ12,14-prostaglandin J_2 (15d-PGJ_2). 15d-PGJ_2 is a reactive cyclopentenone-type prostaglandin D_2 metabolite generated during inflammatory processes, and is a potent inducer of intracellular oxidative stress and directly modulates the activity of several biologically important molecules through the specific alkylation of free sulfhydryl (SH) groups on cysteine residues of the target proteins. An alkylating agent, MNNG, is another potential oxidative stress inducer that reacts with free SH groups, although MNNG is well known to methylate DNA, and effectively induces checkpoint activation through the activation of both ATM and ATR with the subsequent activation of Chk1 and Chk2. We found that MNNG and 15d-PGJ_2 effectively activate ATM in *NBS1*- or *MSH6*- deficient chicken DT40 cells. We further found that ATM is also activated by treating chromatin-free immunoprecipitated ATM with MNNG or 15d-PGJ_2, and that 15d-PGJ_2 binds covalently to ATM. Interestingly, 15d-PGJ_2-induced ATM activation leads to p53 activation and apoptosis, but not to Chk2 or H2AX phosphorylation. These results indicate that ATM is activated through direct modification by free SH-group-modifying reagents independently of DNA damage, resulting in apoptotic downstream response. However, it remains to be established how ATM regulates intracellular oxidative stress and how ATM abnormality leads to various defective manifestations in oxidative stress response. For such analysis, 15d-PGJ_2 might be a useful agent, since ATM activation by 15d-PGJ_2 does not result in the activation of DNA damage-linked Chk1 and Chk2 phosphorylation.

c-ABL tyrosine kinase stabilizes RAD51 chromatin association

Hiroko Shimizu and Ken-ichi Yamamoto

HRR is a major pathway for the resolution of DNA double-strand breaks (DSBs) in the somatic cells of higher eukaryotes. HRR is mediated by RAD51, the eukaryal orthologue of bacterial RecA. A key step in HRR involves the assembly of RAD51 onto DNA substrates at the site of DNA breakage to form an ordered, helical nucleoprotein filament, which catalyzes homologous pairing and the strand exchange reaction. In vertebrate cells of avian, rodent or human origin, RAD51 assembly is marked in cells by the formation of nuclear foci containing RAD51. An important unresolved question concerns the nature of the signaling process that triggers RAD51 assembly at sites of DNA breakage. A c-ABL, ubiquitously expressed non-receptor-type tyrosine kinase, is activated by ionizing radiation (IR) in an ATM-dependent manner and plays important roles in growth arrest and cell death. The results of previous studies indicate that c-ABL is involved in HRR through the phosphorylation of RAD51. We provide evidence that c-ABL, a tyrosine kinase activated by DNA damage which phosphorylates RAD51 on Tyr-315, works at a previously unrecognized, proximal step to initiate RAD51 assembly. We first show that c-ABL associates with chromatin after DNA damage in a manner dependent on its kinase activity. Using RAD51 mutants that are unable to oligomerize to form a nucleoprotein filament, we separate RAD51 assembly on DNA to form foci into two steps: stable chromatin association followed by oligomerization. We show that phosphorylation on Tyr-315 by c-ABL is required for chromatin association of oligomerization-defective RAD51 mutants, but is insufficient to restore oligomerization. Our findings suggest a new model for the regulation of early steps of HRR. We also found that imatinib, a specific inhibitor for the c-ABL tyrosine kinase, effectively inhibited c-ABL-mediated enhancement of RAD51 chromatin association, raising a possibility that the inhibition of RAD51 functions is one of mechanisms by which imatinib exerts its anti-cancer activity as a molecular targeting drug. Our work thus provides useful information for future clinical application of imatinib and related compounds as direct and/or adjuvant therapeutic drugs for haematopoietic malignancies and solid d tumors.

Cell cycle and chromatin control by nuclear small G protein Ran

Naoyuki Hayashi and Ken-ichi Yamamoto

The Ran GTPase system regulates the direction and timing of several cellular events, such as nuclear-cytosolic transport, centrosome formation, and nuclear envelope assembly in telophase. Most nuclear Ran localizes to chromatin, by both RCC1-dependent and independent mechanisms. However, Ran's functions on chromatin and for cell cycle are poorly understood. To gain insight into the Ran system's involvement in chromatin formation, we investigated gene silencing at the telomere in several mutants of the budding yeast, which had defects in genes involved in the Ran system. A temperature-sensitive (Ts) mutation of the RanGAP gene, *rna1-1*, caused reduced silencing at the telomere, assessed by observing the expression of a *URA3* marker inserted at the telomere, and Ts mutations of yeast Ran homologue, *gsp1-1268* and *gsp1-1757*, increased this silencing. In the *rna1-1* mutant, hyperphosphorylated Sir3 protein accumulated. This reduced telomere silencing in *rna1-1* cells was suppressed by a high dosage of the *SIR3* gene. These results suggest that active Ran GTPase opens the heterochromatin structure at the telomere in yeast.
In cell cycle studies using *gsp1* mutants, we found suppression to the hydroxyurea (HU) and ultra violet (UV) sensitivities of the *mec1* mutant, which lacks one of the two ATM family kinases. In UV-irradiated *mec1 gsp1* cells, Rad53 was phospholyrated despite the lack of Mec1. This suppression of the *mec1* phenotype depended on the *TEL1* gene, which encodes the other of the ATM family kinases, given that the triple mutant, *mec1 gsp1 tel1*, was unable to grow. These results indicated that Rad53 was activated by Tel1 in *mec1 gsp1* cells, suggesting that Gsp1 helps regulate the role switching of the ATM family kinases Mec1 and Tel1.

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Division of Molecular Cell Signaling

General Summary of Division of Molecular Cell Signaling

Scaffold proteins of the mammalian MAP kinase (MAPK) cascades are considered having critical roles in spatio-temporal regulation of MAPK pathways by organizing their signaling components into functional modules. We are particularly interested in the functions of these scaffold proteins, mainly c-Jun NH_2-terminal kinase (JNK)/stress-activated protein kinase-associated protein 1 (JSAP1) and its family member JNK-associated leucine zipper protein (JLP); scaffold proteins that participate in JNK MAPK cascades, both *in vitro* and *in vivo*. Our findings in the past three years are summarized as follows:

A) Role of JSAP1-JNK signaling in cerebellar development

During the development of the cerebellum, massive clonal expansion of granule cell precursors (GCPs) occurs in the outer part of the external granular layer (EGL). We have provided evidence that JSAP1 and active JNK were expressed preferentially in the post-mitotic inner EGL progenitors in the developing cerebellum. Moreover, *Jsap1* deficiency resulted in increasing numbers of proliferating GCPs in mouse embryos. Besides, overexpression of JSAP1 in cultured GCPs led to increased numbers of NeuN-positive cells together with the activation of JNK. Together, these data strongly indicated that JSAP1 promotes the cell-cycle exit and differentiation of GCPs by modulating JNK activity in cerebellar development.

B) Expression of JSAP1 and JNK in mouse brain

We studied JSAP1 and JNK expression in developing and adult mouse brains. Our results obtained by *in situ* hybridization and immunohistochemical analyses strongly suggested that JSAP1-JNK signaling plays important roles in developing and adult mouse brains.

C) Function of JLP in the regulation of cell migration

We investigated the function of JLP by deleting it in cultured cells. Our results strongly suggested that JLP regulates cell migration through an interaction with $G_{\alpha 13}$.

D) Function of JLP in mice

We examined the expression of JLP in various mouse tissues, and found that JLP was strongest in the testis. We also investigated the function of JLP by disrupting the *Jlp* gene in mice, and found that the male homozygotes were subfertile. Taken together, these observations may suggest that JLP plays an important role in testis during development.

The scaffold protein JSAP1 regulates proliferation of cerebellar granule cell precursors by modulation JNK signaling

T. Sato, T. Torashima, K. Sugihara, H. Hirai, M. Asano and K. Yoshioka

Cerebellar granule cell precursors (GCPs) proliferate in the outer part of the external granular layer (EGL). They begin their differentiation by exiting the cell cycle and migrating into the inner part of the EGL. Scaffold proteins for mitogen-activated protein kinase (MAPK) pathway are though to function in the spatial and temporal regulation of these pathways by organizing the MAPK signaling components into functional modules. Our group previously identified JNK/stress-activated protein kinase-associated protein 1 (JSAP1, also known as JNK-interacting protein 3 (JIP3)) as a scaffold protein for mammalian JNK MAPK pathway. Here we report that JSAP1, a scaffold protein for JNK signaling pathway, is expressed predominantly in the post-mitotic GCPs of the inner EGL. JSAP1 knockdown or treatment with a JNK inhibitor enhances proliferation of cultured GCPs, but the overexpression of wild-type JSAP1 leads to increased proportions of $p27^{Kip1}$- and NeuN-positive cells, even with saturating concentration of Sonic hedgehog (Shh), a potent GCP mitogen. However, these differentiation-promoting effects on GCPs are attenuated significantly in cells overexpressing a mutant JSAP1 that lacks the JNK-binding domain. Together, these data suggest that JSAP1 antagonizes the mitogenic effect of Shh on GCPs and promotes their exit from the cell cycle and differentiation, by modulating JNK activity.

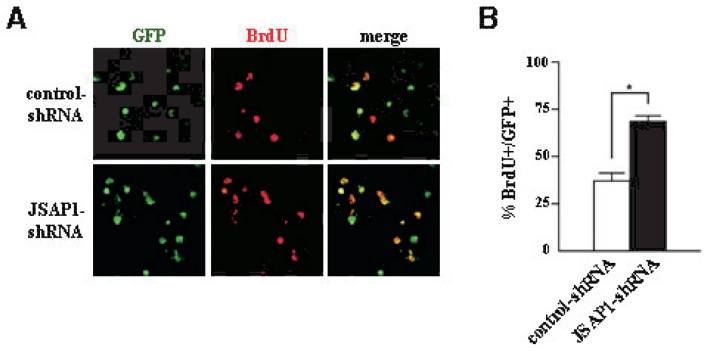

Figure Knockdown of JSAP1 expression enhances the proliferation of cultured GCPs. (A) Double Immunofluorescence of P4 cultured GCPs infected with lentiviruses expressing GFP plus either control-shRNA or JSAP1-shRNA, 72 h after infection. They were stained with antibodies to GFP (green) and BrdU (red). (B) The percentage GFP-positive cells that were BrdU-positive in cultures infected with the control lentivirus or lentivirus containing the JSAP1-shRNA (mean + SEM from 3 experiments, $*p < 0.005$, Student's *t*-test).

Reference:

T. Sato, T. Torashima, K. Sugihara, H. Hirai, M. Asano and K. Yoshioka (2008) Mol. Cell. Neurosci. 39:569-578.

Neural-specific ablation of the scaffold protein JSAP1 in mice causes neonatal death

A. Iwanaga, T. Sato, K. Sugihara, A. Hirao, N. Takakura, H. Okamoto, M. Asano and K. Yoshioka

We previously identified c-Jun NH(2)-terminal kinase (JNK)/stress-activated protein kinase-associated protein 1 (JSAP1, also known as JNK-interacting protein 3) as a scaffolding factor for JNK intracellular signaling pathways. Targeted gene-disruption studies have shown that JSAP1-null mice are unable to breathe and die shortly after birth. Although neural defects might be responsible for their death, there has been no convincing evidence for this. Here we first generated genetically engineered mice carrying a loxP-flanked (floxed) *Jsap1* gene. To evaluate the validity of this deletion as a *Jsap1* conditional knockout (KO), we created mice in which the same exon was deleted in all cell lineages, and compared their phenotypes with those of the *Jsap1* conventional KO mice reported previously. The two KO lines showed indistinguishable phenotypes, i.e., neonatal death and morphological defects in the telencephalon, indicating that the conditional deletion was a true null mutation. We then introduced the floxed *Jsap1* deletion mutant specifically into the neural lineage, and found that the *Jsap1* conditional KO mice showed essentially the same phenotypes as the JSAP1-null mice. These results strongly suggest that the neonatal death of *Jsap1*-deficient mice is caused by defects in the nervous system.

Table Genotype analysis of littermates from crosses of $Jsap1^{flox/flox}$ and $Jsap1^{flox/+}$;*nes-cre* mice

Stage	No. of animals with genotype:				
	Total	*flox/flox*	*flox/+*	*flox/flox;nes-cre*	*flox/+;nes-cre*
E18.5	122	35	28	28	31
P0	56	15	19	9	13
P1	52	15	21	2	14
P2	31	9	7	0	15
Weaned	88	32	26	0	30

Reference: A. Iwanaga, T. Sato. K. Sugihara, A. Hirao, N. Takakura, H. Okamoto, M. Asano and K. Yoshioka (2007) Neurosci. Lett. 429:43-48.

The scaffold protein c-Jun NH_2-terminal kinase-associated leucine zipper protein regulates cell migration through interaction with the G protein $G_{\alpha 13}$

D. Gantulga, B. Tuvshintugs, Y. Endo, T. Takino, H. Sato, S. Murakami and K. Yoshioka

Scaffold proteins for MAP kinase (MAPK) signalling modules play an important role in the specific and efficient signal transduction of the relevant MAPK cascades. Here, we investigated the function of the scaffolding protein c-Jun NH(2)-terminal kinase (JNK)-associated leucine zipper protein (JLP) by depleting it in cultured cells using a short hairpin RNA (shRNA) against human JLP. HeLa and DLD-1 cells stably expressing the shRNA showed a defect in cell migration. The re-expression of full-length shRNA-resistant mouse JLP rescued the impaired cell migration of the JLP-depleted HeLa cells; whereas, a C-terminal deletion mutant of mouse JLP, which failed to bind the G protein $G_{\alpha 13}$, showed little or no effect on the cell migration defect. Furthermore, although a constitutively active $G_{\alpha 13}$ enhanced the migration of control HeLa cells, the $G_{\alpha 13}$-induced cell migration was significantly suppressed in the JLP-depleted HeLa cells. Taken together, these results suggest that JLP regulates cell migration through an interaction with $G_{\alpha 13}$.

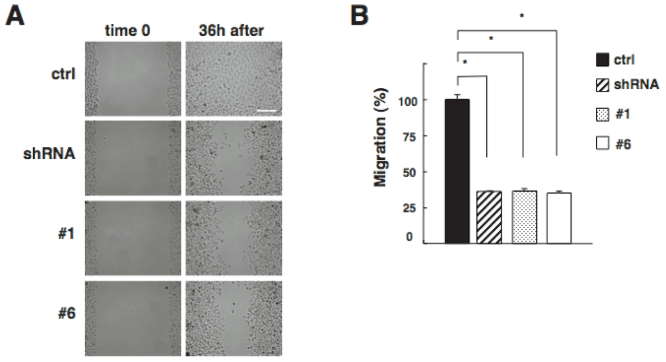

Figure Impairment of cell migration in JLP KD HeLa cells. (A) The control (ctrl) HeLa cells, the pooled JLP KD HeLa cells, and clones #1 and #6 stably expressing JLP-shRNA1 were replated onto collagen-coated dishes at a saturation density. After being scratched, the wounded monolayers were allowed to heal for 36 h. Scale bar, 200 μm. Experiments were performed independently three times, and representative results are shown. (B) The cell migration activity of these cell lines was quantified by a Transwell assay. Values represent the number of migrated pooled KD, clone #1, and clone #6 cells as a percentage of the number of control cells (means $\pm$ SD from three independent experiments, $*p < 0.01$, Student's *t*-test).

Reference: D. Gantulga, B. Tuvshintugs, Y. Endo, T. Takino, H. Sato, S. Murakami and K. Yoshioka (2008) J. Biochem. 144:693-700.

Ablation of the scaffold protein JLP causes reduced fertility in male mice

A. Iwanaga, G. Wang, D. Gantulga, T. Sato, T. Baljinnyam, K. Shimizu, K. Takumi, M. Hayashi, T. Akashi, H. Fuse, K. Sugihara, M. Asano and K. Yoshioka

The specific and efficient activation of mitogen-activated protein kinase (MAPK) signaling modules is mediated, at least in part, by scaffold proteins. c-Jun NH_2-terminal kinase (JNK)-associated leucine zipper protein (JLP) was identified as a scaffold protein for JNK and p38 MAPK signaling modules. JLP is expressed nearly ubiquitously and is involved in intracellular signaling pathways, such as the $G_{\alpha 13}$ and Cdo-mediated pathway, in vitro. To date, however, JLP expression has not been analyzed in detail, nor are its physiological functions well understood. Here we investigated the expression of JLP in the mouse testis during development. Of the tissues examined, JLP was strongest in the testis, with the most intense staining in the elongated spermatids. Since the anti-JLP antibody used in this study can recognize both JLP and sperm-associated antigen 9 (SPAG9), a splice variant of JLP that has been studied extensively in primates, we also examined its expression in macaque testis samples. Our results indicated that in mouse and primate testis, the isoform expressed at the highest level was JLP, not SPAG9. We also investigated the function of JLP by disrupting the *Jlp* gene in mice, and found that the male homozygotes were subfertile. Taken together, these observations may suggest that JLP plays an important role in testis during development, especially in the production of functionally normal spermatozoa.

Table Fertility of *Jlp*-deficient mice

Jlp genotype		No.	No.	Mean litter
Male	Female	crossings	pregnancies (%)	sizes (±SD)
+/-	+/+, +/-	26	25 (96.2)	8.5 (±2.1)
-/-	+/+, +/-	41	10 (24.4)*	6.4 (±1.6)
+/+, +/-	-/-	12	10 (83.3)	6.9 (±1.4)

Mean litter size was calculated as the number of live pups born per number of litters. *$p < 0.01$, chi-square test.

Reference: A. Iwanaga et al. Transgenic Res. (2008) 17:1045-1058.

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Division of Molecular Virology and Oncology

General Summary of Division of Molecular Virology and Oncology

Recent studies have demonstrated that members of the matrix metalloproteinase (MMP) gene family play a central role in the degradation of extra cellular matrix (ECM) macromolecules under various physiological and pathological conditions. Degradation of ECM is one of the first steps for tumor invasion and metastasis, and MMP have been strongly implicated in this step. Membrane type-1 MMP (MT1-MMP, MMP-14) was identified as the first physiological activator of latent MMP-2 (proMMP-2). The role of MT1-MMP in pericellular proteolysis is not restricted to proMMP-2 activation as MT1-MMP is a functional enzyme that can also degrade a number of ECM components and hence can play a direct role in ECM turnover. Accumulating evidences have demonstrated that MT1-MMP expression level is closely associated with invasiveness and malignancy of tumors, suggesting that MT1-MMP is one of the most critical factors for tumor invasion and metastasis. Thus, we believe that MT1-MMP could be a molecular target for diagnosis and therapy of malignant tumors.

A) Pro-MMP-2 Activation by MT1-MMP:

The suggested model for pro-matrix metalloproteinase-2 (proMMP-2) activation by MT1-MMP implicates the complex between MT1-MMP and tissue inhibitor of MMP (TIMP)-2 as a receptor for proMMP-2. Due to the complicated mechanism for pro-MMP-2 activation, physiological significance of pro-MMP-2 activation remained unclear. We created an artificial receptor for pro-MMP-2, which enabled us to examine the mechanism and physiological significance of pro-MMP-2 activation by MT1-MMP.

B) Substrate Specificity of MT1-MMP:

While degradation of ECM is an important aspect of MMP biology, growing evidence has demonstrated specific processing/activation or degradation of cell surface receptors and ligands by MT1-MMP. We have been developing expression cloning strategy to identify MT1-MMP substrates, which were not only ECM components but also cell-surface receptors and ligands.

C) Regulation of Cell Migration by MT1-MMP:

Although MMPs were known to be involved in cell migration, the molecular mechanism had remained elucidated. We demonstrated that degradation of ECM by cell-surface MT1-MMP induces a rapid turn-over of focal adhesion, which stimulates a sustained signal through the MEK/ERK pathways. This sustained ERK activation in turn stimulates cell migration on ECM.

Membrane-type 1 matrix metalloproteinase modulates focal adhesion stability and cell migration.

T. Takinoa, Y. Watanabea, M. Matsuia, H. Miyamoria, T. Kudoa, M. Seiki*, H.Sato (*Dept. Cancer Cell Research, Inst. of Medical Science, University of Tokyo)

Membrane-type 1 matrix metalloproteinase (MT1-MMP) plays an important role in extracellular matrix-induced cell migration and the activation of extracellular signalregulated kinase (ERK). We showed here that transfection of the MT1-MMP gene into HeLa cells promoted fibronectin-induced cell migration, which was accompanied by fibronectin degradation and reduction of stable focal adhesions, which function as anchors for actinstress fibers. MT1-MMP expression attenuated integrin clustering that was induced by adhesion of cells to fibronectin. The attenuation of integrin clustering was abrogated by MT1-MMP inhibition with a synthetic MMP inhibitor, BB94. When cultured on fibronectin, HT1080 cells, which endogenously express MT1-MMP, showed so-called motile morphology with well-organized focal adhesion formation, well-oriented actin-stress fiber formation, and the lysis of fibronectin through trails of cell migration. Inhibition of endogenous MT1-MMP by BB94 treatment or expression of the MT1-MMP carboxyl-terminal domain, which negatively regulates MT1-MMP activity, resulted in the suppression of fibronectin lysis and cell migration. BB94 treatment promoted stable focal adhesion formation concomitant with enhanced phosphorylation of tyrosine 397 of focal adhesion kinase (FAK) and reduced ERK activation. These results suggest that lysis of the extracellular matrix by MT1-MMP promotes focal adhesion turnover and subsequent ERK activation, which in turn stimulates cell migration.

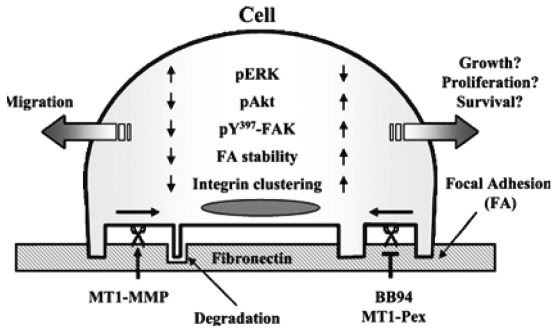

Fig. 1 Model depicting the regulation of cell migration by MT1-MMP. Proteolytic degradation of ECM by MT1-MMP reduces integrin clustering, focal adhesion stability, FAK phosphorylation at Tyr-397 and Akt activation, and induces ERK activation, resulting in the promotion of cell migration. In contrast, MT1-MMP inhibition by BB94 or by expression of the MT1-MMP hemopexin-domain induces FAK phosphorylation and Akt activation, and attenuates ERK activation. Controlled ECM degradation by MT1-MMP seems to play an important role in the regulation of focal adhesion breakdown, which is required for cell migration, invasion, growth, proliferation, and apoptosis.

Reference: T. Takino et al., Exp. Cell Res., 312, 1381-1389 (2006).

Cleavage of Amyloid-β Precursor Protein (APP) by Membrane-Type Matrix Metalloproteinases.

M. Ahmad, T. Takino, H. Miyamori, T. Yoshizaki*, M. Furukawa* and H. Sato (*Dept. Otolaryngology, Med. Sch., Kanazawa Univ.)

Amyloid-β precursor protein (APP) was identified by the expression cloning from a human placenta cDNA library as a gene product which modulates the activity of membrane-type matrix metalloproteinase-1 (MT1-MMP). Co-expression of MT1-MMP with APP in HEK293T cells induced cleavage and shedding of APP ectodomain when co-expressed with APP adaptor protein Fe65. Among MT-MMPs tested, MT3-MMP and MT5-MMP also caused efficient APP shedding. Recombinant APP protein was cleaved by MT3-MMP *in vitro* at the A^{463}-M^{464}, N^{579}-M^{580}, H^{622}-S^{623} and H^{685}-Q^{686} peptide bonds, which included a cleavage site within the amyloid β peptide region known to produce a C-terminal fragment. The Swedish-type mutant of APP, which produces a high level of amyloid β peptide, was more effectively cleaved by MT3-MMP than wild-type APP either in the presence or absence of Fe65; however, amyloid β peptide production was not affected by MT3-MMP expression. Expression of MT3-MMP enhanced Fe65-dependent transactivation by APP fused to the Gal4 DNA-binding and transactivation domains. These results suggest that MT1-MMP, MT3-MMP and MT5-MMP should play an important role in regulation of APP functions in tissues including central nervous system.

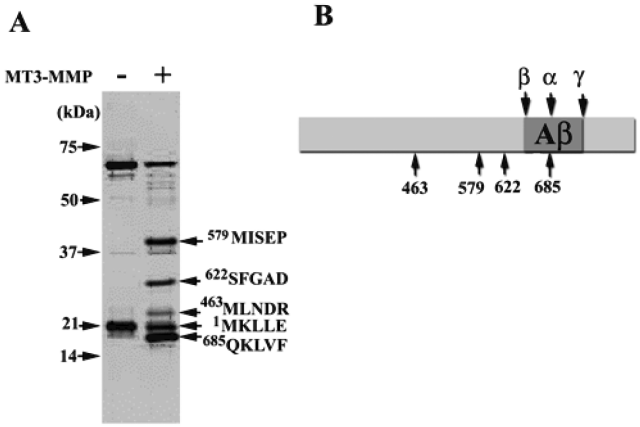

Fig. 1 (A) Recombinant APP protein (5 μg) was incubated with or without recombinant MT3-MMP catalytic domain, separated by 12% SDS-PAGE and stained with Coomassie Brilliant Blue. The N-terminal amino acid sequence of each fragment was determined. (B) MT3-MMP cleavage sites are marked on full length APP in relation to the cleavage sites for α-, β and γ-secretases.

Reference: M. Ahmad et al. J. Biochem., 139, 517-526 (2006).

Substrate choice of membrane-type 1 matrix metalloproteinase is dictated by tissue inhibitor of metalloproteinase-2 levels.

T. Kudo, T. Takino, H. Miyamori, E.W. Thompson* and H.Sato (*Department of Surgery, University of Melbourne)

Although tissue inhibitor of metalloproteinase-2 (TIMP-2) is known to be not only an inhibitor of matrix metalloproteinases (MMPs) but also a co-factor for membrane-type 1 MMP (MT1-MMP)-mediated MMP-2 activation, it is still unclear how TIMP-2 regulates MMP-2 activation and cleavage of substrates by MT1-MMP. In this study we examined the levels of cell-surface MT1-MMP, MMP-2 activation, and cleavage of MT1-MMP substrates in 293T cells transfected with MT1-MMP and TIMP-2 genes. Co-expression of TIMP-2 at an appropriate level increased the level of cell-surface MT1-MMP, both the TIMP-2-bound and free forms, and generated processed MMP-2 with gelatin-degrading activity. In contrast, MT1-MMP substrates testican-1 and syndecan-1 were cleaved by the cells expressing MT1-MMP, which was inhibited by TIMP-2 even at the levels which stimulate MMP-2 activation. These results suggest that TIMP-2 environment determines MT1-MMP substrate choice between direct cleavage of its own substrates and MMP-2 activation.

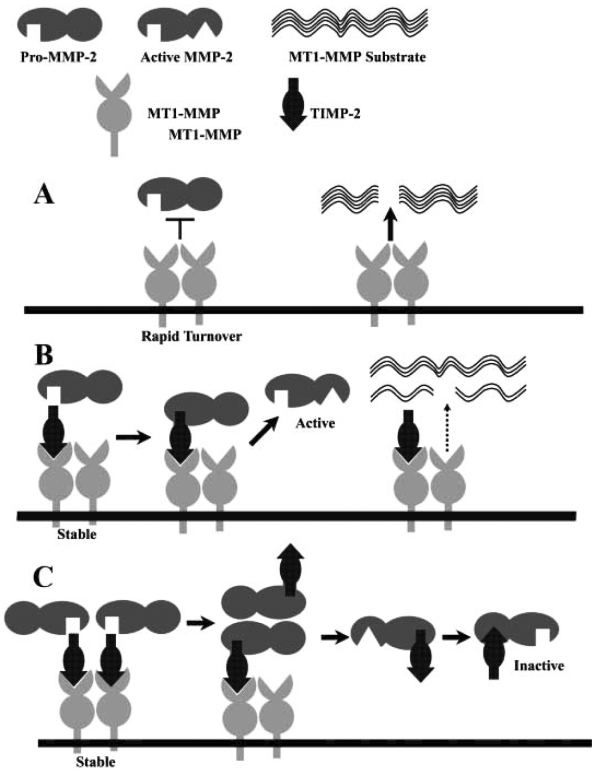

Fig. 1 (A) At low or no TIMP-2, TIMP-2-free MT1-MMP digests its own substrates such as testican-1 but it does not activate pro-MMP-2. TIMP-2-free MT1-MMP shows the most active proteolytic activity, which in turn causes rapid turnover due to intensive auto-degradation. (B) At appropriate levels of TIMP-2, binding of TIMP-2 protects MT1-MMP from auto-degradation, and the complex serves as a receptor for pro-MMP-2, which is then cleaved by adjacent TIMP-2-free MT1-MMP to generate active MMP-2. (C) When MT1-MMP is saturated with TIMP-2, pro-MMP-2 may transiently replace TIMP-2 to generate TIMP-2-free MT1-MMP, which then cleaves pro-MMP-2, but activated MMP-2 is finally blocked by TIMP-2.

Reference: T. Kudo et al. Cancer Sci., 98, 563-568 (2007).

Cleavage of growth differentiation factor 15 (GDF15) by membrane type 1-matrix metalloproteinase abrogates GDF15-mediated suppression of tumor cell growth.

S.H. Abd El-Aziz, Y. Endo, H. Miyamori, T. Takino and H. Sato

Growth Differentiation Factor 15 (GDF15), a TGF- β superfamily member, has been cloned from placenta cDNA library as a gene product that promoted activation of pro-MMP2 mediated by MT1-MMP. Expression of MT1-MMP in HEK293T cells caused cleavage of GDF15 mature form at N^{252}-M^{253} to produce 6-kD C-terminal fragment. Treatment of MCF7 cells with GDF15 induced activation of p53 and enhanced expression of p21, which was abrogated by MT1-MMP expression. Treatment of MCF7 cells with GDF15 caused suppression of cell proliferation. However, proliferation of MCF7 cells trasnfected with MT1-MMP gene was not affected by GDF15 treatment, but was suppressed in the presence of MMP inhibitor BB94. HT1080 cells transfected with GDF15 gene, which endogenously express MT1-MMP, synthesize a high level GDF15 precursor form and a low level mature form, and treatment of cells with BB94 enhanced production of GDF15 mature form. In consistent with GDF15 production, HT1080 cells transfected with GDF15 gene proliferated almost equally with control cells, and addition of BB94 effectively suppressed growth of HT1080 cells transfected with GDF15 gene but not control cells. These results suggest that MT1-MMP contributes to tumor cell proliferation through the cleavage of GDF15 which down-regulates cell proliferation by inducing activation of p53 and p21 synthesis.

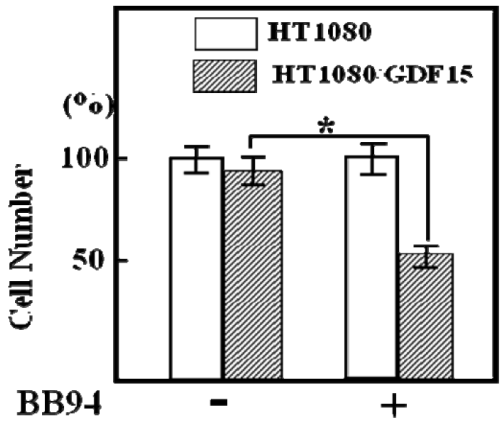

Fig. 1. HT1080 cells stably transfected with control plasmid or GDF15 plasmid were cultured in the presence or absence of BB94 for 24 h, and the cell number was compared. *, P<0.001. Note that inhibition of endogenous MT1-MMP by BB94 induced accumulation of GDF15, which in turn caused growth suppression.

Reference: S.H. Abd El-Aziz et al., Cancer Sci., 98, 1330-1335 (2007).

Inhibition of Membrane-Type 1 Matrix Metalloproteinase at Cell-Matrix Adhesions

T. Takino, H. Saeki, H. Miyamori, T. Kudo and Hiroshi Sato

Membrane-type 1 matrix metalloproteinase (MT1-MMP) has been implicated in tumor invasion and metastasis. We previously reported that extracellular matrix degradation by MT1-MMP regulates cell migration via modulating sustained integrin-mediated signals. In this study, MT1-MMP－expressing cells were plated onto fibronectin-coated plates and monitored for cell-matrix adhesion formation and fibronectin degradation. The fibronectin was degraded and removed in line with the cell migration track. The migrating cells showed a polarized morphology and were in contact with the edge of fibronectin through the leading edge, in which cell-matrix adhesions are concentrated. Expression of MT1-MMP targeted to cell-matrix adhesions by fusing with the focal adhesion targeting (FAT) domain of focal adhesion kinase (FAK) promoted the initial fibronectin lysis at the cell periphery immediately after adhesion. These results suggest that fibronectin is degraded by MT1-MMP located at cell-matrix adhesions, which are concentrated at the leading edge of the migrating cells. To inhibit MT1-MMP at cell-matrix adhesion, the dominant negative form of MT1-MMP (MT1-Pex) was targeted to the cell-matrix adhesion by fusing with the FAT domain (MT1-Pex-FAT). MT1-Pex-FAT accumulated at cellmatrix adhesions and inhibited fibronectin degradation as well as FAK phosphorylation more effectively than parental MT1-Pex. MT1-Pex-FAT was also shown to suppress the invasion of tumor cells into three-dimensional collagen gel more strongly than MT1-Pex. These results suggest that MT1-MMP－mediated extracellular matrix lysis at cell-matrix adhesions induces the establishment of cell polarity, which facilitates cell-matrix adhesion turnover and subsequent cell migration. This model highlights the role of MT1-MMP at the leading edge of migrating cells.

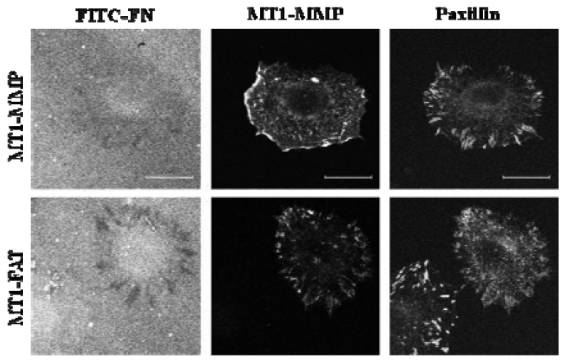

Fig. 1. HeLa cells were transfected with either MT1-MMP or MT1-FAT and seeded on FITC-fibronectin－coated coverslips for 1 h. Cells were fixed and stained for cell-surfaceMT1-MMP (MT1) and anti-paxillin antibody (Paxillin).
Fibronectin lysis was detected as the decrease of FITC-fibronectin.

Reference: T. Takino et al., Cancer Res., 67, 11621-11629 (2007).

Activation of Matrix Metalloproteinase (MMP)-2 By Membrane-type 1-MMP Through An Artificial Receptor For ProMMP-2 Generates Active MMP-2.

Y. Nishida, H. Miyamori, E.W. Thomposon*, T. Takino and H. Sato (*Department of Surgery, University of Melbourne)

The suggested model for pro-matrix metalloproteinase-2 (proMMP-2) activation by membrane-type 1-matrix MMP (MT1-MMP) implicates the complex between MT1-MMP and tissue inhibitor of MMP (TIMP)-2 as a receptor for proMMP-2. To dissect this model and assess the pathological significance of MMP-2 activation, an artificial receptor for proMMP-2 was created by replacing the signal sequence of TIMP-2 with cytoplasmic/transmembrane domain of type II transmembrane mosaic serine protease (MSP-T2). Unlike TIMP-2, MSP-T2 served as a receptor for proMMP-2 without inhibiting MT1-MMP, and generated TIMP-2-free active MMP-2 even at a low level MT1-MMP. Thus, MSP-T2 did not affect direct cleavage of a substrate testican-1 by MT1-MMP, whereas TIMP-2 inhibited it even at the level which stimulated proMMP-2 processing. Expression of MSP-T2 in HT1080 cells enhanced MMP-2 activation by endogenous MT1-MMP, and caused intensive hydrolysis of collagen gel. Expression of MSP-T2 in U87 glioma cells, which express a trace level of endogenous MT1-MMP induced MMP-2 activation, and enhanced cell-associated protease activity, activation of extra-cellular signal-regulated kinase and metastatic ability into chick embryonic liver and lung. MT1-MMP can exert both maximum MMP-2 activation and direct cleavage of substrates with MSP-T2, which cannot be achieved with TIMP-2. These results suggest that MMP-2 activation by MT1-MMP potentially amplifies protease activity, and combination with direct cleavage of substrate causes effective tissue degradation and enhances tumor invasion and metastasis, which highlights the complex role of TIMP-2. MSP-T2 is a unique tool to analyze physiological and pathological roles of MMP-2 and MT1-MMP in comparison with TIMP-2.

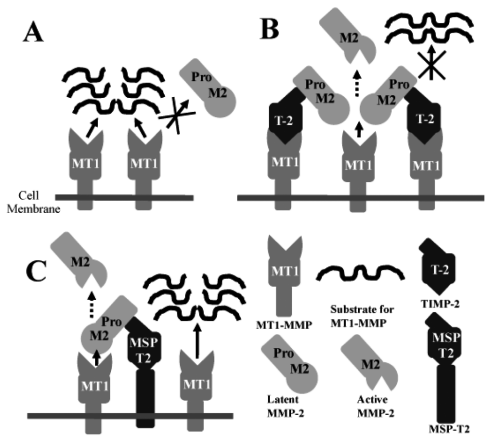

Fig. 1. A, MT1-MMP directly cleaves its substrate. B, in TIMP-2-dependent proMMP-2 activation, the majority of MT1-MMP functions as a receptor for proMMP-2. C, MSP-T2 serves as a receptor for proMMP-2 without inhibiting MT1-MMP, and thus MT1-MMP can perform both proMMP-2 activation and direct cleavage of substrate.
Y. Nishida et al., Cancer Res., 68, 9096-9104 (2008).

DEVELOPMENT OF AN ANTITUMOR ADENOSINE ANALOG, 3'-ETHYNYLADENOSINE

Y. Endo, T. Obata*, T. Sasaki* (*Lab. Bio-organic Chem, Sch. Pharm., Aichi Gakuin Univ.)

Several 2'-deoxyadenosine analogs such as cladribine, fludarabine, and clofarabine are used in the treatment of lymphoid malignancies. These nucleosides are metabolically activated by phosphorylation enzymes including deoxycytidine kinase (dCK), thereby inhibiting DNA polymerases and ribonucleotide reductase, while also showing resistance to adenosine deaminase (ADA). With the aim of developing more potent antitumor nucleosides than 2'-deoxyadenosine analogs reported, 3'-ethynyladenosine (EAdo) was designed as an RNA synthesis inhibitor.[1] We have studied the antitumor mechanisms of EAdo using human solid tumor cell lines. In culture, IC_{50} values of EAdo ranged from 0.05 μM to 2 μM. EAdo also effectively inhibited RNA biosynthesis in tumor cells. The cytotoxic activity of EAdo was markedly reduced by simultaneous treatment with the adenosine kinase (AK) inhibitor ABT-702. Furthermore, EAdo-resistant cells newly established from human gastric cancer cell line (NUGC-3) showed extremely decreased AK activity and expressed aberrant AK mRNA with deletion of 151 bp corresponding Exon 8 and Exon 9. These results indicate that cellular EAdo is metabolically phosphorylated by AK and that the AK gene becomes a molecular target for acquired resistance. On the other hand, we found that EAdo as well as adenosine and its analogs was enzymatically converted to inosine form (EIno) and rapidly inactivated by adenosine deaminase. As a result, the intraperitoneal administration of EAdo was able to prolong the survival time of mice bearing P388 leukemia, but was ineffective in mice bearing sarcoma-180 solid tumor. Thus, to improve the *in vivo* antitumor efficacy of EAdo we synthesized two EAdo derivatives (2-F-EAdo and EAdo-5'-monophosphate, EAMP). Although 2-F-EAdo was highly resistant to adenosine deaminase, its antitumor activity extremely depended on cellular AK activity because 2-F-EAdo showed the cytotoxic effect on only AK-overexpressing tumor cells. On the contrary, EAMP showed the growth inhibitory effect on sarcoma-180 solid tumor transplanted s.c. in mice. We propose that EAdo may be a potent lead compound to develop novel antitumor purine nucleoside analog which is therapeutically available for insensitive tumors to deoxycytidine and deoxyadenosine analogs preferentially phosphorylated by deoxycytidine kinase.

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Abnormal caudal intervertebral disc formation in ADAMTS-1 null mice

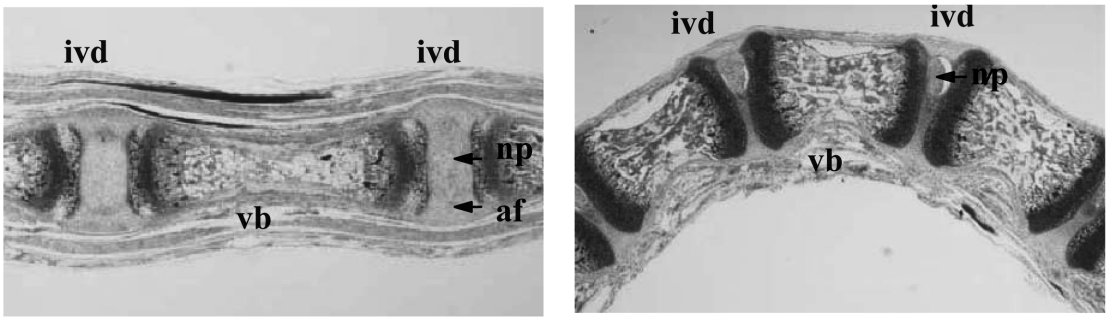
K. Kuno

ADAMTS-1 is a metalloproteinase associated with extracellular matrix that has aggrecan-degrading activity and anti-angiogenic activitiy. We reported previously that ADAMTS-1 null mice display renal anomalies that resemble ureteropelvic junction obstruction. In addition, the ovulatory ability of ADAMTS-1 null mice is significantly impaired. ADAMTS-1 null ovaries also contain a number of unusual follicles without granulosa cell layers. These findings indicated that ADAMTS-1 plays an important role in tissue architecture in the ureteropelvic junction, ovulation, follicular development, and maintenance of the follicle structure in the ovary. In the present study, we conducted a detailed examination of spine morphology in ADAMTS-1 null mice.

We found that about 50% of ADAMTS-1 null mice featured a kinked tail from birth to 3 weeks of age, while this frequency was less than 2 % in heterozygous mice. The tail kinks observed in the ADAMTS-1 null mice were transient and recovered to normal morphology by 4 weeks of age. Histological analysis revealed that the tail kinks in the ADAMTS-1 null mice occurred at the intervertebral disc portion of the caudal vertebra. In control mice, the well-developed nucleus pulposus was located at the center of the caudal intervertebral disc, and the annulus fibrosus symmetrically enclosed the nucleus pulposus. In contrast, the caudal intervertebral disc of ADAMTS-1 null mice has a very small nucleus pulposus that was apart from the center of the caudal intervertebral disc. In addition, ADAMTS-1 protein was strongly expressed in the nucleus pulposus region and was also detected in the annulus fibrosus in the immunohistochemical analysis.

These findings demonstrate that ADAMTS-1, which is produced by nucleus pulposus cells, plays an important role in the regulation of the developmental process of the nucleus pulposus in the caudal intervertebral disc.

ADAMTS-1 +/- **ADAMTS-1 -/-**



ivd: intervertebral disc
vb: vertebral body

np: nucleus pulposus
af: annulus fibrosus

Suppression of collagen-induced arthritis by ADAMTS-1 neutralizing antibodies

K. Kuno, *M. Hakozaki, *K. Hirose

Erosion of cartilage is a major feature of arthritis. Proteolytic cleavage of the cartilage proteoglycan, aggrecan is an important factor in the cartilage destruction seen in joint diseases. Aggrecanases, which belong to the ADAMTS family of proteinases, are responsible for aggrecan degradation. We previously demonstrated that ADAMTS-1 has aggrecan-degrading activity (Kuno K. et al., 2000). In the present study, we examined the effect of neutralizing anti-ADAMTS-1 antibodies on collagen-induced arthritis in mice in an effort to understand the role of ADAMTS-1 in cartilage destruction in arthritis.

To induce collagen-induced arthritis (CIA), DBA/1J mice were immunized intradermally at the base of the tail with 100 μg bovine type II collagen emulsified with an equal volume of complete Freund's adjuvant. Three weeks later, the mice were boosted in the same manner. Antibody administration was started immediately after the first sensitization and was carried out twice a week, for a total of 10 doses.

Unexpectedly, anti-ADAMTS-1 treatment of the CIA mice resulted in a marked reduction in arthritis score, and suppression of paw-swelling. The destruction of cartilage and proliferation of synovium, as well as the infiltration of inflammatory cells, usually observed in the knee joints of CIA mice was markedly decreased by treatment with anti-ADAMTS-1 antibodies as determined by histological analysis (Fig. 1).

We demonstrated that anti-ADAMTS-1 neutralizing antibodies are effective in suppressing both inflammatory symptoms and joint destruction in CIA mice. These results suggest that ADAMTS-1 contributes to the elicitation of inflammation in CIA. Therefore, we consider ADAMTS-1 as a new target for the development of novel therapeutics for inflammatory arthritis.

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Division of Molecular Bioregulation

General Summary of Division of Molecular Bioregulation

Tumor cells and resident cells can produce various chemokines and pro-inflammatory cytokines, during carcinogenesis and metastasis processes. The produced chemokines and pro-inflammatory cytokines can modulate the microenvironment of tumor tissues, thereby affecting carcinogenic and/or metastatic processes. A major research object in our division is to elucidate the roles of these endogenously produced chemokines and pro-inflammatory cytokines in these processes, in order to use these molecules and/or their antagonists for the treatment of cancer.

We originally identified a serine/threonine kinase, Pim-3, as a proto-oncogene expressed selectively in chronic inflammation-mediated hepatoma tissues in mice. Our subsequent studies have unraveled that Pim-3 is enhanced selectively in malignant lesions of endoderm-derived organs and can counteract apoptotic cell death of cancer cells. These observations prompt us to investigate the possibility of the development of an anti-cancer drug by targeting Pim-3.

A) Chemokines and pro-inflammatory cytokines in carcinogenesis and metastasis processes

Tumor cells as well as resident normal cells can produce a wide variety of chemokines and pro-inflammatory cytokines, thereby affecting the course of carcinogenesis and metastasis processes. Until present, we have provided definitive evidence to indicate that tumor necrosis factor receptor p55 has crucial roles in inflammation-mediated colon carcinogenesis, liver metastasis, and lung metastasis. Moreover, we demonstrated that tumor cells and resident normal cells can produce various chemokines and that the produced chemokines have effects on tumor cells as well as inflammatory cells, thereby contributing to carcinogenesis and metastasis processes.

B) Pim-3, a proto-oncogene with serine/threonine kinase activity, in carcinogenesis

Pim-3 is aberrantly expressed in malignant cells and lesions of endoderm-derived organs, including liver, pancreas, colon, and stomach. Pim-3 can inactivate a pro-apoptotic molecule, Bad, by phosphorylating its Ser^{112} residue, thereby preventing apoptotic cell death. Thus, Pim-3 may be a good molecular target for treating tumors, which exhibit the enhanced expression of Pim-3. We are now developing a chemical with a low molecular weight, which can inhibit the cell proliferation of cancer cells by inhibiting Pim-3 kinase.

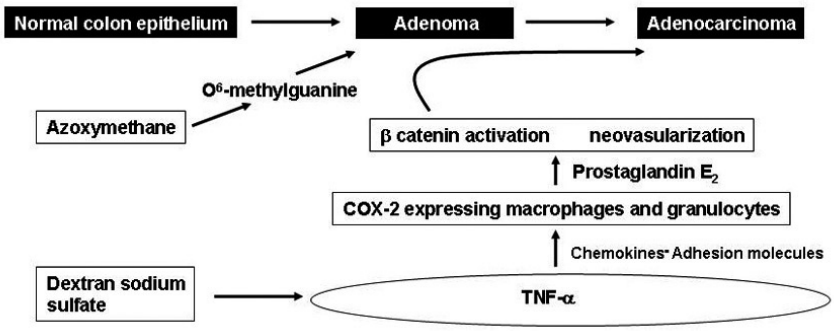
Crucial involvement of tumor necrosis factor in colon carcinogenesis

B.K. Popivanova and N. Mukaida

The risk of colon cancer is 20- to 30-fold higher in patients with ulcerative colitis, than in a control population. These observations suggest the crucial contribution of chronic inflammation to colon carcinogenesis. Multiple colon tumors with nuclear accumulation of β-catenin, develop in mice after an intraperitoneal injection of a generator of O^6-methylguanine, azoxymethane (AOM), followed by a repetitive oral intake of dextran sodium sulfate (DSS) solution. This condition mimics colon carcinoma development in patients with ulcerative colitis. Accumulating evidence indicates the crucial role of the activation of a transcription factor NF-κB in this model, although it remains elusive which factor(s) is responsible for NF-κB activation. We examined the role of tumor necrosis factor (TNF), a potent activator of NF-κB, in this model

Treatment with AOM + DSS caused TNF-α expression and TNF-receptor p55 (TNF-Rp55)-expressing inflammatory cell infiltration in lamina propria and submucosal regions of colon, together with enhanced cyclooxygenase (COX)-2 expression. TNF-Rp55-deficient mice developed much less numbers of colon tumors, along with reduced inflammatory cell infiltration and COX-2 expression. COX-2 was expressed mainly by infiltrating granulocytes and macrophages. The analysis using bone marrow chimeric mice revealed that bone marrow-derived cells were mainly responsible for tumor formation. Thus, locally-produced TNF-α is presumed to recruit TNF-Rp55-expressing granulocytes and macrophages, main producers of COX-2, therby contributing to colon carcinogenesis. Administration of etanercept, a specific antagonist of TNF-α, after multiple tumor formation induced by AOM + DSS, markedly reduced the numbers and sizes of tumors and reduced colonic infiltration of granulocytes and macrophages, COX-2 expression, and tumor neovascularization.

These observations identify TNF-α as a crucial mediator of the initiation and progression of colitis-associated colon carcinogenesis.



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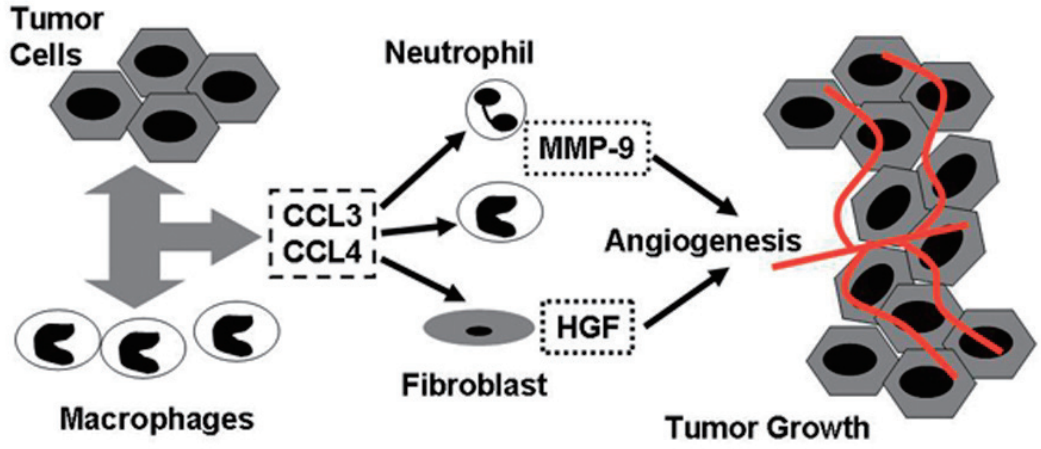
Essential contribution of the CCL3-CCR5 axis to murine lung metastasis process

Y. Wu, T. Baba, and N. Mukaida

Metastasis proceeds through interaction between cancer cells and resident cells such as leukocytes and fibroblasts. An intravenous injection of a mouse renal cell carcinoma, Renca, into wild-type (WT) mice, resulted in multiple metastasis foci in lungs and was associated with intratumoral accumulation of macrophages, granulocytes, and fibroblasts. A chemokine, CCL3, was detected in infiltrating cells and to a lesser degree, tumor cells, together with an infiltration of leukocytes expressing CCR5, a specific receptor for CCL3. A lack of *CCL3* or *CCR5* gene reduced the number of metastasis foci in the lung. The analysis using bone marrow chimeric mice revealed that both bone marrow- and non-bone marrow-derived cells contributed to metastasis formation. CCL3- and CCR5-deficient mice exhibited a reduction in intratumoral accumulation of macrophages, granulocytes, and fibroblasts. Moreover, intratumroal neovascularization, an indispensable process for metastasis, was attenuated in these gene deficient mice. Intrapulmonary expression of matrix metalloproteinase (MMP)-9 and hepatocyte growth factor (HGF) was enhanced in WT mice and the increases were markedly reduced in CCL3- and CCR5-deficient mice. Furthermore, MMP-9 protein was detected in macrophages and granulocytes, the cells which also express CCR5 and *in vitro* stimulation by CCL3 induced macrophages to express MMP-9. Intratumoral fibroblasts expressed CCR5 and HGF protein. Fibroblasts *in vitro* exhibited chemotactic responses and expressed HGF in response to CCL3. Collectively, the CCL3-CCR5 axis appears to regulate intratumoral trafficking of leukocytes and fibroblasts, and MMP-9 and HGF expression, and as a consequence to accelerate neovascularization and subsequent metastasis formation.



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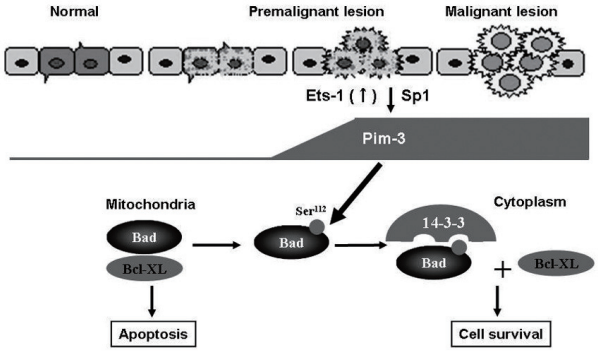
Enhanced expression of a proto-oncogene, Pim-3, with serine/threonine kinase activity, in various types of tumors

Y. Li, B.K. Popivanova, and N. Mukaida.

We previously observed that a member of the proto-oncogene Pim family, Pim-3, which expresses serine/threonine kinase activity, was aberrantly expressed in human and mouse hepatomas but not normal liver. Due to the unavailability of the nucleotide sequence of full-length human Pim-3 cDNA, we cloned full-length Pim-3 cDNA, consisting of 2,392 bp, which encodes a predicted open reading frame consisting of 326 amino acids.

Our subsequent studies demonstrated that Pim-3 gene expression was enhanced in malignant cells and lesions of endoderm-derived organs including pancreas, colon, and stomach, in addition to liver. The analysis on the promoter region of the human *Pim-3* gene revealed that the region up to -264 bp was essential for constitutive *Pim-3* gene expression in human pancreatic cancer cell lines, and the mutation in the Ets-1 and Sp1 binding sites reduced the reporter activities. Consistently, Ets-1 mRNA and protein were constitutively expressed together with Pim-3, in human pancreatic cancer cell lines. These observation would indicate that a transcription factor, Ets-1, can induce aberrant Pim-3 expression and subsequently prevent apoptosis in human pancreatic cancer cells, in collaboration with Sp1.

We further demonstrate that Pim-3 can phosphorylate a pro-apoptotic molecule, Bad, at its Ser^{112} but not Ser^{136} residue in human pancreatic and colon cancer cell lines. Moreover, Pim-3 overexpression can induce the expression of an anti-apoptotic molecule, Bcl-X_L. Thus, Pim-3 can inactivate Bad and maintain the expression of Bcl-X_L and eventually prevent apoptosis of human cancer cells. These observations would indicate that Pim-3 may be a good molecular target for several types of cancer, for example, pancreatic cancer, a representative intractable cancer.



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Other achievements

Patent No. 2008-16078

Intermediate synthetics of stemonamide and their application for the prevention and/or the treatment of cancer (Pending on June 19, 2008)

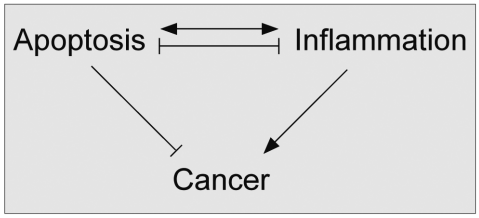
Inventors; Mukaida, N., Ishibashi, H., and Taniguchi., T.

Division of Immunology and Molecular Biology

General Summary of Division of Immunology and Molecular Biology

In the last three years, our study has been aiming to clarify the tripartite relationship among apoptosis, inflammation and cancer. It is generally believed that apoptosis is an important process to prevent oncogenesis, and that inflammation is a causative factor for cancer development. In terms of the relationship between apoptosis and inflammation, it has been said that apoptosis does not induce inflammation. However, recent studies demonstrated that apoptosis and inflammation uses many common protein molecules for signal transduction. For example, Fas ligand, an apoptosis inducing factor, was found to be a potent inducer of inflammation *in vivo*. Furthermore, we previously discovered that in a mouse model of chronic hepatitis that eventually develops hepatic cancer, administration of a neutralizing antibody against Fas ligand suppressed not only the hepatic inflammation but also the cancer development. Based on these results, we are investigating the functions of proteins (including Fas ligand) that play a role at the crossroad of apoptosis and inflammation, aiming to discover new findings useful for cancer therapy.

Tripatite relationship among apoptosis, inflammation and cancer



A) Study for the molecular mechanism of the inflammatory activity of Fas ligand.

Fas ligand is famous as a death factor; however, transplantation of Fas ligand-expressing cells into the peritoneal cavity of a mouse induces peritonitis associated with massive neutrophil infiltration. In addition, Fas ligand induces production of IL-8, a chemokine for neutrophils, in the human embryonic kidney (HEK)-293 cell line. Our previous study revealed that NF-κB plays an important role in the Fas ligand-induced IL-8 production and that caspase-8 is involved in this response. This time, we further demonstrated that AP-1 is also required for optimal IL-8 production, and that caspase-8 and JNK play essential roles in this response.

B) Study for the molecular mechanism of ASC-mediated apoptosis

ASC was originally discovered as a protein that forms a large aggregate in an apoptotic HL-60 human leukemia cell treated with a chemotherapeutic drug. This protein was independently discovered as a product of the gene called TMS1 whose expression in various human cancer tissues were suppressed by DNA methylation. It has been also reported that the expression of ASC is controlled by p53 tumor suppressor, and that ASC is involved in etoposide-induced apoptosis of tumor cells. These results suggest that ASC is a novel tumor suppressor. In addition, ASC was recently identified as an adaptor protein that mediates inflammatory and apoptotic signals from some members of the NLR family (such as cryopyrin and CARD12) that function as cytoplasmic sensors for pathogens and activate cellular innate immune responses. The molecular mechanism of

ASC-mediated apoptosis has been controversially reported. Initially, it was reported to be caspase-9 dependent; however, this notion was recently challenged. Therefore, we have investigated that molecular mechanism of ASC-mediated apoptosis and clearly demonstrated that caspase-8 plays an important role for this response. Just like the extrinsic pathway of apoptosis that is initiated by a death factor, ASC-mediated apoptosis in type-2 cells depends on proteolytic activation of Bid by caspase-8.

Recently, we also discovered that ASC mediates necrotic cell death under some conditions. Our current study is aiming to clarify the molecular mechanism of ASC-mediated necrosis. We also investigate possible use of ASC as a molecular target for cancer therapy.

C) The regulatory mechanisms of NLR proteins

Several members of NLR proteins (including cryopyrin) function as cytoplasmic sensors for pathogens and activators for apoptosis and inflammation as described above. However, we previously discovered other members of NLRs such as PYNOD, PYPAF2 and PYPAF3 function as negative regulators for ASC and caspase-1. This time, we have searched for cryopyrin-binding proteins using the yeast-two hybrid system. As a result, we have identified FAF1 as a novel cryopyrin-binding protein, and found that FAF1 inhibits cryopyrin-mediated NF-κB activation. In addition, we have established PYNOD-transgenic mice, and we are now investigating the *in vivo* functions of PYNOD.

Caspase-8-and JNK-dependent AP-1 activation is required for Fas ligand-induced IL-8 production

Norihiko Matsumoto, Ryu Imamura, and Takashi Suda

Despite a dogma that apoptosis does not induce inflammation, Fas ligand (FasL), a well-known death factor, possesses pro-inflammatory activity. For example, FasL induces nuclear factor κB (NF-κB) activity and interleukin 8 (IL-8)-production by engagement of Fas in human cells. Here, we found that a dominant negative mutant of c-Jun, a component of the activator protein-1 (AP-1) transcription factor, inhibits FasL-induced AP-1 activity and IL-8 production in HEK293 cells. Selective inhibition of AP-1 did not affect NF-κB activation and vice-versa, indicating that their activations were not sequential events. The FasL-induced AP-1 activation could be inhibited by deleting or introducing the lymphoproliferation (lpr)-type point mutation into the Fas death domain (DD), knocking down the Fas-associated DD protein (FADD), abrogating caspase-8 expression with small interfering RNAs (siRNAs), or using inhibitors for pan-caspase and caspase-8 but not caspase-1 or caspase-3. Furthermore, wild-type, but not a catalytically inactive mutant, of caspase-8 reconstituted the FasL-induced AP-1 activation in caspase-8-deficient cells. Fas ligand induced the phosphorylation of two of the three major mitogen-activated protein kinases (MAPKs): extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) but not p38 MAPK. Unexpectedly, an inhibitor for JNK but not for MAPK/ERK kinase inhibited the FasL-induced AP-1 activation and IL-8 production. These results demonstrate that FasL-induced AP-1 activation is required for optimal IL-8 production, and this process is mediated by FADD, caspase-8, and JNK.

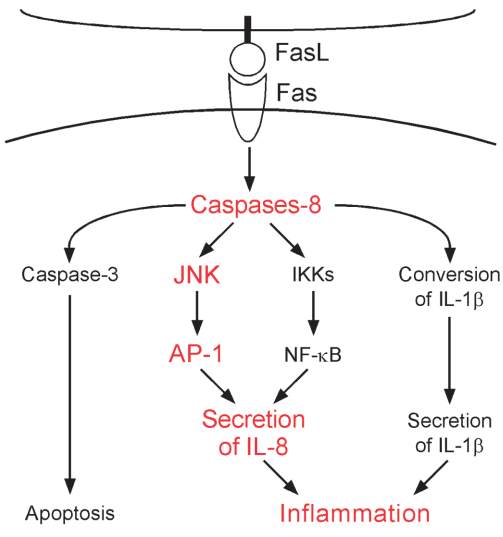


Figure. Fas ligand is well known death factor that induces apoptosis in a caspase-8 dependent manner. We previously demonstrated that stimulation by Fas ligand induces IL-1β secretion in LPS-primed mouse macrophages and IL-8 secretion in human embryonic kidney (HEK)-293 cells. Interestingly, it was found that both of these inflammatory responses are caspase-8 dependent. In this study, we further discovered that activation of Fas by Fas ligand induces AP-1 activation in a caspase-8- and JNK-dependent manner. This AP-1 activation is required for the Fas ligand-induced IL-8 production in HEK293 cells.

Fas-Associated Factor 1 is a negative regulator of PYRIN-containing Apaf-1-like protein 1

Takeshi Kinoshita, Chiaki Kondoh, Mizuho Hasegawa, Ryu Imamura, and Takashi Suda

PYRIN-containing apoptotic protease-activating factor-1-like proteins (PYPAFs, also called NALPs) participate in inflammatory signaling by regulating NF-κB activation and cytokine processing, and have been implicated in autoimmune and inflammatory disorders. However, the precise mechanisms that regulate the signal pathway leading to NF-κB activation are not completely understood. Here, we used yeast-two hybrid assays to identify Fas associated factor 1 (FAF1) as a protein interacting with the pyrin domains of several PYPAFs. In these assays, FAF1 interacted strongly with PYPAF1, PYPAF3, and PYPAF7, moderately with PYPAF2 and PYNOD, but not at all with the pyrin domains of pyrin or the adaptor molecule ASC. The interaction between FAF1 and PYPAF1 in mammalian cells was confirmed by immunoprecipitation assays, and the Fas-interacting domain of FAF1 was critical for this interaction. When coexpressed in HEK293 cells, FAF1 interfere with NF-κB activation induced by PYPAF1 and ASC. A FAF1 mutant lacking the Fas-interacting domain showed significantly reduced ability to inhibit NF-κB activation. Furthermore, down-regulation of endogenous FAF1 protein augmented LPS-induced IL-8 production, a biological marker for NF-κB activation, in monocytic cells. Finally, the level of FAF1 expression in THP-1 cells increased in response to NF-κB stimulation. These findings suggest that FAF1 functions as a negative regulator of an NF-κB signal pathway that involves PYPAF1 and ASC.

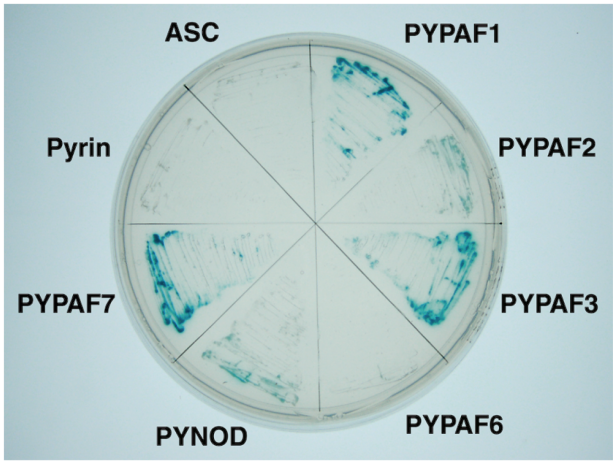


Figure 1. FAF1 interacts with the pyrin domains of PYPAF1, PYPAF2, PYPAF3, PYNOD, and PYPAF7, but not with those of pyrin, ASC and PYPAF6 in Yeast-two hybrid assays.

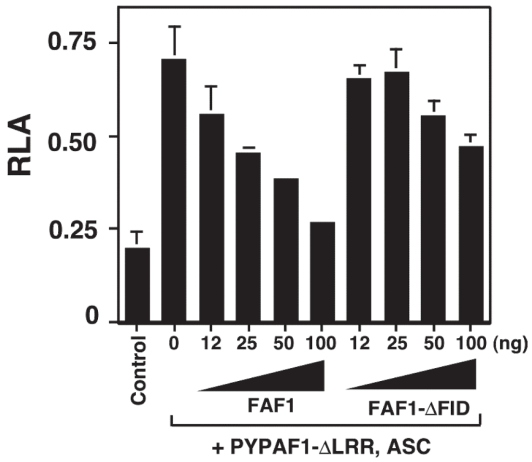


Figure 2. Cotransfection of a constitutive active mutant of PYPAF1 (PYPAF1- ΔLRR) and ASC induces NF-κB activation as revealed by a luciferase reporter assay in HEK293 cells. This NF-κB activation was inhibited by cotransfection of FAF1.

Expression of NLRP7 (PYPAF3) protein in endometrial cancer tissues

Satoshi Ohno*, Takeshi Kinoshita*, Yumiko Ohno, Toshinari Minamoto, Nobutaka Suzuki, Masaki Inoue and Takashi Suda (*Both authors equally contributed to this work)

Nucleotide-binding domain and leucine-rich repeat-containing family, pyrin domain-containing 7 (NLRP7) (pyrin-containing apoptotic protease activating factor-1-like protein 3; PYPAF3, NACHT domain-, leucine-rich repeat, and pyrin domain-containing 7; NALP7) has been thought to contribute to innate immunity and inflammation. Although expression of NLRP7 in human seminoma tissues and several cancer cell lines has been demonstrated, the pathophysiological and prognostic importance in cancer tissues has not been defined. In this study, a series of 70 endometrial cancer cases that had undergone curative resection was studied to determine the correlation between NLRP7 expression and clinico-pathological characteristics in human endometrial cancer tissue. Tissue specimens were evaluated for NLRP7 by immunohistochemistry. NLRP7 expression was positive in cancer cells in 7 cases (10%). There was a statistical relationship between the depth of tumor invasion and NLRP7 expression (p=0.0326). NLRP7 expression showed a trend for being associated with poor prognosis. Conclusion: Tumor-produced NLRP7, associated with myometrial invasion, might provide additional prognostic information in endometrial cancer patients.

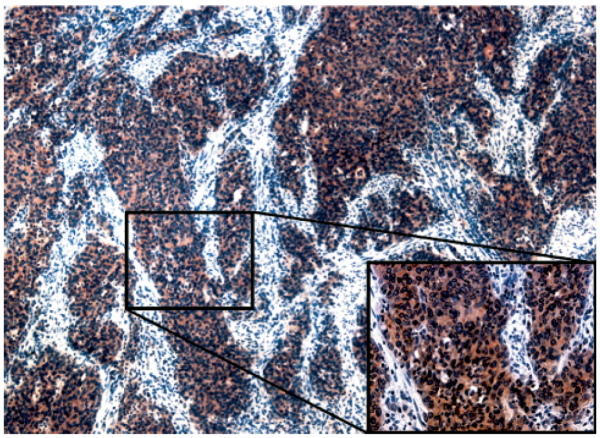


Figure 1. Representative sections of endometrial cancer with immunohistochemical staining of NLRP7. Strong cytoplasmic staining is observed in the invasion front of the tumor (×40; inset, ×200).

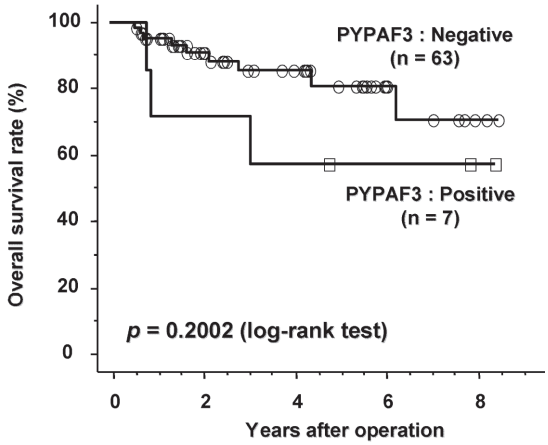


Figure 2. The Kaplan-Meier survival curves of 70 patients with endometrial carcinoma in relation to NLRP7 expression are shown.

Mechanism of ASC-mediated apoptosis: Bid-dependent apoptosis in type II cells

M. Hasegawa, K. Kawase, N. Inohara, R. Imamura, W-C. Yeh, T. Kinoshita, and T. Suda

ASC is an adaptor molecule that mediates apoptotic and inflammatory signals, and implicated in tumor suppression. However, the mechanism of ASC-mediated apoptosis has not been well elucidated. Here, we investigated the molecular mechanisms of ASC-mediated apoptosis in several cell lines using a CARD12-Nod2 chimeric protein that transduces the signal from muramyl dipeptide into ASC-mediated apoptosis. Experiments using dominant-negative mutants, small-interfering RNAs, and peptide inhibitors for caspases indicated that caspase-8 was generally required for ASC-mediated apoptosis, while a requirement for caspase-9 depended on the cell type. In addition, CLARP/FLIP (a natural caspase-8 inhibitor) suppressed ASC-mediated apoptosis, and *Clarp-/-* mouse embryonic fibroblasts were highly sensitive to ASC-mediated apoptosis. Bax-deficient HCT116 cells were resistant to ASC-mediated apoptosis as reported previously, although we failed to observe colocalization of ASC and Bax in cells. Like Fas-ligand-induced apoptosis, the ASC-mediated apoptosis was inhibited by Bcl-2 and/or Bcl-XL in type-II but not type-I cell lines. Bid was cleaved upon ASC activation, and suppression of endogenous Bid expression using small-interfering RNAs in type-II cells reduced the ASC-mediated apoptosis. These results indicate that ASC, like death receptors, mediates two types of apoptosis depending on the cell type, in a manner involving caspase-8.

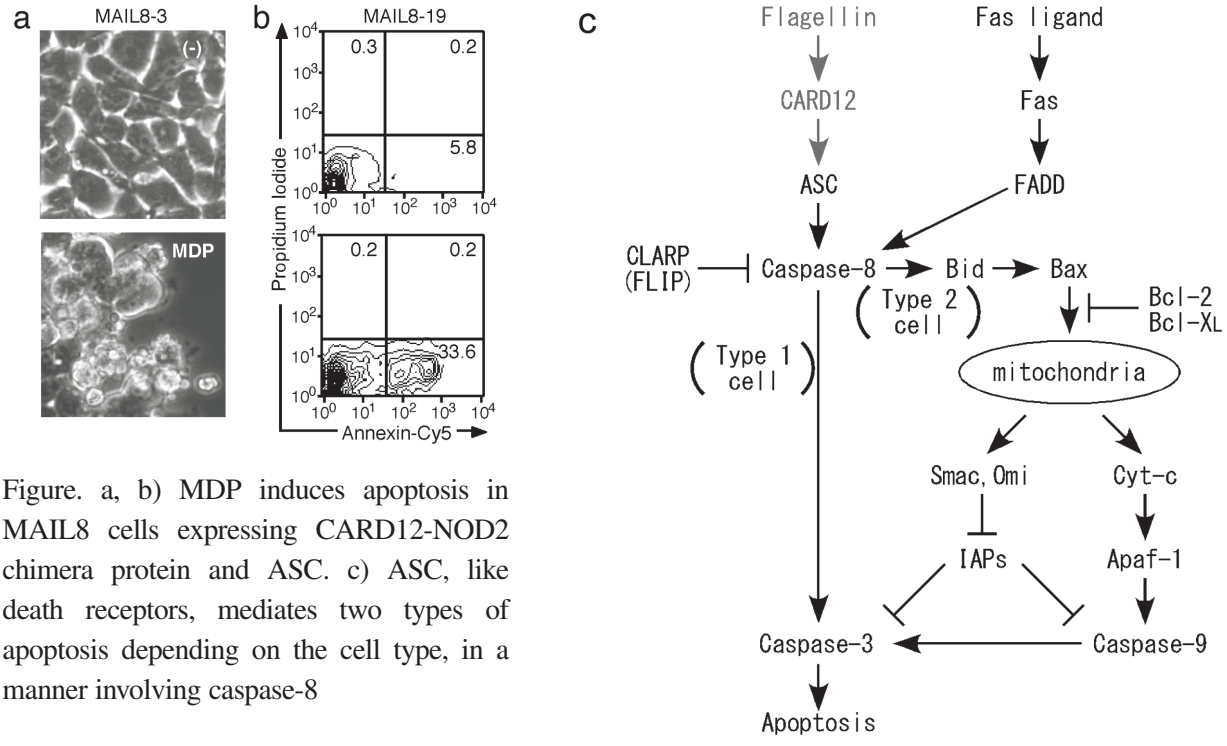


Figure. a, b) MDP induces apoptosis in MAIL8 cells expressing CARD12-NOD2 chimera protein and ASC. c) ASC, like death receptors, mediates two types of apoptosis depending on the cell type, in a manner involving caspase-8

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Division of Molecular Genetics

General Summary of Division of Molecular Genetics

Stem cells are defined as cells that have the ability to perpetuate through self-renewal, and develop into mature cells of a particular tissue through differentiation. Appropriate controls of stem cell functions are critical for maintaining tissue homeostasis. It has been suggested that dysregulation of stem cell could lead to cell senescence or cancer. Most cancers comprise heterogeneous populations of cells with marked differences in their proliferative potential as well as the ability to reconstitute the tumor on transplantation. It has been proposed that a rare population of tumor-initiating cells, conceptually termed cancer stem cells, is responsible for initiation and recurrence of tumor, although it is still controversial whether all tumors follow cancer stem cell theory. We have been dedicated mainly to basic researches on the mechanisms of stem cell regulation in both normal and malignant tissues. To understand molecular mechanisms by which stem cells are regulated, several approaches have been done as follows.

A) Identification of molecules essential for self-renewal of tissue stem cells.

Normal tissue stem cells are defined by three common properties: 1. Presence of an extensive capacity for self-renewal that allows maintenance of the undifferentiated stem cell pool over the life time of the host; 2. Strict regulation of stem-cell number; 3. Ability to undergo a broad range of differentiation events to clonally reconstitute all of the functional elements within the tissues. Since hematopoiesis provides the most well-characterized stem cell system, roles of several molecules on self-renewal of stem cells have been analyzed using hematopoietic system in this division. Further, for some molecules, other type of stem cells including germ or neural stem cells have been analyzed to address a question whether self-renewal of several tissue stem cells is regulated by common molecules or signaling pathways.

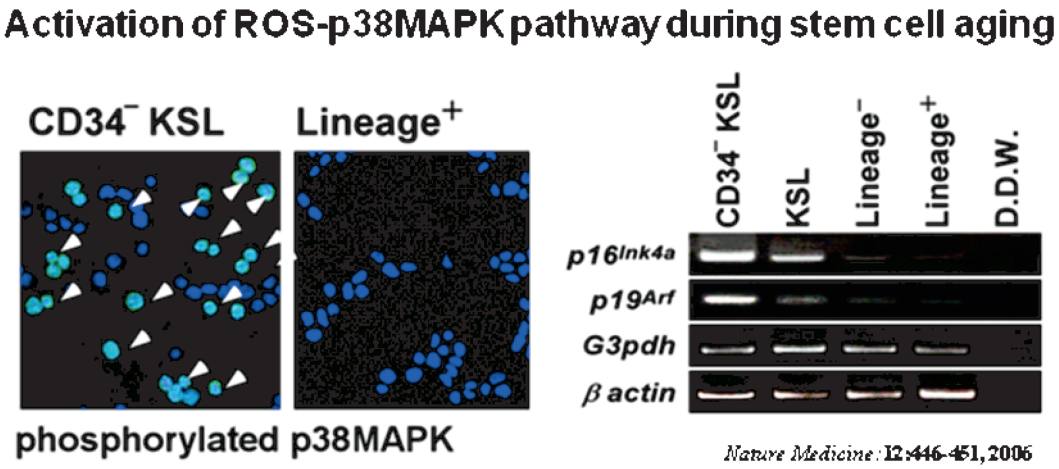
B) Analysis of heterogeneity in malignant tissues by stem-cell marking system

It has been still controversial whether all tumors, in particular aggressive solid tumors, follow cancer stem cell theory. We have been investigating whether the cells within a highly aggressive solid tumor are stochastically or hierarchically organized, in aggressive tumors. To do so, we have established a system for identification or visualization of stem cells by using promoter activity of genes that highly express in tissue stem cells. The heterogeneity in malignant tissues has been analyzed by using our stem-cell marking system.

Reactive oxygen species act through p38 MAPK to limit the lifespan of hematopoietic stem cells.

A. Hirao, K. Ito, K. Naka, T. Suda

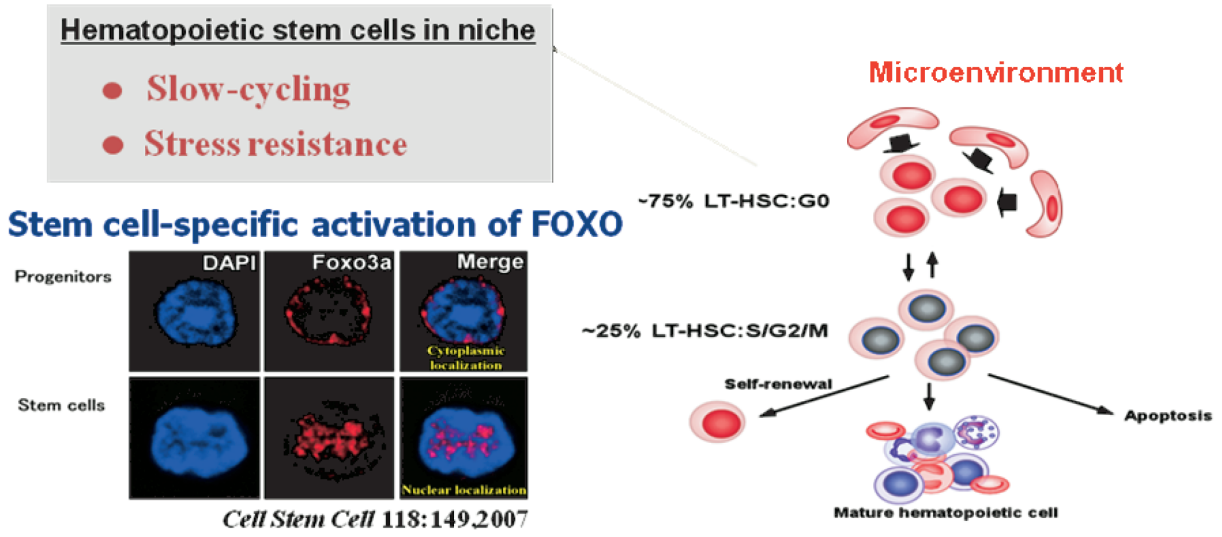
Hematopietic stem cells (HSC) in adult mouse bone marrow are quiescent, while mobilized HSCs are cycling rapidly following depletion of mature and progenitor cells by bleeding, infection, cytotoxic reagents or irradiation. Although interaction between microenvironments and HSCs contributes to maintenance of quiescence of HSCs resulting in the long-term self-renewal, mechanisms are not understood. Here we show that activation of p38 MAPK in response to increasing levels of reactive oxygen species (ROS) limits the lifespan of HSCs in vivo. In Atm(-/-) mice, elevation of ROS levels induces HSC-specific phosphorylation of p38 MAPK accompanied by a defect in the maintenance of HSC quiescence. Inhibition of p38 MAPK rescued ROS-induced defects in HSC repopulating capacity and in the maintenance of HSC quiescence, indicating that the ROS-p38 MAPK pathway contributes to exhaustion of the stem cell population. Furthermore, prolonged treatment with an antioxidant or an inhibitor of p38 MAPK extended the lifespan of HSCs from wild-type mice in serial transplantation experiments. These data show that inactivation of p38 MAPK protects HSCs against loss of self-renewal capacity. Our characterization of molecular mechanisms that limit HSC lifespan may lead to beneficial therapies for human disease.



Foxo3a is essential for maintenance of the hematopoietic stem cell pool.

K. Miyamoto, K. Naka, A. Hirao

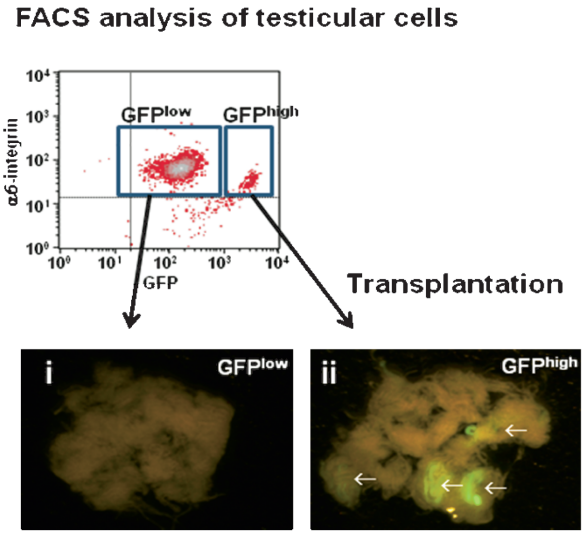
Hematopoietic stem cells (HSCs) are maintained in an undifferentiated quiescent state within a bone marrow niche. Here we show that Foxo3a, a forkhead transcription factor that acts downstream of the PTEN/PI3K/Akt pathway, is critical for HSC self-renewal. We generated gene-targeted Foxo3a(-/-) mice and showed that, although the proliferation and differentiation of Foxo3a(-/-) hematopoietic progenitors were normal, the number of colony-forming cells present in long-term cocultures of Foxo3a(-/-) bone marrow cells and stromal cells was reduced. The ability of Foxo3a(-/-) HSCs to support long-term reconstitution of hematopoiesis in a competitive transplantation assay was also impaired. Foxo3a(-/-) HSCs also showed increased phosphorylation of p38MAPK, an elevation of ROS, defective maintenance of quiescence, and heightened sensitivity to cell-cycle-specific myelotoxic injury. Finally, HSC frequencies were significantly decreased in aged Foxo3a(-/-) mice compared to the littermate controls. Our results demonstrate that Foxo3a plays a pivotal role in maintaining the HSC pool.



Identification of stem cells during prepubertal spermatogenesis via monitoring of nucleostemin promoter activity.

M. Ohmura, K. Naka, A. Hirao

The nucleostemin (NS) gene encodes a nucleolar protein found at high levels in several types of stem cells and tumor cell lines. The function of NS is unclear but it may play a critical role in S-phase entry by stem/progenitor cells. Here we characterize NS expression in murine male germ cells. Although NS protein was highly expressed in the nucleoli of all primordial germ cells, only a limited number of gonocytes showed NS expression in neonatal testes. In adult testes, NS protein was expressed at high levels in the nucleoli of spermatogonia and primary spermatocytes but at only low levels in round spermatids. To evaluate the properties of cells expressing high levels of NS, we generated transgenic reporter mice expressing green fluorescent protein (GFP) under the control of the NS promoter (NS-GFP Tg mice). In adult NS-GFP Tg testes, GFP and endogenous NS protein expression were correlated in spermatogonia and spermatocytes but GFP was also ectopically expressed in elongated spermatids and sperm. In testes of NS-GFP Tg embryos, neonates, and 10-day-old pups, however, GFP expression closely coincided with endogenous NS expression in developing germ cells. Our results support the existence in neonatal testes of spermatogonial stem cells with long-term repopulating capacity. Furthermore, our data show that NS expression does not correlate with cell-cycle status during prepuberty, and that strong NS expression is essential for the maintenance of germline stem cell proliferation capacity. We conclude that NS is a marker of undifferentiated status in the germ cell lineage during prepubertal spermatogenesis.



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Other achievements

The fourth JSPS (Japan Society for the Promotion of Science) PRIZE

Division of Genetics

General Summary of Division of Genetics

In the past three years (2006-2008), we have established gastric cancer mouse model (*K19-Wnt1/C2mE: Gan* mice) thorough simultaneous activation of Wnt and PGE_2 signaling pathways in gastric mucosa. Using *Gan* mouse model, we have investigated the role of host inflammatory response and angiogenesis in gastric tumorigenesis. We have also evaluated the chemopreventive or anti-cancer effects of several compounds on gastric tumorigenesis of *Gan* mice in collaboration with pharmaceutical companies. To investigate the effects of PGE_2 in other tissues, we constructed another transgenic mice expressing PGE_2 in pancreatic islets and examined the relationship between PGE_2-induced responses and diabetes.

A) *Gan* mice develop gastric tumors that recapitulate human gastric cancer.

It has been established that induction of COX-2 and mPGES-1 is found in variety of cancer tissues including gastrointestinal cancer. COX-2 and mPGES-1 cooperate to biosynthase PGE_2. On the other hand, Wnt/β-catenin activation is one of the major causes for gastrointestinal development. *Gan* mice that express COX-2, mPGES-1, and Wnt1 simultaneously in gastric mucosa develop gastric adenocarcinoma. Microarray analyses indicate that gene expression profile of *Gan* mouse tumor is similar to that found in human gastric cancer.

Gan mice are thus unique model that recapitulates human gastric cancer, and useful for clinical as well as basic research on gastric tumorigenesis.

B) Inflammation promotes gastrointestinal tumorigenesis through Wnt activation.

Accumulating evidence has indicated that inflammatory responses play a key role in tumor development. We investigated the role of macrophages in gastrointestinal tumorigenesis, and found that depletion of macrophages results in suppression of intestinal tumor growth. Moreover, TNF-α secreted from activated macrophages promotes Wnt/β-catenin signaling in gastric tumor cells. These results suggest that inflammation promotes tumor cell growth by activation of Wnt/β-catenin signaling.

C) Gastric tumors stimulate stromal fibroblasts resulting in activation of angiogenesis.

In the *Gan* mouse tumor tissues, angiogenesis is significantly enhanced. Using *Gan* mice and other transgenic strains expressing only PGE_2 or Wnt1 in the stomach, we found that tumor epithelial cells stimulate stromal fibroblasts to be myofibroblasts. Activated myofibroblasts express angiogenic factors including VEGF, which causes enhancement of angiogenesis.

Carcinogenesis in mouse stomach by simultaneous activation of the Wnt and PGE_2 signaling pathways

H. Oshima and M. Oshima

Accumulating evidence indicates that prostaglandin E_2 (PGE_2), a downstream product of cyclooxygenase 2 (COX-2), plays a key role in gastric tumorigenesis. The Wnt pathway is also suggested to play a causal role in gastric carcinogenesis. To investigate the role of Wnt and PGE_2 in gastric cancer, we have generated transgenic mice that activate both pathways, and examined their phenotypes. We constructed *K19-Wnt1* transgenic mice expressing *Wnt1* in the gastric mucosa using the keratin 19 promoter. We then crossed *K19-Wnt1* mice with another transgenic line *K19-C2mE* to obtain *K19-Wnt1/C2mE* (*Gan*) compound transgenic mice. The *K19-C2mE* mice express COX-2 and microsomal prostaglandin E synthase-1 (mPGES-1) in the stomach, showing an increased gastric PGE_2 level. *K19-Wnt1* mice had a significant suppression of epithelial differentiation, and developed small preneoplastic lesions consisting of undifferentiated epithelial cells with macrophage accumulation (Fig. 1*C*). Importantly, additional expression of COX-2 and mPGES-1 converted the benign lesions in the *K19-Wnt1* mice into invasive gastric adenocarcinomas by 30 weeks of age (Fig. 1*B*). Notably, we found mucous cell metaplasia in the glandular stomach of the *K19-Wnt1/C2mE mice* (Fig. 1*D*) as early as 5 weeks of age, long before the adenocarcinoma development.

In conclusion, Wnt signaling keeps the gastric progenitor cells undifferentiated. Simultaneous activation of both Wnt and PGE_2 pathways causes gastric adenocarcinomas through the metaplasia-carcinoma sequence.

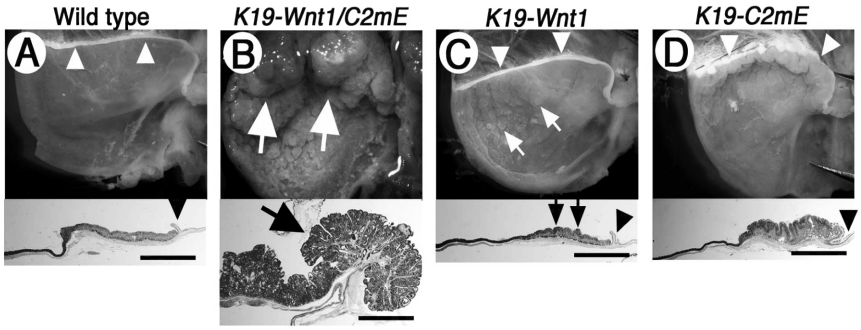

Fig. 1. Gastric phenotypes of mouse models (*top*) and H&E staining (*bottom*). Genotypes are indicated on each panel. Arrows in B indicate gastric tumors of *Gan* mouse.

Reference: Oshima H, *et al*. Gastroenterology, 131: 1086, 2006.

Activated macrophages promote Wnt signaling through TNF-α in gastric tumor cells.

K. Oguma, H. Oshima and M. Oshima

The canonical Wnt signaling pathway (Wnt/β-catenin pathway) operates by stabilizing β-catenin. The activation of Wnt/β-catenin signaling plays a key role in gastrointestinal tumorigenesis as well as in normal intestinal stem cells. It has been suggested that the promotion of Wnt/β-catenin activity beyond the threshold is important for carcinogenesis. We herein investigated the role of macrophages in promotion of Wnt/β-catenin activity in gastric tumorigenesis. We found β-catenin nuclear accumulation in macrophage infiltrated dysplastic mucosa of the *K19-Wnt1* mouse stomach. Moreover, macrophage depletion in $Apc^{\Delta 716}$ mice resulted in the suppression of intestinal tumorigenesis. These results suggested the role of macrophages in the activation of Wnt/β-catenin signaling, which thus leads to tumor development. Importantly, the conditioned medium of activated macrophages promoted Wnt/β-catenin signaling in gastric cancer cells, which was suppressed by the inhibition of tumor necrosis factor (TNF)-α (Fig. 1). Furthermore, treatment with TNF-α induces GSK3β phosphorylation, which resulted in the stabilization of β-catenin. We also found that *Helicobacter* infection in the *K19-Wnt1* mouse stomach caused mucosal macrophage infiltration and nuclear β-catenin accumulation. These results suggest that macrophage-derived TNF-α promotes Wnt/β-catenin signaling through inhibition of GSK3β, which may contribute to tumor development in the gastric mucosa. Accordingly, the present results suggest that suppression of macrophage infiltration and its activation by anti-inflammatory drugs or inhibitors for PGE_2 pathway is a possible strategy for chemoprevention against gastric cancer.

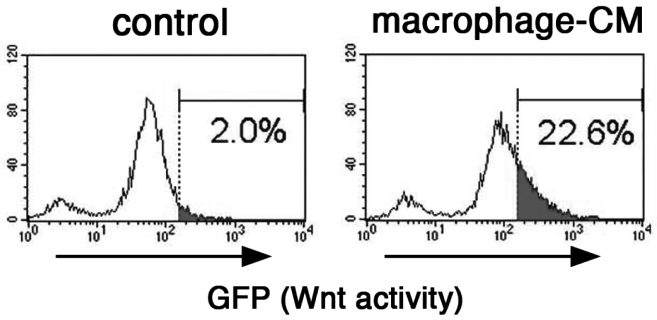

Fig. 1. FACS analyses of GFP intensity corresponding to Wnt activity of control gastric cancer cells (*left*) and stimulated cancer cells with conditioned medium derived from activated macrophages (*right*).

Reference: Oguma K, *et al*. EMBO J, 27: 1671, 2008.

Stromal fibroblasts activated by tumor cells promote angiogenesis in mouse gastric cancer.

X Guo, H. Oshima and M. Oshima

Myofibroblasts constitute important niche for tumor development through the promotion of angiogenesis. However, the mechanism of stromal fibroblast activation in tumor tissues has not been fully understood. A gastric cancer mouse model (*Gan* mice) was recently constructed by simultaneous activation of PGE_2 and Wnt signaling in the gastric mucosa. Because both the PGE_2 and Wnt pathways play a role in human gastric tumorigenesis, *Gan* mouse model therefore, recapitulates the molecular etiology of human gastric cancer. Microvessel density increased significantly in *Gan* mouse tumors (Fig. 1). Moreover, the expression of vascular endothelial growth factor A (VEGFA) was predominantly induced in the stromal cells of gastric tumors. Immunohistochemistry suggested that VEGFA-expressing cells in the stroma were α-smooth muscle actin (SMA)-positive myofibroblasts (Fig. 1). Bone marrow transplantation experiments indicated that subset of gastric myofibroblasts were derived from bone marrow. Importantly, the α-SMA index in cultured fibroblasts increased significantly when stimulated with the conditioned medium (CM) of *Gan* mouse tumor cells, indicating that gastric tumor cells activate stromal fibroblasts. Furthermore, CM of *Gan* mouse tumor cells induced VEGFA expression both in embryonic and gastric fibroblasts, which further accelerated the tube formation of human umbilical vein endothelial cells *in vitro*. Notably, stimulation of fibroblasts with PGE_2 and/or Wnt1 did not induce VEGFA expression, thus suggesting that factors secondarily induced by PGE_2 and Wnt signaling in the tumor cells are responsible for activation of stromal fibroblasts. Such tumor cell-derived factors may therefore be an effective target for chemoprevention against gastric cancer.

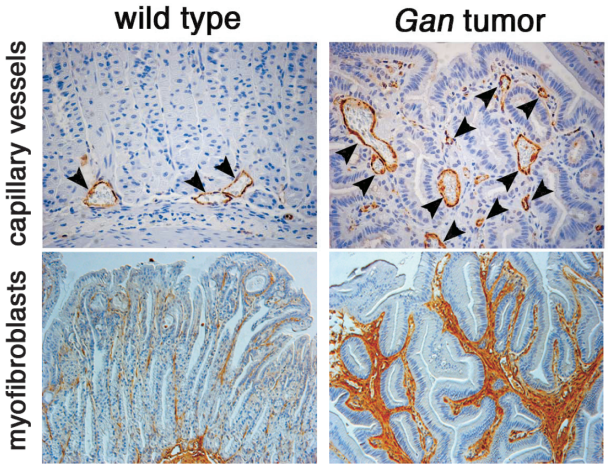

Fig. 1. Immunohistochemistry of wild-type mouse stomach (*left*) and *Gan* mouse gastric tumors (*right*) to show significantly increased capillary vessels (*top*) and myofibroblasts (*bottom*) in *Gan* tumor tissues.

Reference: Guo X, *et al*. J Biol Chem, 283: 19864, 2008.

Destruction of pancreatic β-cells by transgenic induction of PGE_2 in the islets.

H. Oshima and M. Oshima

Type 2 diabetes mellitus is characterized by insulin resistance of peripheral tissues and dysfunction of pancreatic β-cells. Furthermore, the number of pancreatic β-cells decreases as a secondary effect of advanced type 2 diabetes, although molecular mechanism has not been elucidated. Recently, it has been shown that hyperglycemic conditions induce the expression of cyclooxygenase-2 (COX-2) in pancreatic islets and increase the downstream product prostaglandin E_2 (PGE_2). To investigate whether high glucose-induced PGE_2 has an adverse effect on pancreatic β-cells, we generated transgenic mice (*RIP-C2mE*) that express COX-2 and microsomal prostaglandin E synthase-1 (mPGES-1) in their β-cells using the rat insulin-2 gene promoter (RIP). The homozygous *RIP-C2mE* (*Tg/Tg*) mice showed severe hyperglycemia from 6 weeks of age. Although the heterozygous *RIP-C2mE* (*Tg/-*) mice showed normal blood glucose levels throughout their lifetime, this level increased significantly compared with that of wild-type mice when glucose was loaded. The relative number of β-cells to the total islet cell number was reduced to 54% and 14% in the *RIP-C2mE*(*Tg/-*) and (*Tg/Tg*) mice, respectively, whereas that in the wild-type mice was 84% (Fig. 1). Importantly, the proliferation rate in the islets of the *RIP-C2mE* (*Tg/Tg*) mice at four weeks of age decreased significantly in comparison to that in the wild-type mice. Because β-cells replicate not only during the postnatal period but also in the adult pancreas at a basal level, it is possible that increased PGE_2 signaling thus contributes to the reduction of the pancreatic β-cell mass through inhibition of proliferation, thereby aggravating diabetes further.

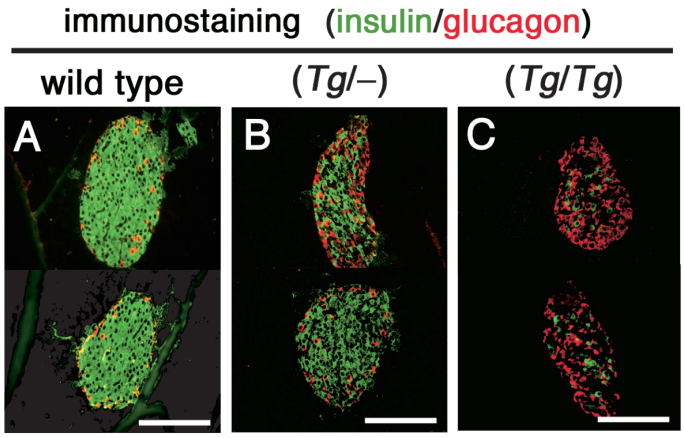

Fig. 1. Immunohistochemistry of insulin (*green*) and glucagons (*red*) to detect β-cells and α cells, respectively, in wild-type, heterozygous *RIP-C2mE*, and homozygous *RIP-C2mE* mice. Note significant decrease of β-cells in (*Tg/Tg*) mouse islets.

Reference: Oshima H, *et al*. J Biol Chem, 281: 29330, 2006.

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Division of Stem Cell Medicine

General Summay of Division of Stem Cell Medicine

In 2006, Dr. Nishimura was appointed to the professor of Division of Stem Cell Medicine. Our goal is to understand the mechanisms of tissue homeostasis driven by stem cell systems and to understand physiological or pathological conditions resulting from stem-cell system defects. Our studies started from our previous discovery of "melanocyte stem cells" (MSC), which supply melanocytes (pigment cells) required for hair pigmentation. The system has a number of advantages for stem cell research. Localization of MSCs and their niche can be visualized and the functional levels of the system cell system can be easily monitored. By taking those advantages, we have demonstrated that the stem cell niche plays a dominant role in stem cell fate determination. We searched for molecular mechanisms responsible for the phenomenon using a candidate approach with mouse coat color genetics. We have identified MITF, a master regulator for melanocyte development and its downstream effector, BCL2 as essential for MSC maintenance. In the past three years, we have identified the niche cells for MSCs and niche-derived extrinsic factors key for MSC maintenance. Through those approaches, we are trying to understand the underlying mechanisms for tissue homeostasis, ageing and cancer development.

A) Identification of niche cells and niche-derived factors for MSC maintenance.

Organization of the stem cell niche is fundamental for stem cell maintenance. However, it is largely unknown in majority of stem cell systems. We have identified hair follicle stem cells as functional niche-cells for MSCs and the niche-derived TGF-b as a key extrinsic factor for MSC renewal/maintenance. Furthermore, we demonstrated the Endothelin signaling regulates the expression and transcriptional activity of MITF, a master transcription factor for melanocyte development and also for MSC maintenance.

B) Mechanisms for MSC ageing and quality control of stem cell pools.

Physiological hair graying is the most obvious outward sign of aging even in normal mammals. We previously demonstrated that physiological hair graying is caused by incomplete self-renewal/maintenance of MSCs. However, it is still not known why self-renewal of MSCs becomes defective during the course of ageing. Accumulation of DNA damage is currently implicated in somatic stem cell ageing and appearance of age-related phenotypes, while little is known about the fate of stem cells under the genotoxic stress and its overall outcome. Our recent data indicated that stem cell differentiation but not stem cell apoptosis nor senescence is the major fate of MSCs under irrepairable/excessive genotoxic stress.

Epistatic connections between microphthalmia-associated transcription factor and endothelin signaling in Waardenburg syndrome and other pigmentary disorders

Kayo Sato-Jin, Emi K. Nishimura, David E. Fisher and Genji Imokawa

Waardenburg Syndrome (WS) is an inherited sensorineural deafness condition in humans due to melanocyte deficiencies in the inner ear and forelock. Mutation of MITF is known to produce WS type IIA whereas mutations of either endothelin (EDN) or its receptor endothelin receptor B (EDNRB) produce WS type IV. However, a link between MITF haploinsufficiency and EDN signaling has not yet been established. We demonstrated mechanistic connections between EDN and MITF and their functional importance in melanocytes. Addition of EDN to cultured human melanocytes stimulated the phosphorylation of MITF in an EDNRB-dependent manner, which was completely abolished by a MEK inhibitor. The expression of MITF-M mRNA transcripts was markedly augmented after incubation with EDN1, and was followed by increased expression of MITF protein. Upregulated expression of MITF was found to be mediated via both the MAPK-RSK-CREB and PKC-PKA-CREB pathways. Additionally EDNRB expression itself was seen to be dependent upon MITF. The functional importance of these connection is illustrated by the ability of EDN to stimulate expression of melanocytic pigmentation and proliferation markers in an MITF-dependent fashion. Collectively these data provide mechanistic and epistatic links between MITF and EDN/EDNRB, critical melanocytic survival factors and Wardenburg Syndrome genes.

Reference:
Sato-Jin K*, Nishimura E.K.*, Akasaka E, Huber W, Nakano H, Miller A, Du J, Wu M, Hanada K, Sawamura D, Fisher DE, and Imokawa G. (*: co-first author)
FASEB J. 22(4):1155-68. 2008

Key role for transforming growth factor-β in melanocyte stem cell immaturity and quiescence

Emi K. Nishimura, Misa Suzuki, Jürgen Roes, Friedrich Beermann, David E. Fisher

Organization of the stem cell niche is fundamental for stem cell maintenance. We previously demonstrated that the stem cell niche plays a dominant role in stem cell fate determination in melanocyte stem cell (MSC) systems. However, the niche cells and niche-derived factors responsible for stem cell maintanence is largely unknown not only in hair follicles but also in many other stem cell systems. Melanocyte stem cells in the bulge area of hair follicles are responsible for hair pigmentation and when defective, result in hair graying. We analyzed the process of MSC entry into the quiescent state and showed that niche-derived transforming growth factor b (TGF-b) signaling plays an important role in this process. TGF-b not only induces reversible cell cycle arrest, but downregulates MITF, the master regulator of melanocyte differentiation, and its downstream melanogenic genes, *in vitro*. TGF-b signaling is activated in MSCs when they reenter the quiescent non-cycling state during hair cycles and this process is Bcl2-dependent for MSC survival in-vivo. Furthermore, targeted TGF-b type II receptor (TGFBRII) deficiency in the melanocyte lineage causes incomplete maintenance of MSC immaturity and resultant hair graying. These data demonstrate that the TGF-b signaling pathway is a key niche factor for melanocyte stem cell quiescence and immaturity.

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Division of Translational and Clinical Oncology

General Summary of Division of Translational and Clinical Oncology

The mission of the division centers on laboratory and clinical research to develop the novel strategies and modalities for clinical management (diagnosis and treatment) and prevention of cancer in the gastrointestinal and respiratory tracts. Research projects are based on molecular and cellular characteristics of individual tumor types that are relevant to their biological properties including metastatic potential, recurrence and outcome. Our current efforts are focused on the following projects. We are intended to translate as much the achievements created from these studies as possible to the fields responsible for diagnosis and treatment of cancer patients in clinical setting.

A) Molecular pathology and translational research for oncogenic signaling networks

(1) Novel mechanisms underlying deregulated Wnt/β-catenin signaling
RNA *trans*-factor CRD-BP (coding region determinant-binding protein) is a previously unrecognized transcription target of β-catenin/Tcf complex, and stabilizes mRNA of β-TrCP (β-transducin repeats-containing protein), NF-κB and c-Myc. CRD-BP is a novel cancer target that integrates multiple oncogenic signaling pathways.

(2) Pathologic properties of glycogen synthase kinase 3β (GSK3β)-mediated cellular signaling
GSK3β supports and promotes tumor cells' survival and proliferation, and protects them from undergoing apoptosis in cancers of the major digestive organs. The results warrant proposing this kinase as a novel target in cancer treatment (PCT/JP 2006/300160).

B) Development of tailored chemotherapy based on pharmacogenetics

Thymidylate synthase (TS) is a target of fluoropyrimidines including 5-FU. TS has the unique gene polymorphisms (VNTR and SNP) in the 5'-UTR. Frequent LOH has been found in TS locus. TS gene polymorphisms and LOH are linked with the gene expression and can be of clinical use for tailored chemotherapy.

C) Translational research of DNA methylation markers

Both promoter hypermethylation and global hypomethylation occur simultaneously in cancer. The profile of the DNA methylation is characteristic as molecular signature in individual cancer, influencing patients' outcome. Tailored medicine (diagnosis, treatment and prevention) can be developed using the methylation markers.

D) Establishment of tissue material resources of human gastrointestinal cancer

The material resource is important for all types of translational research for diagnosis, treatment as well as molecular, cellular and biological cancer research.

CRD-BP mediates stabilization of βTrCP1 and c-myc mRNA in response to β-catenin signalling

Noubissi FK, Elcheva I, Bhatia N, Shakoori A, Ougolkov A, Liu J, Minamoto T, Ross J, Fuchs SY, Spiegelman VS.

Although constitutive activation of β-catenin/Tcf signalling is implicated in the development of human cancers, the mechanisms by which the β-catenin/Tcf pathway promotes tumorigenesis are incompletely understood. Messenger RNA turnover has a major function in regulating gene expression and is responsive to developmental and environmental signals. mRNA decay rates are dictated by *cis*-acting elements within the mRNA and by *trans*-acting factors, such as RNA-binding proteins. Here we show that β-catenin stabilizes the mRNA encoding the F-box protein βTrCP1, and identify the RNA-binding protein CRD-BP (coding region determinant-binding protein) as a previously unknown target of β-catenin/Tcf transcription factor. CRD-BP binds to the coding region of βTrCP1 mRNA. Overexpression of CRD-BP stabilizes βTrCP1 mRNA and elevates βTrCP1 levels (both in cells and in vivo), resulting in the activation of the Skp1-Cullin1-F-box protein ($SCF^{\beta TrCP}$) E3 ubiquitin ligase and in accelerated turnover of its substrates including IκB and β-catenin. CRD-BP is essential for the induction of both βTrCP1 and c-Myc by β-catenin signalling in colorectal cancer cells. High levels of CRD-BP that are found in primary human colorectal tumours exhibiting active β-catenin/Tcf signalling implicates CRD-BP induction in the upregulation of βTrCP1, in the activation of dimeric transcription factor NF-κB and in the suppression of apoptosis in these cancers.

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Noubissi FK, Elcheva I, Bhatia N, Shakoori A, Ougolkov A, Liu J, Minamoto T, Ross J, Fuchs SY, Spiegelman VS. CRD-BP mediates stabilization of βTrCP1 and c-myc mRNA in response to β-catenin signalling. Nature 2006;441(7095):898-901.

Adjuvant immunochemotherapy with protein-bound polysaccharide K for colon cancer in relation to oncogenic β-catenin activation.

Yamashita K, Ougolkov AV, Nakazato H, Ito K, Ohashi Y, Kitakata H, Yasumoto K, Omote K, Mai M, Takahashi Y, Minamoto T.

PURPOSE: Protein-bound polysaccharide K (PSK) is an immunotherapeutic agent that promotes apoptosis by inhibiting nuclear factor (NF)-κB activation in cancer cells. We previously showed that oncogenic β-catenin activates NF-κB and inhibits apoptosis by up-regulating β-transducin repeat-containing protein. We investigated whether the activation state of β-catenin in the primary tumor is associated with differences in survival rates of patients with colon cancer undergoing immunochemotherapy with 5-fluorouracil (5-FU) plus PSK vs. chemotherapy with 5-FU alone. METHODS: We assessed the activation states of β-catenin and NF-κB in the primary tumors of 202 colon cancer patients, and analyzed the data in terms of the clinicopathologic characteristics and survival of patients undergoing the two forms of adjuvant therapy. RESULTS: We found two distinct patterns of nuclear accumulation of activated β-catenin in the tumor cells: diffuse nuclear accumulation (NAd) in 89 cases (44 percent) and selective nuclear accumulation at the tumor invasion front (NAinv) in 18 cases (9 percent). NF-κB activation was found in 64 cases (32 percent). In patients with NAd-type β-catenin activation, immunochemotherapy significantly improved recurrence-free survival, cancer death survival, and overall survival rates compared with patients receiving chemotherapy alone. No survival benefit was found in cases with NAinv-type β-catenin activation or no activation. Similarly, immunochemotherapy favored the survival of patients with NF-κB activation. Multivariate analysis established the TNM stage and administration of PSK as independent prognostic factors in the patients with NAd-type β-catenin activation. CONCLUSIONS: The presence of NAd-type β-catenin activation identifies patients with colon cancer who respond better to immunotherapy with PSK.

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Detection of active fraction of glycogen synthase kinase 3β in cancer cells by nonradioisotopic *in vitro* kinase assay.

Mai W, Miyashita K, Shakoori A, Zhang B, Yu ZW, Takahashi Y, Motoo Y, Kawakami K, Minamoto T.

Glycogen synthase kinase 3β(GSK3β) is a well-known marker and potential therapeutic target in non-insulin-dependent diabetes mellitus and Alzheimer's disease. Our recent demonstration that GSK3β has a previously unrecognized role in colorectal cancer facilitates the development of a nonradioisotopic *in vitro* kinase assay (NRIKA) for detecting GSK3β activity in gastrointestinal cancer cells. The NRIKA uses a sequential combination of immunoprecipitations to isolate GSK3β in sample cells' lysates, and an in vitro kinase reaction that uses recombinant β-catenin protein (substrate) and nonradioisotopic ATP, followed by immunoblotting to detect β-catenin phosphorylated in serine 33, 37 and/or threonine 41 residues. The NRIKA detected higher expression of active GSK3β in stomach, colon, pancreas and liver cancer cell lines than in human embryonic kidney cells (HEK293) considered nonneoplastic. Inhibition of cancer cell-derived GSK3β activity by GSK3β inhibitors (SB-216763, AR-A014418) was detected by the NRIKA. GSK3β inhibition attenuated survival and proliferation and induced apoptosis in all types of cancer cells but not in HEK293. These findings supported the idea that the pathologic roles of GSK3β are definite and common in various types of cancer. The NRIKA provides a basis for evolving a high-throughput tool for testing substances for GSK3β inhibition, and for screening and identifying novel GSK3β inhibitors with a view to discovering drugs for treatment of cancer as well as non-insulin-dependent diabetes mellitus and Alzheimer's disease.

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Inhibition of glycogen synthase kinase 3β activity attenuates proliferation of human colon cancer cells in rodents

Shakoori A, Mai W, Miyashita K, Yasumoto K, Takahashi Y, Ooi A, Kawakami K, Minamoto T.

The authors' recent discovery that glycogen synthase kinase 3β (GSK3β) participates in colon cancer cells' survival and proliferation prompted us to investigate whether GSK3β inhibition alters proliferation of colon cancer cells in vivo. Groups of four or five athymic mice (Balb/c, nu/nu) with subcutaneous xenografts of SW480 human colon cancer cells were treated with dimethyl sulfoxide (DMSO) or different doses (1, 2 and 5 mg/kg body weight) of either small-molecule GSK3β inhibitor (SB-216763 and AR-A014418) by intraperitoneal injection three times per week for 5 weeks. Compared with DMSO (a diluent of the GSK3β inhibitors) as a control, either GSK3β inhibitor significantly inhibited proliferation of cancer cell xenografts in the rodents in a dose-dependent manner. Histochemical and immunohistochemical analysis of tumor xenografts demonstrated a significant, dose-dependent decrease in fractions of proliferating cells and an increase in the incidence of apoptosis of cancer cells in mice treated with either GSK3β inhibitor. No adverse events or effects were observed in the rodents during the course of treatment, except for rare lethal accidents due to intraperitoneal injection. Morphological examination showed no apparent pathologic changes in major organs including the lungs, liver, pancreas, kidneys, spleen and large bowel of rodents treated with DMSO and the GSK3β inhibitors. The results indicate that the GSK3β inhibitors would be a novel class of therapeutic agent for colon cancer.

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Shakoori A, Mai W, Miyashita K, Yasumoto K, Takahashi Y, Ooi A, Kawakami K, Minamoto T. Inhibition of GSK-3β activity attenuates proliferation of human colon cancer cells in rodents. Cancer Sci 2007;98(9):1388-1393.

Methylation levels of LINE-1 repeats and CpG island loci are inversely related in normal colonic mucosa

Iacopetta B, Grieu F, Phillips M, Ruszkiewicz A, Moore J, Minamoto T, Kawakami K.

Hypermethylation of CpG island loci within gene promoter regions is a frequent event in colorectal cancer that is often associated with transcriptional silencing and has been referred to as CIMP+. DNA hypomethylation can occur in concert with CIMP+, although these two phenomena appear not to be related in colorectal cancer. The authors investigated here whether the methylation level of LINE-1 repeats, a surrogate marker for genomic methylation, was associated with the level of CpG island methylation in colorectal cancers and in matching normal colonic mucosa from 178 patients. The MethyLight assay was used to quantitate the methylation of CpG islands within the MLH1, P16 (INK4A), TIMP3, DAPK, APC, ER and MYOD genes. A real-time, methylation-specific polymerase chain reaction assay was also used to quantitate the methylation of LINE-1 repeats. In colorectal cancer, no associations were seen between methylation levels in LINE-1 repeats and CpG island loci, including a new CpG island panel that was recently proposed for CIMP+. In normal colonic mucosa, however, the methylation level of LINE-1 repeats was inversely correlated with CpG-island methylation of the MLH1, P16, TIMP3, APC, ER and MYOD genes. The methylation level of LINE-1 repeats in normal colonic mucosa also showed significant associations with common polymorphisms in the methylene tetrahydrofolate reductase and methylene tetrahydrofolate dehydrogenase genes involved in methyl group metabolism. Further investigation of genomic and CpG island methylation in normal colonic mucosa and the possible influences of environmental and genetic factors may provide new insights into the development of CIMP+ colorectal cancer.

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Iacopetta B, Grieu F, Phillips M, Ruszkiewicz A, Moore J, Minamoto T, Kawakami K. Methylation levels of LINE-1 repeats and CpG island loci are inversely related in normal colonic mucosa. Cancer Sci. 2007 Sep;98(9):1454-1460.

Low expression of gamma-glutamyl hydrolase mRNA in primary colorectal cancer with the CpG island methylator phenotype.

Kawakami K, Ooyama A, Ruszkiewicz A, Jin M, Watanabe G, Moore J, Oka T, Iacopetta B, Minamoto T.

The CpG island methylator phenotype (CIMP+) in colorectal cancer (CRC) is defined as concomitant and frequent hypermethylation of CpG islands within gene promoter regions. We previously demonstrated that CIMP+ was associated with elevated concentrations of folate intermediates in tumour tissues. In the present study, we investigated whether CIMP+ was associated with a specific mRNA expression pattern for folate- and nucleotide-metabolising enzymes. An exploratory study was conducted on 114 CRC samples from Australia. mRNA levels for 17 genes involved in folate and nucleotide metabolism were measured by real-time RT-PCR. CIMP+ was determined by real-time methylation-specific PCR and compared to mRNA expression. Candidate genes showing association with CIMP+ were further investigated in a replication cohort of 150 CRC samples from Japan. In the exploratory study, low expression of gamma-glutamyl hydrolase (GGH) was strongly associated with CIMP+ and CIMP+-related clinicopathological and molecular features. Trends for inverse association between GGH expression and the concentration of folate intermediates were also observed. Analysis of the replication cohort confirmed that GGH expression was significantly lower in CIMP+ CRC. Promoter hypermethylation of GGH was observed in only 5.6% (1 out of 18) CIMP+ tumours and could not account for the low expression level of this gene. CIMP+ CRC is associated with low expression of GGH, suggesting involvement of the folate pathway in the development and/or progression of this phenotype. Further studies of folate metabolism in CIMP+ CRC may help to elucidate the aetiology of these tumours and to predict their response to anti-folates and 5-fluorouracil/leucovorin.

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Division of Functional Genomics

General Summary of Division of Functional Genomics

Division of Functional Genomics has been started at April 2007 as one of the laboratories of Molecular and Cellular Targeting Translational Oncology Center. To develop the novel target-based cancer therapeutics, a detailed knowledge of the genes and signaling pathways mutated in cancer will be required. However, the heterogeneity and complexity of genomic alterations in most human cancers hamper straightforward identification of cancer-causing mutations. We use the retrovirus-infected mice as a model system for identifying novel cancer genes efficiently. Retroviruses induce tumors through activation of proto-oncogenes or inactivation of tumor suppressor genes as a consequence of retroviral integrations into host genome. Thus the viral integration sites provide powerful genetic tags for cancer gene identification. We are exploring the novel molecular targets for cancer treatment based on functional characterization of the cancer genes isolated by high-throughput screens using retroviral insertional mutagenesis. Once these genes are identified, we use gene knockout and transgenic mice to understand how these genes function in tumorigenesis, and to develop new animal models for human cancer.

A) Identification of novel tumor suppressor genes using retroviral insertional mutagenesis in mice with genomic instability

Generally, most retroviral integrations tagged proto-oncogenes rather than tumor suppressor genes. In order to isolate tumor suppressor genes efficiently, we utilized the Bloom syndrome model mice that have a high rate of mitotic recombination and LOH so that viral integrations could be efficiently homozygous. More than 20 candidate tumor suppressor genes were isolated in the screen, and they included the known tumor suppressor genes (Rb family, Cdk inhibitors etc.) and also novel interesting candidates (JmjC domain family).

B) Involvement of protein methyltransferases and demethylases in oncogenesis

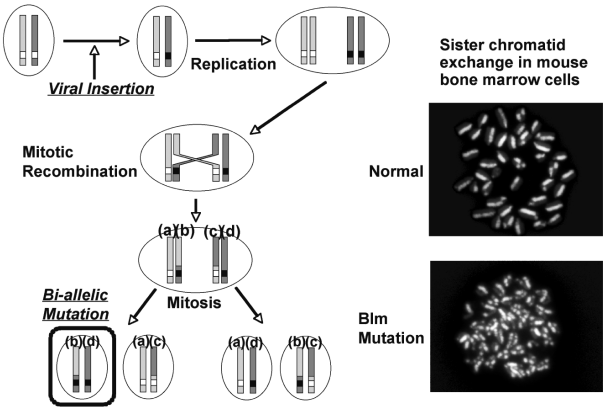
We have so far identified 17 histone methyltransferase genes and 11 histone demethylase genes as potential oncogenes or tumor suppressor genes by retroviral tagging. Among histone modifications, acetylation has been unambiguously associated with cancer, and the inhibitor of histone deacetylases has been developed as an anti-cancer drug. However, a clear correlation of histone methylation and cancer has not been conclusively demonstrated yet. Our findings provide a unique opportunity to explore the novel relationship between the regulation of protein methylation and oncogenesis.

Efficient identification of candidate tumor suppressor genes using retroviral insertional mutagenesis in mice with genomic instability

K. Minehata[+], K. Akagi*, N.A. Jenkins*, N.G. Copeland* and T. Suzuki
([+]HMRO, Kyoto University Graduate School of Medicine, *Mouse Cancer Genetics Program, NCI-Frederick, USA)

Retroviral insertional mutagenesis in mice is one of the important strategies for efficient and high-throughput identification of cancer-causing genes. However, there was one limitation to the use of retroviral tagging for gene discovery. Viral integrations primarily identify proto-oncogenes rather than tumor suppressor genes. This is presumably because a single retroviral induced mutation is sufficient to activate an oncogene and initiate a tumor, while two mutations are needed to inactivate a tumor suppressor gene. To overcome this limitation, we utilized the mice that have genomic instability so that viral integration in one allele could be efficiently rendered homozygous. Mice carrying mutations in the Bloom syndrome (Blm) gene have a high frequency of spontaneous mitotic recombination and loss of heterozygosity (LOH). Thus, MuLV (murine leukemia virus)-infected mice with Blm mutation are thought to be more apt to carry retroviral integrations in tumor suppressor genes.

In fact, the MuLV-infected Blm mutant mice showed earlier onset of lymphoma than Blm wild type mice. Using the high-throughput retroviral tagging, we have isolated so far more than 150 candidate disease genes including the genes whose coding regions are consistently disrupted by multiple retroviral integrations. Most of the 'disrupted' candidate genes showed the evidences of bi-allelic mutations in the tumors. Those genes included the known genes that were reported to function as tumor suppressors (Rb family, Cdk inhibitors etc.), and also novel interesting candidate genes (JmjC domain family). Thus, the MuLV-infected mouse with genomic instability is a useful animal model that makes us enable to isolate candidate tumor suppressor genes efficiently.



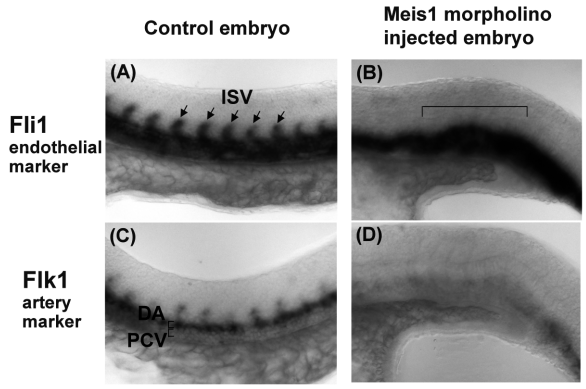
Efficient induction of bi-allelic mutations in retrovirus-infected Bloom syndrome model mice

The roles of Meis1 oncogene products on the development of endothelial cells in zebrafish

K. Minehata[+], A. Kawahara[+] and T. Suzuki
([+]HMRO, Kyoto University Graduate School of Medicine)

Meis1 (myeloid ectopic viral insertion site1) encodes a homeobox domain transcription factor, and was originally identified as one of the common viral integration sites in acute myeloid leukemia of retrovirus-infected mice. Meis1 is also frequently found up-regulated along with Hox genes in human leukemias and cooperates with Hox genes to accelerate the onset of leukemia. In addition to the roles of Meis1 on leukemogenesis, its function on normal hematopoietic development has been also investigated. Previous reports showed that Meis1-deficient mice died by embryonic day 14.5 with the reduction of megakaryocytes and definitive hematopoietic stem cells (HSCs) suggesting that Meis1 is involved in the generation of definitive HSCs.

Zebrafish is a useful model organism for studies of hematopoietic and vascular development, and the developmental processes are definitively conserved between zebrafish and mammals. Thus we use zebrafish in order to investigate the function of Meis1 during normal development. Knockdown of *meis1* by antisense *meis1*morpholino led to the impairment of intersegmental vessel formation in the zebrafish embryo. In *meis1* morphants, the expression of an artery marker (*ephrinB2*) was reduced in dorsal aorta, and the expression of vein markers (*flt4, ephB4*) was expanded in dorsal aorta and posterior cardinal vein, suggesting the severe defects on artery development. Furthermore, the expression of *vascular endothelial growth factor* (*vegf*) receptor, *flk1*, was significantly decreased in these embryos. Interestingly, *flk1* morpholino-injected embryos exhibited similar defects as *meis1* morphants. Thus, these results implicate that *meis1* is a novel regulator involved in vascular development and endothelial cell differentiation, presumably affecting the *vegf* signaling pathway.



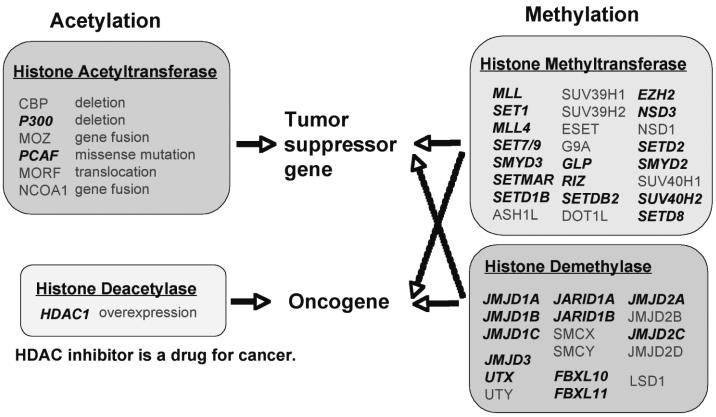
Meis1 oncogene product is involved in vascular(ISV) development and artery(DA) differentiation in zebrafish

Involvement of protein methyltransferases and demethylases in oncogenesis

A. Ishimura, M. Terashima, K. Nishida, K. Yokota and T. Suzuki

From the tumors of the MuLV-infected Blm mice, we isolated more than twenty candidate tumor suppressor genes that showed the evidences of bi-allelic mutations in the tumors. Those genes included the known tumor suppressor genes and also novel interesting candidates such as Jmjd5 and Fbxl10, the genes encoding JmjC domain containing proteins. Knockdown of Jmjd5 or Fbxl10 expression by each shRNA was shown to confer a mutator phenotype to the cells, suggesting both genes are excellent candidates for tumor suppressor genes.

Recently, several laboratories have shown that the JmjC domain is the signature motif for histone demethylase. Post-translational modification of histones such as acetylation and methylation has been implicated in multiple biological processes including transcription, DNA replication, DNA repair and heterochromatin formation. By the large-scale retroviral insertional mutagenesis, we have so far identified 17 histone methyltransferase genes and 11 histone demethylase genes as potential oncogenes or tumor suppressor genes. These findings provide a unique opportunity to explore the relationship between histone methylation and cancer. Among histone modifications, acetylation has been unambiguously associated with cancer, however, a clear correlation of methylation and cancer has not been conclusively demonstrated yet. Since the inhibitors of histone deacetylases have been developed as anti-cancer drugs, we expect that histone methylation may be the important field to find the new molecular targets for cancer treatment. We have recently found that the expressions of some methyl-modifying enzymes are also deregulated in human lung cancer. Now we are trying to clarify the molecular mechanism of cancer induced by the deregulation of protein methylation.



Target genes identified by retroviral tagging from mouse tumors

Protein methytransferases and demethylases are good candidates of novel molecular targets for cancer

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Division of Tumor Dynamics and Regulation

General Summary of Division of Tumor Dynamics and Regulation

Growth factors and their receptors of tyrosine kinase play fundamental roles in diverse processes, including development, morphogenesis, cell growth, and tissue regeneration. Because of this, aberrant regulation of receptor tyrosine kinases participates in the development and progression of a variety of human cancers. Among malignant behavior of tumors, tumor metastasis is the most important factor affecting survival of cancer patients. Approximately 90% of all cancer death arise from the metastatic spread of primary tumors. Although mutational alterations and epigenetic events that occur in oncogenes and tumor suppressor genes are of a genetical background for tumorigenesis, invasive and metastatic behavior of tumor cells is largely regulated by extracellularly acting growth factors. Among them, hepatocyte growth factor (HGF) particularly affects metastatic behavior in a wide variety of cancer cells.

The signal exchange, cell-cell communications, and the genetic programs that function during tissue regeneration are evoked in cancer tissues. Tumors are likely to be wounds that do not heal (Dvorak, 1986). In normal tissues, HGF-Met signaling plays diverse roles in organogenesis and healing of wounded tissues, but aberrant regulation in HGF-Met signaling confers highly invasive and metastatic characteristic in a variety of cancer cells. Despite the appreciation of the clinical relevance of tumor metastasis, therapeutic intervention that can efficiently prevent metastasis has yet appeared. If metastatic tumors can be suppressed to non-metastatic tumors, the rate of cancer cures is considered to show much improvement. Inhibition of HGF-Met signaling has been considered to provide ways to prevent cancer metastasis.

We discovered NK4 as a competitive inhibitor against HGF-Met. NK4 is a fragment of HGF, consisting of an N-terminal hairpin domain and four kringle domains. NK4 binds to the Met but does not activate Met, thereby competitively inhibiting HGF-dependent activation of Met. We thereafter found that NK4 functions as an angiogenesis inhibitor, and that this angiostatic activity of NK4 is probably independent of its original activity as an HGF-antagonist. Based on unique biological actions of NK4, anti-cancer effect of NK4 has been demonstrated in different types of malignant tumors in mice. We recently addressed therapeutic effect of NK4 on malignant tumors such as colon cancer and malignant pleural mesothelioma. On the other hand, based on advances in bioinformatics and protein crystallography, new approaches to discover small molecule inhibitors for specific target proteins have progressed. Application of such new drug discovery technologies is exciting to discover molecules targeting HGF-Met.

HGF and its receptor Met tyrosine kinase play roles particularly in dynamic morphogenesis, cellular locomotion, and epithelial-mesenchymal interaction/transition during development and

tissue regeneration. In the adult, HGF-Met signaling is activated in response to tissue injuries for regeneration of various organs, including the liver. Administration of HGF suppresses the onset of pathology, facilitates tissue regeneration, and improves pathology such as tissue fibrosis. For the clinical development of HGF as regenerative medicine, clinical trials of HGF for treatment of skin ulcer and acute renal failure started in 2009.

During basic research to know mechanisms for tissue regeneration by HGF, we found that HGF exerts biological activity in "the injured tissue-selective manner," even when HGF is administered systemically. The injured tissue-selective activation of the Met receptor suggests that in the injured tissue Met is activated upon stimulation with HGF, but in non-injured (normal) tissues and the regenerated tissue after wounding, Met receptor activation is suppressed even after stimulation with HGF. Understanding of the mechanism by which Met activation is regulated in response to tissue injury or intactness may be a clue to know the fundamental question on how the mass and organization of tissues are maintained before and after regeneration. Likewise, loss-of-function in the negative regulation of Met activation is considerable as a possible mechanism for an aberrant activation of Met in cancer.

A) Anti-cancer Approach with NK4 and Anti-angiogenic Mechanism of NK4

The mechanism by which NK4 inhibits angiogenesis remained unknown. We recently found that NK4 inhibited extracellular assembly of fibronectin, by which integrin-dependent anchoring signal transduction was inhibited by NK4 in endothelial cells. Based on unique bifunctional characteristic of NK4, therapeutic approaches with NK4 have been examined in experimental models, including colon cancer, pleural malignant mesothelioma, multiple myeloma, and hepatoma. In these models, NK4 protein administration or NK4 gene therapy inhibited tumor invasion and metastasis, and angiogenesis-dependent tumor growth.

B) Suppression of Met/HGF Receptor Activation by the Met Juxtamembrane function and Cell-Cell Contact

We previously showed that Met activation is suppressed by Ser985 phosphorylation in the juxtamembrane (JM) domain of Met. Since the Met deleted with the JM domain naturally exists as a splicing variant, the JM Ser985 phosphorylation may play an important physiological role. On the other hand, because the contact inhibition is fundamental characteristic of normal cells, we hypothesized that Met activation might be regulated by cell-cell contact. We found the suppressive mechanism for Met activation by cell-cell contact, using hepatocytes in primary culture. The cell-cell contact up-regulated expression of LAR protein tyrosine phosphatase and LAR dephosphorylated Met, by which Met activation was suppressed even after stimulation with HGF. Therefore, we found two different mechanisms involved in suppression of Met activation even when

cells are stimulated with HGF. The one is cell-cell contact-dependent inactivation of the Met by LAR. The other is suppression of Met activation by the JM Ser985 phosphorylation.

C) In Silico-Based Drug Discovery Targeting HGF-Met

HGF-Met pathway is a hot target in worldwide discovery of molecular target therapy of cancer. To discover new small molecule inhibitors for HGF-Met pathway, a research group (K. Matsumoto as a head researcher) was organized by researchers of different specialties (bioinformatics, protein chemistry, protein crystallography). In this research, small molecule inhibitors as lead compounds were discovered by successive in silico-based drug discovery techniques. Analysis of the crystal structure of the complex composed of a target protein and a compound is in progress for potentiation and optimization of lead compounds.

Anti-cancer Approach with NK4 and Anti-angiogenic Mechanism of NK4: Inhibition of Endothelial Fibronectin Assembly and Colon Cancer Metastasis

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We previously showed that NK4 inhibited angiogenic responses driven by VEGF and bFGF, as well as HGF. However, elucidation of the mechanism by which NK4 inhibits angiogenesis has remained. In vascular endothelial cells, NK4 allowed activation of VEGF receptor and Erk1/2, whereas NK4 inhibited cyclin D1 expression and Rb phosphorylation. NK4 inhibited cell surface assembly of fibronectin and integrin-dependent signaling pathway. Endothelial cell inhibition by NK4 occurred regardless of Met/HGF receptor expression. These results suggest that NK4 may bind to a cell surface molecule different from the Met and that the binding of NK4 to this molecule may inhibit cell surface assembly of fibronectin (Fig. 1).

When mouse colon cancer cells (MC-38) were inoculated into the spleen, the cancer cells metastasized to the liver and subsequently invaded into surrounding liver. In this model, hepatic gene expression of NK4 suppressed hepatic metastasis and intrahepatic growth of metastases. In situ Met tyrosine phosphorylation was inhibited by NK4 and this was associated with inhibition of invasion of metastases in the liver. NK4 inhibited tumor angiogenesis and this was associated with suppression of tumor growth in the liver (Fig. 2). The invasive and metastatic behavior of cancer leads to difficulty in attaining a long-term survival. We propose that simultaneous targeting of both and the HGF-mediated invasion-metastasis and tumor angiogenesis may prove to be a new approach for treatment of malignant tumor.

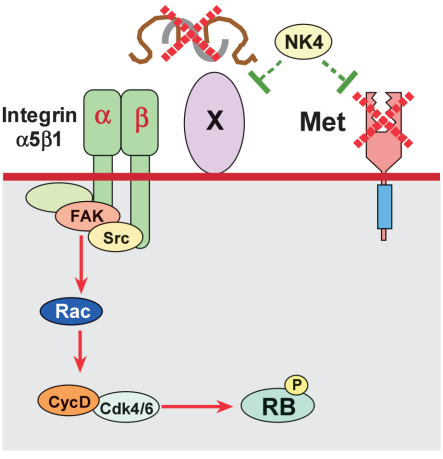

Fig. 1. Possible mechanism for angioinhibitory action of NK4.

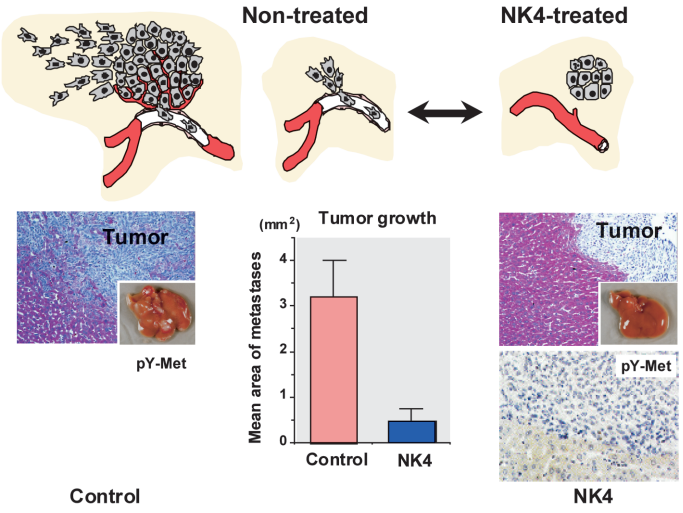

Fig. 2. Inhibition of metastasis and invasive growth of colon cancer by NK4 in mice. NK4 inhibited liver metastasis (inset) and growth of metastases (middle graph). NK4 inhibited Met phosphorylation in cancer cells (lower panels) and this was associated with inhibition of invasion of cancer cells.

Anti-cancer Approach with NK4 and Anti-angiogenic Mechanism of NK4: NK4 Gene Therapy for Malignant Mesothelioma

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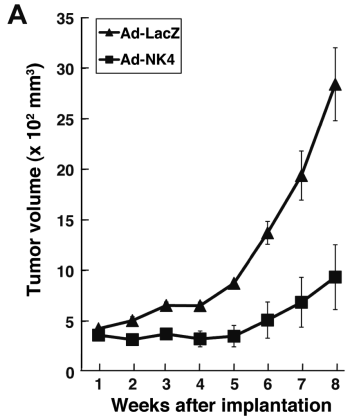
Malignant pleural mesothelioma is highly invasive, diffuse neoplasm arising from mesothelial-lined surfaces in the pleural cavity. The exposure to asbestos is causative for the development of malignant pleural mesothelioma and this disease is expected to increase dramatically over the next few decades. New approaches for the treatment of malignant pleural mesothelioma are clearly needed. We studied on HGF-Met in migration and invasion of malignant mesothelioma cells and therapeutic approach by NK4 gene therapy in mice.

In seven human malignant mesothelioma cell lines in culture, HGF stimulated Met tyrosine phosphorylation and migration of the cells. NK4 inhibited HGF-induced Met phosphorylation and migration. Among seven different human malignant mesothelioma lines, EHMES-10 cells formed growing tumor, when they were subcutaneously implanted into nude mice. Therapeutic effect of NK4 was examined in mice using recombinant adenovirus (Ad-NK4) for expression of NK4 gene. Ad-NK4 was intratumorally administered with an interval of 7 days. The growth of subcutaneous tumor was inhibited in Ad-NK4 treated mice as compared to control mice given Ad-LacZ (Fig. 1A). Analysis of blood vessels in tumor tissues indicated that NK4 inhibited the tumor angiogenesis, thereby suppressing angiogenesis-dependent tumor growth. In collagen gel culture of EHMES-10 cells, HGF enhanced invasion of the cells, whereas NK4 inhibited invasion and growth of the cells (Fig. 1B).



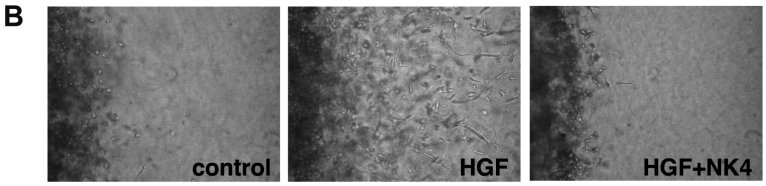


Fig. 1. Inhibition of subcutaneous mesothelioma growth by Ad-NK4 (A) and inhibition of invasion of malignant mesothelioma in collagen gel (B).

HGF-Met pathway plays a significant role in migration and invasion of malignant pleural mesothelioma cells. Together with angioinhibitory action of NK4, therapeutic value of NK4 and its combined therapy for treatment of malignant pleural mesothelioma is considerable. Further study on Ad-NK4 therapy in an orthotopic implantation model of malignant mesothelioma and its preclinical safety test are ongoing for clinical study of NK4 gene therapy.

Suppression of Met/HGF Receptor Activation by the Met Juxtamembrane Function and Cell-Cell Contact

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Inhibition of cell proliferation by cell-cell contact is a fundamental characteristic of normal cells by which cellular adhesion successfully maintains highly organized tissue architecture. Proliferation of normal hepatocytes is tightly regulated by cell-cell contact. Hepatocytes do not undergo DNA synthesis even in the presence of excess amount of HGF when the cells are in tight cell-cell contact. Under the sparse condition, HGF induced prolonged Met tyrosine phosphorylation and a marked mitogenic response. Under the confluent condition wherein hepatocytes were in tight cell-cell contact, HGF induced transient Met tyrosine phosphorylation and failed to induce mitogenic response. The activity and expression of the protein tyrosine phosphatase, LAR increased specifically in confluent hepatocytes and not in sparse hepatocytes. LAR and Met were associated, and LAR dephosphorylated Met. Specific inhibition of the LAR expression prolonged activation of Met and released contact inhibition. Thus functional association of LAR and Met underlies the inhibition of Met-mediated signaling through the dephosphorylation of Met, which specifically occurs under the confluent condition (Fig. 1A).

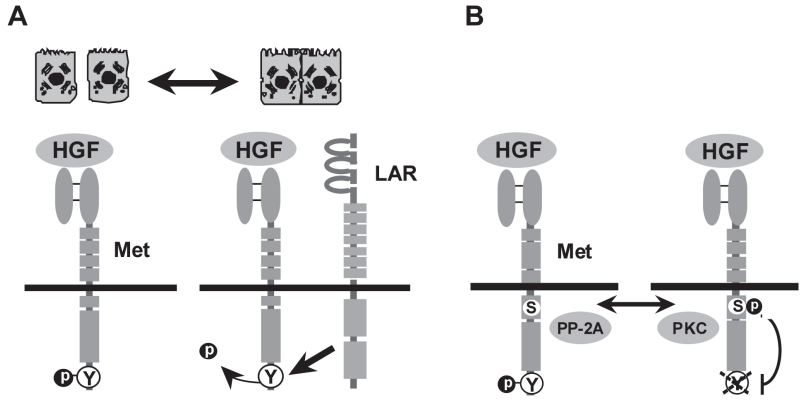

Fig. 1. Suppression of Met receptor activation through cell-cell contact (A) and juxtamembrane Ser985 phosphorylation (B).

In addition to the regulation of Met by cell-cell contact, Met activation is negatively regulated by phosphorylation of Ser985 in the juxtamembrane domain of Met (Fig. 1B). The Met juxtamembrane domain is consisted of highly conserved 47 amino acid residues and the Met lacking the juxtamembrane domain naturally exists as a splicing variant. We showed that Ser985 is phosphorylated by protein kinase-C (PKC) and dephosphorylated by protein phosphatase-2A (PP-2A). Importantly, HGF-dependent Met activation is suppressed by Ser985 phosphorylation. Physiological significance of the negative regulatory mechanisms for Met-dependent signal transduction remains to be further defined, however, we speculate that the Met negative regulation may participate in "the injured tissue-selective" activation of Met. Instead, the loss-of-function in the Met negative regulation may possibly related to malignant progression of tumor cells.

In Silico-Based Drug Discovery Targeting HGF-Met

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([1]Cancer Research Institute, Kanazawa Univ., [2]Graduate School of Science, Osaka Prefecture Univ., [3]Graduate School of Pharmaceutical Sciences, Kyoto Univ., [4]Biological Information Research Group, Advanced Industrial Science and Technology, [5]Graduate School of Engineering and [6]Center for Advanced Science and Innovation, Osaka Univ.)

To discover small molecule inhibitors for HGF-Met pathway, we organized a research group contributed by researchers in different fields and specialties (K. Matsumoto as a head researcher). In this research, specific structures involved in functional association between HGF and Met, and in their dimerization for Met activation were selected as target structures. First, about 3,000,000 chemical compounds were screened using successive combination of virtual techniques (docking simulation method, clustering technique of compounds, etc) and hundreds of compounds were selected (Fig. 1). These compounds were further screened by physical binding assay, ELISA assay, and in vitro biological assay. Through these screening, several lead compounds were obtained. These lead compounds inhibited biological activity of HGF.

Next to potentiate and optimize selected lead compounds, analysis of the crystal structure of the complex composed of a target protein and a compound is in progress (Fig. 2). Based on this "structure-based drug design", lead compounds will be potentiated and optimized.

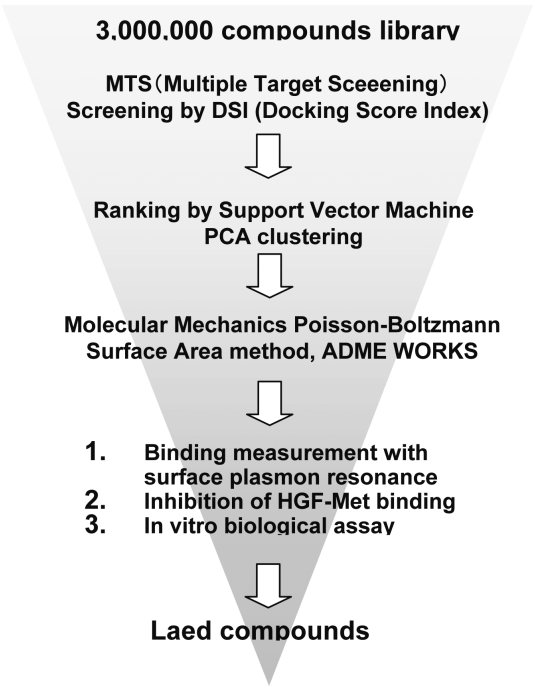

Fig. 1. Outline for screening of 3,000,000 chemical compounds, using in silico virtual screening techniques, biochemical and biological assay.

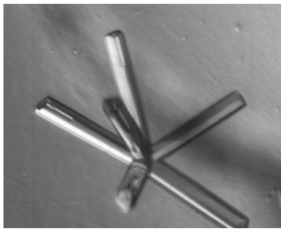

Fig. 2. Crystal of the complex composed of a target protein and selected compound.

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Other achievements

<Award>

Nature Medicine-AnGesMG BioMedical Award (Main Award): K. Matsumoto (2006)
"Discovery and therapeutic approach of NK4, an anti-cancer molecule in the 21st century"

<Granted patent>

1. Title: Asthma preparation
   Inventors: Kanehiro A, Nakamura T, Tanimoto M, Itoh W, Matsumoto K.
   Patent number: GB2414395
   Granted date: 10 / 31 / 2007

<Applied patents>

1. Title:Reconstruction of neural function by use of HGF in transplantation of olfactory mucosal epithelial cells for treatment of spinal cord injury.
   Application number: 2006-061624
   Date of application: 03 / 07 / 2006

2. Title:Variants of HGF precursor protein and their active proteins.
   Application number: 2006-116498
   Date of application: 04 / 20 / 2006

Division of Medical Oncology and Surgical Oncology

General Summary of Division of Medical Oncology and Surgical Oncology

The major obstacles of cancer treatment are metastasis and drug resistance.

The goal of our translational research is the establishment of novel molecular targeted therapeutics for overcoming metastasis and drug resistance of malignant solid tumors, such as lung cancer, pleural mesothelioma, gastric cancer and pancreatic cancer. Our main achievements are as follows.

A) Discovery of novel resistance mechanism to gefitinib in lung cancer harboring EGFR activating mutation.

Lung cancer with epidermal growth factor receptor (EGFR)-activating mutations responds favorably to the EGFR tyrosine kinase inhibitors gefitinib and erlotinib. However, 25% to 30% of patients with EGFR-activating mutations show intrinsic resistance, and the responders invariably acquire resistance to gefitinib. We demonstrated that hepatocyte growth factor (HGF), a ligand of MET oncoprotein, induces gefitinib resistance of lung adenocarcinoma cells with EGFR-activating mutations by restoring the phosphatidylinositol 3-kinase/Akt signaling pathway via phosphorylation of MET, but not EGFR or ErbB3. These findings suggest that inhibition of HGF-MET signaling may be a considerable strategy for more successful treatment with gefitinib.

B) Development of novel molecular targeted therapy for lung cancer metastasis.

Bone metastases occur in more than one-third of patients with advanced lung cancer and are difficult to treat. We previously established osteolytic bone metastasis model of human small cell lung cancer in natural killer cell-depleted SCID mice. Using this metastasis model, we showed that combined use of anti-parathyroid hormone-related protein (PTHrP) neutralizing antibody insensify the therapeutic effect of a third-generation bisphosphonate, zoledronate. In addition, follistatin (FST), an inhibitor of activin, could inhibit the production of multiple-organ metastasis, predominantly by inhibiting the angiogenesis.

C) Development of novel molecular targeted therapy for malignant mesothelioma.

Malignant pleural mesothelioma (MPM) is an aggressive malignancy, which has a poor prognosis with a median survival of less than 1 year. We recently established clinically relevant orthotopic implantation model of MPM. Using this model, we demonstrated that combined use of the antihuman VEGF neutralizing antibody, bevacizumab, enhanced therapeutic effect of pemetrexed. Moreover, vandetanib, a triple inhibitor of EGFR, VEGFR, and RET, showed dramatic therapeutic activity against MPM expressing RET oncogenic rearrangement and VEGF. Furthermore, we found that Lysophosphatidic acid (LPA), one of the simplest natural phospholipids, was a critical factor on

proliferation though its' receptor LPA_1, and on motility though LPA_2, suggesting novel therapeutic targets against MPM.

D) Development of novel molecular targeted therapy for malignant ascites of gastric cancer.

Peritoneal carcinomatosis is a frequent cause of death in patients with advanced gastric carcinoma. We demonstrated that the CXCR4/CXC12 axis facilitated the development of peritoneal carcinomatosis from gastric carcinoma. These findings suggest that CXCR4 may be a potential therapeutic target for peritoneal carcinomatosis of gastric carcinoma.

E) Early detection and molecular targeted therapy for pancreatic cancer.

Pancreatic cancer is the most chemo-refractory neoplasm, so early detection is essential for improvement the prognosis. By the highly sensitive methylation-specific polymerase chain reaction (MSP) and quantitative MSP (Q-MSP) assay using the pure pancreatic juice (PPJ), we found that promoter methylation of TFPI-2 in the PPJ could be a useful marker in the diagnosis of pancreatic cancer using an endoscopically feasible approach.

Hepatocyte growth factor induces gefitinib resistance of lung adenocarcinoma with epidermal growth factor receptor-activating mutations.

Yano S, Wang W, Li Q, Matsumoto K, Sakurama H, Nakamura T, Ogino H, Kakiuchi S, Hanibuchi M, Nishioka Y, Uehara H, Mitsudomi T, Yatabe Y, Nakamura T, Sone S.

Lung cancer with epidermal growth factor receptor (EGFR)-activating mutations responds favorably to the EGFR tyrosine kinase inhibitors gefitinib and erlotinib. However, 25% to 30% of patients with EGFR-activating mutations show intrinsic resistance, and the responders invariably acquire resistance to gefitinib. Here, we showed that hepatocyte growth factor (HGF), a ligand of MET oncoprotein, induces gefitinib resistance of lung adenocarcinoma cells with EGFR-activating mutations by restoring the phosphatidylinositol 3-kinase/Akt signaling pathway via phosphorylation of MET, but not EGFR or ErbB3. Strong immunoreactivity for HGF in cancer cells was detected in lung adenocarcinoma patients harboring EGFR-activating mutations, but no T790M mutation or MET amplification, who showed intrinsic or acquired resistance to gefitinib. The findings indicate that HGF-mediated MET activation is a novel mechanism of gefitinib resistance in lung adenocarcinoma with EGFR-activating mutations. Therefore, inhibition of HGF-MET signaling may be a considerable strategy for more successful treatment with gefitinib.

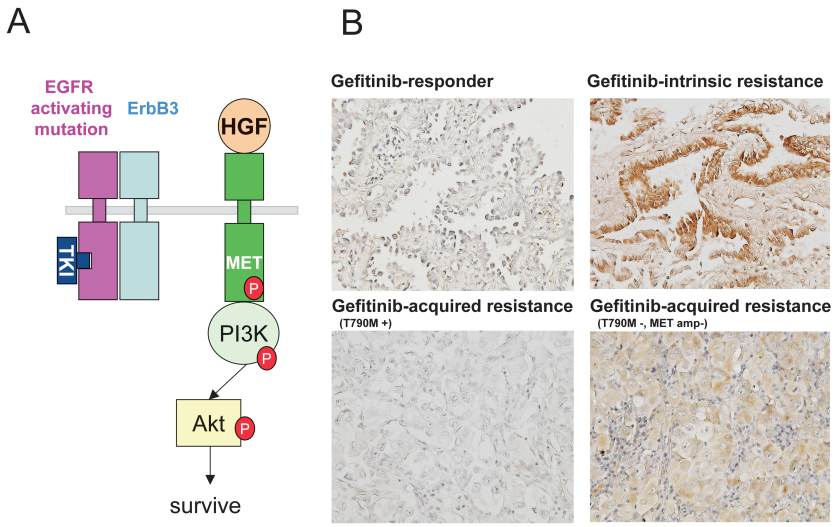
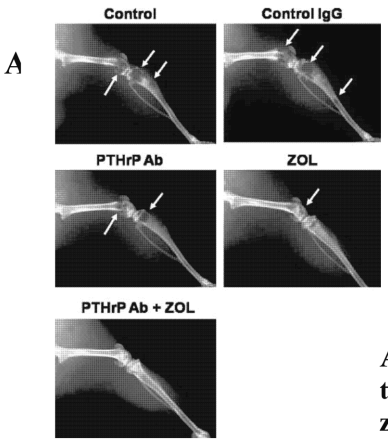


Figure A Signal transduction pathway of HGF-induced gefitinib resistance. HGF restores the phosphatidylinositol 3-kinase/Akt signaling pathway via phosphorylation of MET, but not EGFR or ErbB3. **B HGF expression in tumors from lung cancer patients**. Strong immunoreactivity for HGF in cancer cells was detected in lung adenocarcinoma patients harboring EGFR-activating mutations, but no T790M mutation or MET amplification, who showed intrinsic or acquired resistance to gefitinib.

Intensification therapy with anti-parathyroid hormone-related protein antibody plus zoledronic acid for bone metastases of small cell lung cancer cells in severe combined immunodeficient mice.

Yamada T, Muguruma H, Yano S, Ikuta K, Ogino H, Kakiuchi S, Hanibuchi M, Uehara H, Nishioka Y, Sone S.

Bone metastases occur in more than one-third of patients with advanced lung cancer and are difficult to treat. We showed previously the therapeutic effect of a third-generation bisphosphonate, minodronate, and anti-parathyroid hormone-related protein (PTHrP) neutralizing antibody on bone metastases induced by the human small cell lung cancer cell line, SBC-5, in natural killer cell-depleted severe combined immunodeficient mice. The purpose of our current study was to examine the effect of the combination of PTHrP antibody and zoledronic acid, which has been approved to treat bone metastases, against bone metastases produced by SBC-5 cells expressing PTHrP. Treatment with PTHrP antibody and/or zoledronic acid did not affect the proliferation of SBC-5 cells *in vitro*. Repeated treatments with either PTHrP antibody or zoledronic acid inhibited the formation of osteolytic bone metastases of SBC-5 cells but had no effect on metastases to visceral organs. Importantly, combined treatment with PTHrP antibody and zoledronic acid further inhibited the formation of bone metastases. Histologic assays showed that, compared with either PTHrP antibody or zoledronic acid alone, their combination decreased the number of tumor-associated osteoclasts and increased the number of apoptotic tumor cells. These findings suggest that this novel dual-targeting therapy may be useful for controlling bone metastases in a subpopulation of small cell lung cancer patients.



A. Inhibition of bone metastasis by treatment with PTHrP antibody and/or zoledronic acid in NK cell-depleted SCID mice.

Follistatin suppresses the production of experimental multiple-organ metastasis by small cell lung cancer cells in natural killer cell-depleted SCID mice.

Ogino H, Yano S, Kakiuchi S, Muguruma H, Ikuta K, Hanibuchi M, Uehara H, Tsuchida K, Sugino H, Sone S.

PURPOSE: Follistatin (FST), an inhibitor of activin, regulates a variety of biological functions, including cell proliferation, differentiation, and apoptosis. However, the role of FST in cancer metastasis is still unknown. Previous research established a multiple-organ metastasis model of human small cell lung cancer in natural killer cell-depleted SCID mice. In this model, i.v. inoculated tumor cells produced metastatic colonies in multiple organs including the lung, liver, and bone. The purpose of this study is to determine the role of FST in multiple-organ metastasis using this model. EXPERIMENTAL DESIGN: A human FST gene was transfected into the small cell lung cancer cell lines SBC-3 and SBC-5 and established transfectants secreting biologically active FST. The metastatic potential of the transfectants was evaluated using the metastasis model. RESULTS: FST-gene transfection did not affect the cell proliferation, motility, invasion, or adhesion to endothelial cells *in vitro*. I.v. inoculated SBC-3 or SBC-5 cells produced metastatic colonies into multiple organs, including the lung, liver, and bone in the natural killer cell-depleted SCID mice. FST transfectants produced significantly fewer metastatic colonies in these organs when compared with their parental cells or vector control clones. Immunohistochemical analyses of the liver metastases revealed that the number of proliferating tumor cells and the tumor-associated microvessel density were significantly less in the lesions produced by FST transfectants. CONCLUSIONS: These results suggest that FST plays a critical role in the production of multiple-organ metastasis, predominantly by inhibiting the angiogenesis. This is the first report to show the role of FST in metastases.

The therapeutic efficacy of anti vascular endothelial growth factor antibody, bevacizumab, and pemetrexed against orthotopically implanted human pleural mesothelioma cells in severe combined immunodeficient mice.

Li Q, Yano S, Ogino H, Wang W, Uehara H, Nishioka Y, Sone S.

PURPOSE: Malignant pleural mesothelioma (MPM) is an aggressive malignancy, which has a poor prognosis with a median survival of less than 1 year. The vascular endothelial growth factor (VEGF) has been reported to be an ideal therapeutic target, and a multitargeted antifolate, pemetrexed, has been clinically used for the treatment of MPM. EXPERIMENTAL DESIGN: We examined the therapeutic efficacy of the antihuman VEGF neutralizing antibody, bevacizumab, in combination with pemetrexed against two different human MPM cells, EHMES-10 and MSTO-211H, orthotopically inoculated into severe combined immunodeficient mice. RESULTS: Bevacizumab inhibited a VEGF-induced proliferation of the human endothelial cells in a dose-dependent manner, but it had no effect on the proliferation of the two MPM cell lines *in vitro*. The orthotopically inoculated EHMES-10 cells (VEGF high expressing) produced thoracic tumors and a large volume of bloody pleural effusion, whereas the MSTO-211H cells (VEGF low expressing) produced thoracic tumors and a small volume of bloody effusions. Treatment with bevacizumab effectively inhibited the production of thoracic tumors and dramatically prevented the production of pleural effusion by the EHMES-10 cells but not the MSTO-211H cells. Treatment with bevacizumab reduced the number of enlarged tumor-associated vessels and proliferating tumor cells. Moreover, treatment with bevacizumab in combination with pemetrexed more effectively suppressed the formation of the pleural effusion and prolonged the survival compared with the control and monotherapy in the EHMES-10 cell-bearing severe combined immunodeficient mice. CONCLUSIONS: These results suggest that the combined use of bevacizumab and pemetrexed may therefore be promising for controlling the progression of MPM highly expressing VEGF.

Lysophosphatidic acid stimulates the proliferation and motility of malignant pleural mesothelioma cells through lysophosphatidic acid receptors, LPA_1 and LPA_2.

Yamada T, Yano S, Ogino H, Ikuta K, Kakiuchi S, Hanibuchi M, Kanematsu T, Taniguchi T, Sekido Y, Sone S.

Lysophosphatidic acid (LPA) is one of the simplest natural phospholipids. This phospholipid is recognized as an extracellular potent lipid mediator with diverse effects on various cells. Although LPA is shown to stimulate proliferation and motility via LPA receptors, LPA_1 and LPA_2, in several cancer cell lines, the role of LPA and LPA receptors for malignant pleural mesothelioma (MPM) has been unknown. MPM is an aggressive malignancy with a poor prognosis. Therefore, the development of novel effective therapies is needed urgently. In this study, we investigated the effect of LPA on the proliferation and motility of MPM cells. We found that all 12 cell lines and four clinical samples of MPM expressed LPA_1, and some of them expressed LPA_2, LPA_3, LPA_4 and LPA_5. LPA stimulated the proliferation and motility of MPM cells in a dose-dependent manner. Moreover, LPA-induced proliferation was inhibited by Ki16425, an inhibitor of LPA_1, and small interfering RNA against LPA_1, but not LPA_2. Interestingly, LPA-induced motility was inhibited by small interfering RNA against LPA_2, but not LPA_1, unlike a number of previous reports. These results indicate that LPA is a critical factor on proliferation though LPA_1, and on motility though LPA_2 in MPM cells. Therefore, LPA and LPA receptors, LPA_2 as well as LPA_1, represent potential therapeutic targets for patients with MPM.

A

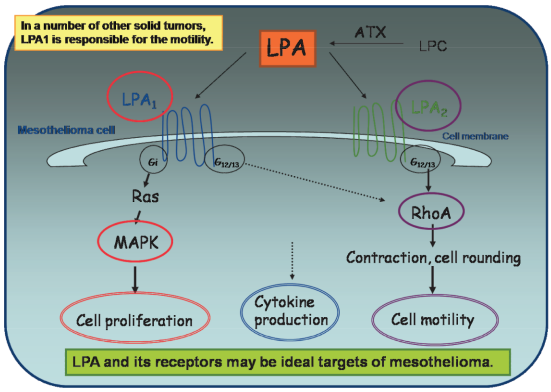


Figure A Signal transduction pathway of LPA-induced metastasis of MPM cells. LPA stimulated the proliferation via LPA_1 and facilitated the motility via LPA_2 in MPM cells.

Novel dual targeting strategy with vandetanib induces tumor cell apoptosis and inhibits angiogenesis in malignant pleural mesothelioma cells expressing RET oncogenic rearrangement.

Ogino H, Yano S, Kakiuchi S, Yamada T, Ikuta K, Nakataki E, Goto H, Hanibuchi M, Nishioka Y, Ryan A, Sone S.

Malignant pleural mesothelioma (MPM) is an aggressive malignancy with a poor prognosis, therefore development of novel effective therapies is urgent. In the present study, we investigated the therapeutic efficacy of vandetanib (ZD6474), an inhibitor of VEGFR-2, EGFR and RET tyrosine kinases, in an orthotopic model of MPM. We found that a human MPM cell line, EHMES-10, expressed RET/PTC3 oncogenic rearrangement and a large amount of VEGF. Vandetanib induced the apoptosis and inhibited the proliferation of EHMES-10 cells *in vitro* (IC(50)=0.3 microM). Once-daily oral treatment with vandetanib inhibited tumor angiogenesis, and reduced significantly the growth of thoracic tumors and the production of pleural effusions, resulting in the prolonged survival of mice in EHMES-10 orthograft model. In contrast, the selective EGFR tyrosine kinase inhibitor, gefitinib, had no effect against EHMES-10 cells both *in vitro* and *in vivo*. Our results suggest that using vandetanib to target RET-dependent tumor cell proliferation and survival and VEGFR-2-dependent tumor angiogenesis may be promising against MPM expressing RET oncogenic rearrangement and VEGF.

Role of the CXCL12/CXCR4 axis in peritoneal carcinomatosis of gastric cancer.

Yasumoto K, Koizumi K, Kawashima A, Saitoh Y, Arita Y, Shinohara K, Minami T, Nakayama T, Sakurai H, Takahashi Y, Yoshie O, Saiki I.

Peritoneal carcinomatosis is a frequent cause of death in patients with advanced gastric carcinoma. Because chemokines are now considered to play an important role in the metastasis of various malignancies, we hypothesized that they may be involved in the development of peritoneal carcinomatosis by gastric carcinoma. Human gastric carcinoma cell lines, which were all highly efficient in generating malignant ascites in nude mice upon i.p. inoculation, selectively expressed CXCR4 mRNA and protein. In particular, NUGC4 cells expressed CXCR4 mRNA at high levels and showed vigorous migratory responses to its ligand CXCL12. CXCL12 enhanced proliferation and rapid increases in phosphorylation of protein kinase B/Akt and extracellular signal-regulated kinase of NUGC4 cells. We also showed that AMD3100 (a specific CXCR4 antagonist) effectively reduced tumor growth and ascitic fluid formation in nude mice inoculated with NUGC4 cells. Additionally, we examined human clinical samples. Malignant ascitic fluids from patients with peritoneal carcinomatosis contained high concentrations of CXCL12 (4.67 ng/mL). Moreover, immunohistochemical analysis showed that 22 of 33 primary gastric tumors with peritoneal metastasis were positive for CXCR4 expression (67%), whereas only 4 of 16 with other distant metastasis were positive (25%). Notably, 22 of 26 CXCR4-expressing primary tumors developed peritoneal metastases (85%). CXCR4 positivity of primary gastric carcinomas significantly correlated with the development of peritoneal carcinomatosis ($P < 0.001$). Collectively, our results strongly suggest that the CXCR4/CXC12 axis plays an important role in the development of peritoneal carcinomatosis from gastric carcinoma. Thus, CXCR4 may be a potential therapeutic target for peritoneal carcinomatosis of gastric carcinoma.

Usefulness of endoscopic ultrasonography for the diagnoses of chronic pancreatitis.

Ohtsubo K, Okai T, Tsuchiyama T, Mouri H, Yamaguchi Y, Watanabe H, Motoo Y, Sawabu N.

We report usefulness of endoscopic ultrasonography (EUS) for the diagnoses of chronic pancreatitis (CP). We evaluated EUS features of hyperechoic foci, hyperechoic strand, lobular out gland margin, lobularity, cyst, stone, ductal dilatation, side branch dilatation, duct irregularity, hyperechoic duct margins, atrophy, localized swelling in cases with CP (30 definite and 6 probable) diagnosed by computed tomography (CT) or endoscopic retrograde cholangiopancreatography (ERCP). Hyperechoic foci, hyperechoic strand, lobularity, hyperechoic duct margins in definite or probable CP were recognized in more than 80% cases. Lobular out gland margin was observed in 14 (47%) of 30 cases with definite CP, although none with probable CP (P=0.06). In conclusions, hyperechoic foci, hyperechoic strand, lobularity, hyperechoic duct margins are useful for screening of CP, and lobular out gland margin would be reliable finding in definite CP.

Fig. 1

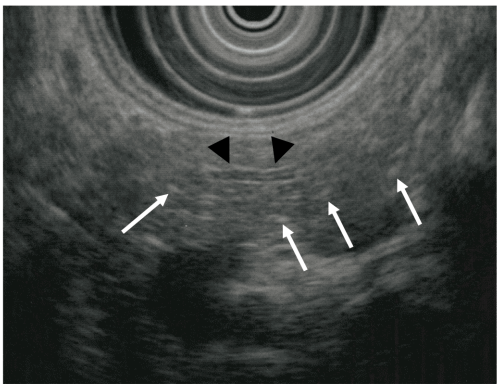


Fig. 2

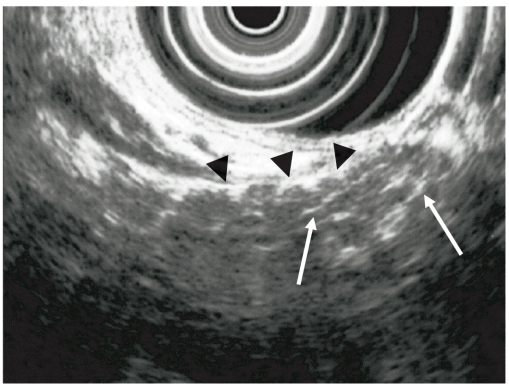


Fig. 1 Endoscopic ultrasonography showing hyperechoic foci (arrow) and hyperechoic duct margins (arrowhead).

Fig. 2 Endoscopic ultrasonography showing hyperechoic strand (arrow) and lobular out gland margin (arrowhead).

Diagnostic utility of aberrant methylation of tissue factor pathway inhibitor 2 in pure pancreatic juice for pancreatic carcinoma.

Jiang P, Watanabe H, Okada G, Ohtsubo K, Mouri H, Tsuchiyama T, Yao F, Sawabu N.

The tissue factor pathway inhibitor 2 (TFPI-2) is a Kunitz-type serine proteinase inhibitor. Recently, the aberrant methylation of TFPI-2 was detected frequently in pancreatic carcinoma (PCa) tissues but not in normal pancreatic tissues. We analyzed the aberrant methylation of TFPI-2 in the pure pancreatic juice (PPJ) aspirated endoscopically from patients with various pancreatic diseases. Using the highly sensitive methylation-specific polymerase chain reaction (MSP) and quantitative MSP (Q-MSP) assay, we investigated the aberrant methylation of TFPI-2 in nine human PCa cell lines and in the PPJ from patients with PCa, intraductal papillary mucinous neoplasms (IPMN) and chronic pancreatitis (CP). The incidence of aberrant TFPI-2 methylation was seven (77.8%) of nine PCa cell lines by Q-MSP. In cell lines, the expression of TFPI-2 mRNA by quantitative reverse transcription-polymerase chain reaction showed an inverse correlation to the aberrant methylation of TFPI-2. The incidence of aberrant TFPI-2 methylation in the PPJ was 21 (58.3%) of 36 PCa patients, three (17.6%) of 17 IPMN and one (4.8%) of 21 CP by MSP assay. Using a suitable cut-off value of 2.5 according to the receiver operating characteristic curve, the incidence of aberrant TFPI-2 methylation in the PPJ by real-time MSP was 18 (62.1%) of 29 PCa patients, one (5.1%) of 17 IPMN and three (14.3%) of 21 CP, respectively. The incidence of quantitative TFPI-2 hypermethylation in the PPJ with PCa was significantly higher than that with IPMN ($P < 0.001$) or CP ($P < 0.001$). Moreover, the aberrant methylation rate of TFPI-2 in the PPJ was 100%, as observed (6/6) in the PCa patients with liver metastasis, and 86.7% (26/30) in stages IVa + IVb of PCa by Q-MSP assay. These results suggest that promoter methylation of TFPI-2 in the PPJ may be a useful marker in the diagnosis and progression of PCa using an endoscopically feasible approach.

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Meetings and Seminars

第27回 がん研究所セミナープログラム

2006(平成18)．2．8～9
金沢大学医学部記念館2階ホール，金沢大学がん研究所会議室

◆細胞周期制御研究分野
○サマリー　善岡克次
○アミロイド前駆体APPに結合するFe65L2の転写に及ぼす影響　棚橋浩

◆組織分子構築研究分野
○サマリー　向田直史
○Pim-3の膵臓癌での過剰発現の意義の検討　藤井千文

◆分子薬理学研究分野
○サマリー　大島正伸
○組織マクロファージが胃癌発生に及ぼす影響の研究　大島浩子

◆遺伝子・染色体研究分野
○サマリー　平尾敦
○組織幹細胞特異的発現分子を指標とするがん幹細胞同定マウスモデルの構築　仲一仁

◆細胞分子病態研究分野
○サマリー　山本健一
○c-Ablファミリー分子のクロマチンにおけるRad51の機能制御の意義　清水弘子
○アルキル化剤，活性酸素によるATMの活性化機構の解析　小林昌彦

◆遺伝子診断研究分野
○サマリー　源利成
○新たながん標的キナーゼGSK3βの活性・機能解析と大腸癌制御への応用　源利成

◆分子標的薬剤開発センター
○サマリー　須田貴司
○抗腫瘍性ヌクレオシドに対する感受性規定因子の同定と応用　遠藤良夫
○ADAMTS-1の卵胞生育過程，損傷治癒過程における役割の解析　久野耕嗣
○Fasリガンド刺激による炎症誘導作用の分子機構　今村龍
○Fas-associated factor1はPYPAF1で誘導されるNF-κB活性化を抑制する　木下健

第28回　がん研究所セミナープログラム

2007(平成19)．2．15，3．8
金沢大学がん研究所会議室，金沢大学医学部記念館2階ホール

◆腫瘍制御研究分野

○サマリー　源　利成
○GSK3βは消火器がん治療の新しい分子ターゲットである　源　利成
○エピジェネティクスを標的にするがん診断・治療法の開発　川上和之

◆腫瘍外科研究分野

○サマリー　高橋豊
○Tumor dormancy therapyの新たな展開－癌を標的とする，放射線，化学，免疫療法の併用－　高橋豊
○胃癌に対する腹腔鏡手術と工学技術の応用　－当教室におけるこれまでの取り組み－　表和彦
○胃癌腹膜播種成立における特異的ケモカインの同定とその臨床応用　安本和生

◆腫瘍内科研究分野

○サマリー　渡邊弘之
○膵液中がん関連遺伝子のメチル化異常の検索による膵腫瘍診断へのアプローチ　渡邊弘之
○各種膵疾患における膵液中，*ppENK*のメチル化異常に関する検討　；MSP法とpyrosequencing法を対比して　大坪公士郎
○当科における内視鏡的胆道ドレナージの検討　毛利久継
○MCP-1が誘導する肝癌自殺遺伝子治療の免疫賦活化の検討　土山智也

◆細胞機能統御研究分野

○サマリー　佐藤博
○がん浸潤における細胞運動とMMP活性発現の協調的制御機構の解析　滝野隆久
○抗腫瘍性ヌクレオシドに対する感受性規定因子の同定と応用　遠藤良夫
○ADAMTS-1中和抗体によるコラーゲン誘導性関節炎の抑制　久野耕嗣
○新規MT1-MMP活性検出法の応用　宮森久志

◆シグナル伝達研究分野

○サマリー　善岡克次
○JNKの転写因子MafBに及ぼす影響　棚橋浩

第29回　がん研究所セミナープログラム

金沢大学大学院医学系研究科　がん医科学専攻　ジョイントセミナー

2008(平成20). 3 . 25～ 3 . 26
医学部Ｆ棟 第２講義室

◆細胞機能統御研究分野

○サマリー	佐　藤　　博
○がん細胞運動とMT1-MMP活性発現の協調的制御機構の解析	滝　野　隆　久
○抗腫瘍性シトシンヌクレオシドの感受性予測マーカーの開発と応用	遠　藤　良　夫
○ADAMTS-1遺伝子欠損マウスにおける尾椎椎間板形成異常	久　野　耕　嗣

◆分子生体応答研究分野

○サマリー	向　田　直　史
○自己免疫寛容誘導に関わる胸腺樹状細胞の役割とそのメカニズムの解明	馬　場　智　久

◆免疫炎症制御研究分野

○サマリー	須　田　貴　司
○抑制性Apaf-1様分子PYNODの機能解析	今　村　　龍
○Flagellin刺激下のヒトマクロファージ様細胞株における caspase-1非依存的なinterleukin-1β分泌	木　下　　健

◆遺伝子・染色体構築研究分野

○サマリー	平　尾　　敦
○マウス白血病モデルを用いた白血病幹細胞の自己複製機構の解析	仲　　一　仁

◆幹細胞医学研究分野

○サマリー	西　村　栄　美
○ニッチにおける組織幹細胞の維持機構の解析	田　所　優　子
○ポリコーム遺伝子群による転写抑制型クロマチンの伝播機構についての解析	青　戸　隆　博

◆腫瘍遺伝学研究分野

○サマリー	大　島　正　伸
○マウスモデルを用いた胃がんの分子病理発生の解析	大　島　浩　子

◆ゲノム分子病態研究分野

○サマリー	山　本　健　一

International Symposium on Tumor Biology in Kanazawa 2006

Program Schedule

January 19 Fri.

Opening Remarks : **Hiroshi Sato** (Cancer Res. Inst., Kanazawa Univ.)

Section A: Tumor environment

Chairperson: **Naofumi Mukaida** (Cancer Res. Inst., Kanazawa Univ.)

Thaddeus S. Stappenbeck (Washington Univ. School of Medicine, USA)

Maintenance and Repair of the Colon Epithelial Barrier

Okio Hino (Juntendo Univ. School of Medicine)

Cancer Environment -Intentional delay of carcinogenesis

Chairperson: **Katsuji Yoshioka** (Cancer Res. Inst., Kanazawa Univ.)

Masayuki Miyasaka (Osaka Univ. Graduate School of Medicine,)

Chemokines in tumor progression and metastasis

Masanobu Ohshima (Cancer Res. Inst., Kanazawa Univ.)

COX-2 Pathway and Gastric Tumrigenesis

Section B: Stem cell and regeneration

Chairperson: **Atsushi Hirao** (Cancer Res. Inst., Kanazawa Univ.)

Mari Dezawa (Kyoto Univ. Graduate School of Medicine.)

Bone marrow stromal cells: applications for neuro-degenerative, neuro-traumatic and muscle degenerative diseases

Atsushi Iwama (Graduate School of Medicine, Chiba Univ.)

Molecular mechanisms involved in the leukemic stem cell self-renewal

Section C: Molecular Target in cancer therapy

Chairperson: **Nobuyuki Takakura** (Cancer Res. Inst., Kanazawa Univ.)

Mitsuo Wakasugi (Faculty of Pharmaceutical Sciences, Kanazawa Univ.)

Functional analysis of DDB in DNA repair and cell growth

Takashi Minami (Labs for Systems Biology and Medicine, RCAST, The Univ. of Tokyo)

Identification of Down Syndrome Critical Region-1 as an Auto-inhibitory factor, from the Systematic analysis of Agonists-treated endothelium

Hideyuki Saya (Faculty of medical and Pharmaceutical Sciences, Kumamoto Univ.)

Dynamics and molecular mechanisms of anti-cancer therapies

Closing Remarks : **Hiroshi Sato** (Cancer Res. Inst., Kanazawa Univ.)

International Symposium on Tumor Biology in Kanazawa 2007

Program Schedule

January 25, Thu.

Opening Remarks : **Hiroshi Sato** (Cancer Res. Inst. Kanazawa Univ.)

Ⅰ. Molecular Targets in Human Cancer

Chairperson: **Takashi Suda** (Cancer Res. Inst., Kanazawa Univ.)

Kenji Yokoi (Graduate School of Medicine, Kanazawa Univ.)

Phosphoproteome analysis of resistance to the EGFR and VEGFR tyrosine kinase inhibitor in human pancreatic cancer cells

Kazuo Yasumoto (Cancer Res. Inst., Kanazawa Univ.)

Role of the CXCL12/CXCR4 axis in peritoneal carcinomatosis of gastric cancer -CXCR4, a potential therapeutic target for gastric cancer dissemination-

Toshinari Minamoto (Cancer Res. Inst., Kanazawa Univ.)

GSK3β , a novel therapeutic target in gastrointestinal cancer

Ⅱ. Cellular Targets in Cancer (1)

Chairperson: **Katsuji Yoshioka** (Cancer Res. Inst. Kanazawa Univ.)

Kensuke Yamauchi (Graduate School of Medicine, Kanazawa Univ.)

Development of real-time subcellular dynamic multicolor imaging in live mice

Su-Jae Lee (Korea Institute of Radiological & Medical Sciences)

Reactive oxygen species in malignant cellular transformation, mitotic checkpoint control, and apoptotic cell death

Ⅲ. Cellular Targets in Cancer (2)

Chairperson: **Atsushi Hirao** (Cancer Res. Inst. Kanazawa Univ.)

Kazuhito Naka (Cancer Res. Inst. Kanazawa Univ.)

A study on establishment of a mouse model for identifying leukemic stem cells

Masaki Mori (Medical Institute of Bioregulation, Kyushu University)

Cancer stem cell research in solid cancer

Ⅳ. Signaling Deregulation in Cancer

Chairperson: **Masanobu Oshima** (Cancer Res. Inst. Kanazawa Univ.)

Vladimir S. Spiegelman (University of Wisconsin School of Medicine and Public Health, Madison, WI)

Mechanisms of stabilization of βTrCP1 and c-myc mRNA in response to Wnt/β-catenin

signaling

Tesshi Yamada (National Cancer Center Research Institute)

Proteomic analysis of β-catenin-mediated colorectal carcinogenesis

Akira Kikuchi (Graduate School of Biomedical Science, Hiroshima University)

Regulation of cellular functions by Wnt signaling and tumorigenesis due to its abnormalities

Serge Y. Fuchs (University of Pennsylvania)

Activation of β-Trcp E3 ubiquitin ligases in human cancers: mechanisms and outcomes

Closing Remarks : **Masayoshi Mai** (Professor Emeritus, Kanazawa University)

International Symposium on Tumor Biology in Kanazawa 2008

Program Schedule

January 24, Thu.

Opening Remarks : **Hiroshi Sato** (Cancer Res. Inst., Kanazawa Univ.)

Session 1.

Chairperson: **Kunio Matsumoto** (Cancer Res. Inst., Kanazawa Univ.)

Shigeki Higashiyama (Ehime University)

Inner Nuclear Membrane Translocation of Plasma Membrane-anchored Growth Factors; Its molecular mechanisms and pathophysiological roles in tumor cells

NeilA. Bhowmick (Vanderbilt University)

TGF-β in tumor-stromal interactions

Session 2.

Chairperson: **Takeshi Suzuki** (Cancer Res. Inst., Kanazawa Univ.)

Takuro Nakamura (Japanese Foundation for Cancer Research)

Identification of New Molecular Pathways in Carcinogenesis by Retroviral Tagging

Shyam K. Sharan (National Cancer Institute)

A Mouse Embryonic Stem Cell Functional Based Assay to Study Variants Identified in Human Breast Cancer Susceptibility Genes

Session 3.

Chairperson: Emi Nishimura (Cancer Res. Inst., Kanazawa Univ.)

Kyu-Sil Choi (Wonkwang University)

In Vivo Assessment of Tumor Angiogenesis Using Molecular Imaging

Hideki Taniguchi (Yokohama City University)

Excessive Self-renewal of Hepatic Stem Cells Drives Cancer Initiation

Hiroko Ohshima(Cancer Res. Inst., Kanazawa Univ.)

Novel Gastric Cancer Mouse Model by Transgenic Expression of Wnt1 and Prostaglandin E2

Session 4.

Chairperson: **Naofumi Mukaida** (Cancer Res. Inst., Kanazawa Univ.)

Masanori Hatakeyama (Hokkaido University)

Cellular Targets of *Helicobacter pylori* CagA in Gastric Carcinogenesis

Xueguang Zhang (Suzhou University)

Expression of CD40 on Glioma and the Relationship with Tumor Angiogenesis

Masaaki Nakaya (Kanazawa Univ. Graduate School of Medical Science)
Cell Polarity Regulation and Cancer Metastasis

Closing Remarks : **Hiroshi Sato** (Cancer Res. Inst., Kanazawa Univ.)

Organization and Personnel

as of February, 2009

Cancer Research Institute
Successive Directors and Acting Directors of the Institute

Dr.Hajime Okamoto	(岡本　肇)	June	1, 1967	～	March	31, 1968
Dr.Tachiomaru Ishikawa	(石川太刀雄丸)	April	1, 1968	～	March	31, 1971
Dr.Ryo Itoh	(伊藤　亮)	April	1, 1971	～	April	1, 1978
Dr.Saburo Koshimura	(越村　三郎)	April	2, 1978	～	April	1, 1982
Dr.Yoriaki Kurata	(倉田　自章)	April	2, 1982	～	April	1, 1984
Dr.Motoichi Hatano	(波田野基一)	April	2, 1984	～	March	31, 1988
Dr.Shunsuke Migita	(右田　俊介)	April	1, 1988	～	March	31, 1990
Dr.Tadanori Kameyama	(亀山　忠典)	April	1, 1990	～	March	31, 1993
Dr.Morinobu Takahashi	(高橋　守信)	April	1, 1993	～	March	31, 1997
Dr.Masayoshi Mai	(磨伊　正義)	April	1, 1997	～	March	31, 2001
Dr.Ken-ichi Yamamoto	(山本　健一)	April	1, 2001	～	March	31, 2005
Dr.Hiroshi Sato	(佐藤　博)	April	1, 2005	～	Present	

Department of Molecular Cancer Cell Biology

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Kunio Matsumoto

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