

**CANCER
RESEARCH
INSTITUTE
REPORT**

2000 - 2002

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Institute Report

2000—2002

SCIENTIFIC REPORTS

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**Division
of
Molecular Biology**

General Summary of Division of Molecular Biology

After the reorganization of the division, the division has been dedicated to basic researches on Hepatitis B Virus (HBV) and Hepatitis C Virus (HCV), the two predominant causes of chronic hepatitis and eventual hepatocellular carcinoma (HCC) among the world. We chose two different targets of the viruses, functions of HBV X protein (HBx) to elucidate its oncogenic role, and molecular properties of HCV NS5B, the central catalytic enzyme of HCV RNA replication, to find better strategies to eradicate HCV infection. Recently the third project on telomere and telomerase with *S. cerevisiae* as well as human has been started after when Dr. N. Hayashi has joined as a research associate. The project may elucidate positive roles of these virus infections in immortalization and overcome of crisis of cells, both of which tightly link to tumorigenesis.

A) Projects on HBx

We proposed that HBx acts as a transcriptional coactivator through direct interaction among HBx, RPB5 (a pol II subunit), and TFIIB. Recently DNA-binding ability of RPB5 was detected and 4 essential residues for the binding were determined. A general transcription factor, TFIIF, was found to bind RPB5 through the middle part of RAP30 in which we identified two essential amino acid residues (126R, 131Q) for the interaction, strongly suggesting that RPB5 and RAP30 are responsible for the association of pol II and TFIIF. A novel protein, RPB5-mediating protein (RMP), was cloned and identified to be a functional antagonist of HBx. The subcellular localization of RMP is regulated by the coiled-coil domain for cytoplasmic localization, and NLS for nuclear localization. Recently, we identified an interacting partner of coiled-coil domain of RMP.

B) Projects on HCV

We have established a method to purify soluble bacterial recombinant NS5B, and identified novel 5 amino acid residues essential for its RdRP activity. Two of the 5 residues are important for oligomeric interaction of NS5B, which is an essential step for RdRP synthesis. We found that the C-terminal 21 aa of NS5B contained a membrane-anchoring domain. NS5B has a nucleolar targeting signal, which may function to recruit nucleolar component(s) to replication complex of HCV. Additionally NS5A, a putative cofactor of RdRP, was determined to interact with NS5B through two discontinuous regions in vivo and in vitro. A systematic alanine scanning of NS5B elucidated the critical 5 amino acid residues identified newly for RdRP, indicating the unique role of the loop region of NS5B.

C) Telomere and telomerase

We focused on yeast *CDC13*, which encodes a binding protein of single stranded G-rich sequence of telomere and *STMI* encoding a binding protein to G:G quadruplex. *STMI* interacted directly with *CDC13* and suppressed Ts⁻ growth of *cdc13-1* on multicopy. On the other hand, we screened protein phosphatase mutation affecting telomere functions and cellular life span. We established the expression and purification of recombinant human telomerase reverse-transcriptase (hTERT) and succeeded in reconstitution of telomerase activity in vitro with the soluble form of hTERT and telomerase RNA (TR) transcribed in vitro. We found that hTERT can oligomerize in vivo and in vitro through two independent regions out of the central part, palm and fingers subdomain. Oligomerization would be another step of regulation of telomerase activity. We are currently focusing further purification of the recombinant hTERT to characterize subcellular localization and functions of hTERT.

We are partly collaborating with Drs. S. Kaneko, K. Kobayashi at the 1st. Int. Med., Med. School, and K. Yoshioka at Cancer Res. Inst., Kanazawa Univ., and internationally with Drs. R. Roeder at Rockefeller Univ., U.S.A., B. Yen at UCSF, U.S.A., D. Yul, at KRIBB, Korea, K. Kim at Seoul Univ., Korea, X. Zhang and H. Tang at Sichuan Univ., China, B. Dashnyam at Biotech. Inst., Mongolia, and W. Hahn at Harvard Univ., U.S.A.

Direct interaction between the subunit RAP30 of TFIIF and RNA polymerase subunit 5 (RPB5) which contributes to the association between TFIIF and RNA polymerase II

Wenxiang Wei, Dorjbal Dorjsuren, Yong Lin, Weiping Qin, Takahiro Nomura, Naoyuki Hayashi and Seishi Murakami

General transcription factor TFIIF, heteromeric tetramer of RAP30 and RAP74, assembles in initiation complex and associates with Pol II but Pol II subunit responsible for the interaction remains unclear. We examined whether TFIIF interacts with RPB5, the exposed domain of which binds HBx and a novel regulatory protein, RPB5-mediated protein (RMP).

The results demonstrate that RPB5 directly binds RAP30 *in vitro* using the purified recombinant proteins, and *in vivo* in COS1 cells transiently expressing recombinant RAP30 and RPB5. The RAP30-binding region was mapped within the middle part (aa 47-120) of RPB5, which partly overlaps the HBx-binding region. Although the middle parts (aa 101-170) and the N-terminal part of RAP30 independently bind RPB5, the latter was not involved in the RPB5-binding when RAP30 was present in TFIIF complex. By scanning the middle part of RAP30 by clustered alanine-substitution mutants, then point alanine-substitutions pinpointed two residues critical for the RPB5-binding in *in vitro* and *in vivo* assays. Wild, but not Y124A and Q131A, in GST-RAP30 forms coexpressed with FLAG-RAP74 efficiently recovered endogenous RPB5 to the FLAG-RAP74-bound anti-FLAG M2 resin. The recovered endogenous RPB5 is assembled in pol II as demonstrated immunologically. Interestingly, coexpression of the middle part of RPB5 and wild RAP30 in GST-forms inhibited recovery of endogenous pol II to the FLAG-RAP74-bound M2 resin, strongly suggesting that the RAP30-binding region of RPB5 inhibited the association of TFIIF and pol II. Taken together, the exposed domain of RPB5 interacts with RAP30 of TFIIF and is important for the association between pol II and TFIIF.

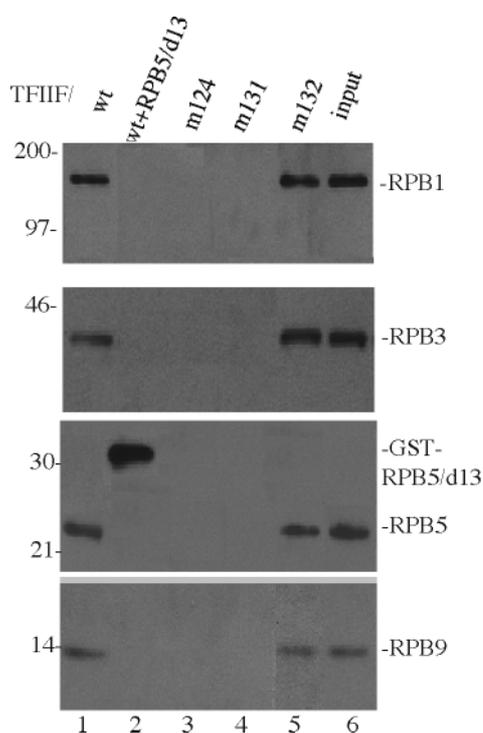


Figure RAP30 is involved in the association of Pol II and TFIIF *in vivo*.

TFIIF mutants defect in binding with endogenous Pol II. COS1 cells were transfected with mammalian expression plasmids of pNKFLAG-RAP74 and pNKGST-RAP30 or its mutants as indicated on the top. In lane 2 additional expression vector of pNKGSTRPB5/d13 was co-transfected. About 2.5mg of total lysates were immunoprecipitated with 20 μ l of anti-FLAG M2 antibody-bound resin. After being washed, the recovered proteins were eluted, fractionated by 12.5% SDS-PAGE and detected by western blot analysis with antibodies against RPB1, RPB3, RPB5 and RPB 9 as indicated to the right Lane 6 shows 3% of input of lysate used for lane 1.

DNA-binding ability of RNA polymerase subunit 5 (RPB5)

Shijun Zhang, Luvsanjav Delgermaa, and Seishi Murakami

RPB5 is a RNA pol II subunit, which is located at the tip of lower jaws of pol II. Recent studies show that RPB5 is in close contact to promoter DNA when Pol II is recruited into the preinitiation complex. It interacts with the basal transcription factors IIB and IIF, and transcriptional regulatory factors such as Hepatitis B Virus X protein (HBx) and a novel regulatory protein, RMP (RPB5-mediating protein), through the middle part. We examined whether RPB5 bind with non-specific DNA.

The results of DNA binding assay using non-specific DNA cellulose demonstrated that RPB5 bound double stranded DNA in vitro, but not single stranded one. By analyzing DNA-binding of truncated forms of RPB5, the DNA-binding required the middle part (aa 47-120) of RPB5. Scanning of the middle part of RPB5 by clustered alanine-substitutions and further point alanine-substitutions pinpointed three sequences and finally 4 residues, V74, V82, T111 and S113, are critical for the DNA-binding. The former two residues may not contribute directly to DNA-binding since the residues seem to be not exposed according to the crystal model. Interestingly, two proline residues, P80 and P112, predicted to be closer to DNA, have no contribution to the DNA-binding. The binding ability is not stronger compared with RAP30's DNA binding in the same experiment condition. DNA-binding of RPB5 can be drastically inhibited in the presence of RAP30 missing the C-terminal DNA-binding domain, RMP and HBx. Interestingly, all of them can interact with RPB5 through the same domain for the DNA-binding, suggesting a possibility that these proteins may regulate DNA-binding ability of RPB5 in transcription

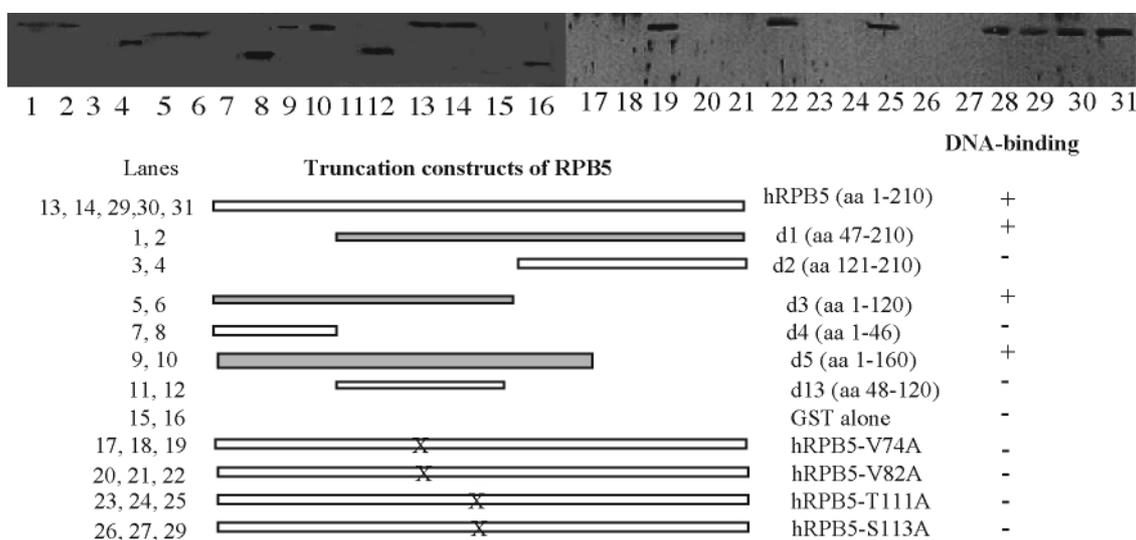


Figure DNA-binding ability of RPB5

Binding ability to double stranded DNA-cellulose was examined. Lanes 2, 4, 6, 8, 10, 12, 14, 16, 19, 22, 25, 28, 31, are 5% of input proteins, respectively. For the experiments with the point mutant proteins were independently repeated twice. Bound proteins were applied 12.5% SDS-PAGE and subjected to Western blotting with anti-RPB5 antibody.

Subcellular localization of RMP, RNA polymerase subunit 5-mediating protein.

Luvsanjav Delgermaa, Naoyuki Hayashi, Takahiro Nomura, Dorjsuren Dorjbal, Bumbei Dashnyam, and Seishi Murakami

We previously identified a novel protein, RMP, which associates with RNA polymerase II through RPB5 and negatively modulates activated transcription *in vivo*. We confirmed that endogenous RMP is mostly in cytoplasm although a minor portion was in nucleus. Therefore, a various versions of RMP constructs fused to GFP were transiently expressed in HLE cells and was cytologically examined by a confocal fluorescent microscopy. The N-terminal half of RMP localizes exclusively in cytoplasm, and the C-terminal half is exclusively nucleus. A NLS at aa 339-344 was identified to be functional since a mutated NLS abolished the nuclear localization of RMP deleting the N-terminal portion. A region responsible for the cytoplasmic localization of RMP was mapped within aa 88 - 118 which well coincided with a predicted coiled-coil domain (aa 83-124). The domain by itself changed subcellular localization of TFIIB from nucleus to cytoplasm when it was fused to GFP-TFIIB. The negative regulatory function of RMP was found to be dependent upon nuclear distribution of RMP since the ability of RMP to corepress the activated transcription by GalVP16 *in vivo* was abolished by the mutation of NLS, and was much augmented by deleting CLS. These results demonstrate that two sequences, the NLS and the coiled-coil domain, are both important for the subcellular localization of RMP. We recently identified a putative interacting partner with the coiled-coil domain by a yeast two-hybrid selection.

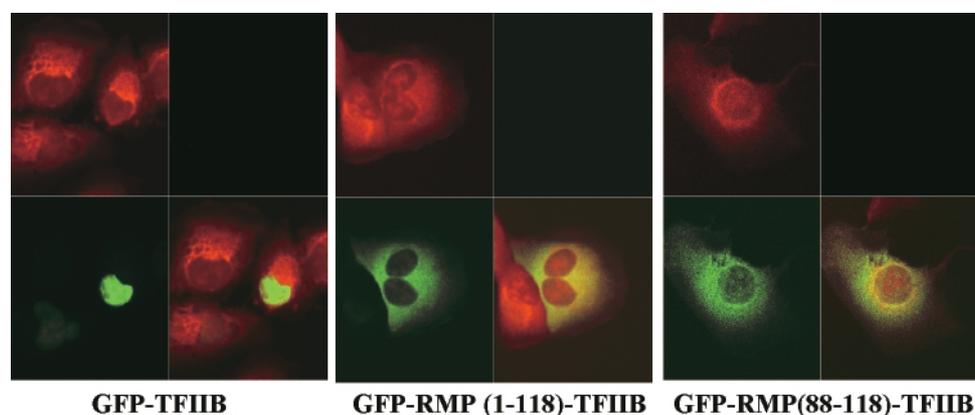


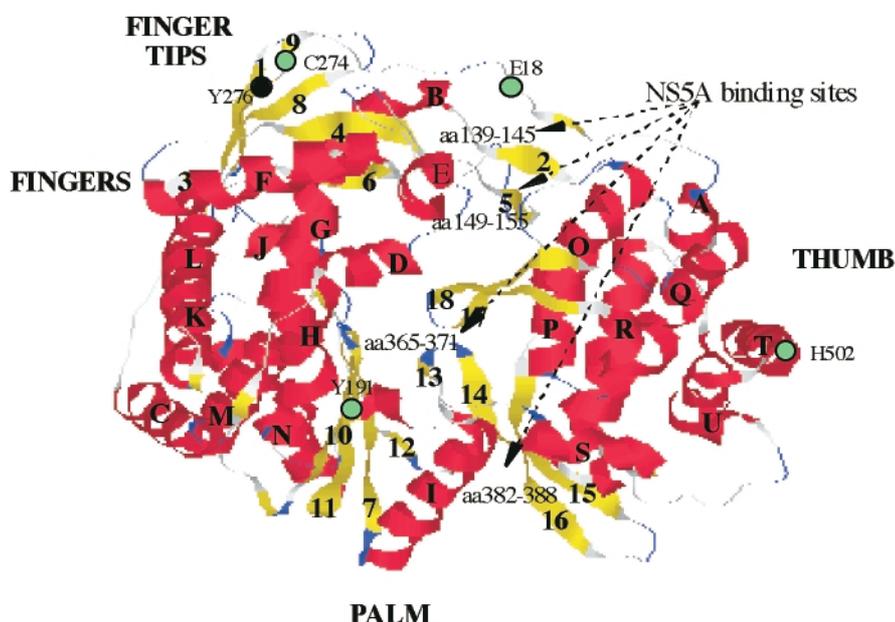
Figure The coiled-coil domain of RMP acts as a cytoplasmic localization sequence.

The N-terminal regions spanning the coiled-coil domain were fused to GFP-TFIIB which was a typical nuclear protein as shown in left panel. Aa 1-118 or the coiled-coil domain alone changed subcellular localization of GFP-TFIIB from nuclear to cytoplasmic as shown in center and right panels. These proteins were transiently expressed in HLE cells and subjected to confocal analyses.

Mutational analysis of the structure and functions of Hepatitis C Virus RNA-dependent RNA polymerase (RdRP)

Weiping Qin, Tatsuya Yamashita, Yukihiro Shirota, Yong Lin, Wenxiang Wei, and Seishi Murakami

HCV NS5B is RdRP, a central catalytic enzyme for HCV replication. To further understand the structure and functions of NS5B, we introduced a series of 27 clustered and 19 point substitution mutations within and outside the ready-known motifs conserved among RdRP by alanine scanning and investigated effects of these mutants on putative properties of NS5B. The GST-fused form of NS5Bt, deleting the C-terminal membrane-anchoring domain, were bacterially expressed and purified as reported previously. Four clustered mutants, cm20t, cm194t, cm2t and cm3t, are defective in RdRP activity. By further analysis with point mutations within these regions, E18, Y191, C274, Y276, and H502, were found to be critical for the RdRP activity. By RNA filter binding assay, 3 sequences (aa 149-155, aa 220-226 and aa 276-280) were important for single strand RNA binding, but finally Y276 was the only residue essential for template/primer-binding. In light of the crystal structure models recently reported, our result indicated that the longer loop in the N-terminal region and the helix located at the top of thumb play important roles in the catalytic activity of RdRP of NS5B. These two substructures are unique among RdRPs and the other reverse transcriptases reported implies the uniqueness of HCV RdRP not only in structure but also in function.



The newly identified 5 residues essential for RdRP activity of NS5B.

The 5 residues (circle in green or black color) are E18 at the long loop, Y191 at palm, C274, and Y276 at finger tip, and at thick thumb, respectively. Y276 (black circle) is defective in template/primer-binding prerequisite to RdRP activity. E18 and H502 residues are far from the Catalytic pocket as reported (Qin et al., Hepatol., 2001).

Oligomeric interaction of Hepatitis C Virus NS5B is critical for catalytic activity of RNA dependent RNA polymerase.

Weiping Qin, Hong Luo, Takahiro Nomura, Naoyuki Hayashi, Tatsuya Yamashita, and Seishi Murakami

HCV NS5B is an RNA-dependent RNA polymerase (RdRP), a central catalytic enzyme for HCV replication, which has the "palm and fingers" substructure. We recently identified 5 novel residues critical for RdRP activity (Hepatology, 33:728-737 (2001)). Among them, E18 and H502, far from the catalytic center, may be involved in conformational change(s) for RdRP activity as addressed in some "palm and fingers" enzymes. We examined the possibility that NS5B is oligomerized, and we could detect interaction between two different tagged NS5B proteins *in vitro* and transiently expressed in mammalian cells. By scanning 27 clustered and then point alanine substitutions *in vivo* and *in vitro*, E18 and H502 were found to be critical for the homomeric interaction *in vivo* and *in vitro*, strongly suggesting a close relationship between the oligomerization and RdRP activity of NS5B. All mutants with substitutions at these two residues failed to bind wild type NS5B, however E18H interacted with H502E *in vitro* and *in vivo*. Interestingly, the NS5B protein with either E18H or H502E did not exhibit RdRP activity, but a mixture of the two mutant proteins did. These results clearly indicate that these two residues of HCV NS5B are critical for the oligomerization that is prerequisite to RdRP activity.

Table Effects of NS5B mutants defective in oligomerization on the RdRP activity

Proteins	UMP(cmp) n=3	Relative ³ percentage (%)
WT	39,200±3,800	100
Nonfused NS5Bt	36,000± 3,200	91
E18A	1,100 ±100	2.5
E18D	800 ±100	2
H502A	900 ±110	2.5
H502K	1,200 ±110	3
H502R	900 ±110	2.5
E18H	2,000±20	5
H502E	1,800±200	4
E18H+H502E	28,000±1,000	70
G317V	1,200± 200	5
G317V+WT	38,200±3,900	100
Y276A+WT	37,480±3,800	95
GST	200 ± 40	0.5
GST+WT	38,000±4,000	100

¹ All experiments were independently done three times. The RdRP activity of wild GST-NS5Bt was taken as the standard.

Hepatitis C Virus NS5A binds RNA-Dependent RNA polymerase NS5B and modulates RdRP activity

Yukihiro Shirota, Hong Luo, Weiping Qin, Shuichi Kaneko, Tatsuya Yamashita, Kenichi Kobayashi, and Seishi Murakami

HCV NS5B is RNA-dependent RNA polymerase (RdRP), the essential catalytic enzyme for HCV replication. Recently, NS5A has been reported to be important for the establishment of HCV replication *in vivo* by the adaptive mutations although its role in viral replication remains uncertain. Here, we report that purified bacterial recombinant NS5A and NS5B directly interact each other *in vitro* detected by glutathione-S-transferase (GST) pull-down assay. Furthermore, complex formation of these proteins transiently coexpressed in mammalian cells was detected by coprecipitation. Using terminally and internally truncated NS5A, two discontinuous regions of NS5A (aa 105-162 and aa 277-334) outside of the adaptive mutations were identified to be independently essential for the binding both *in vivo* and *in vitro*. We examined effect of His-NS5A on RdRP activity of the soluble recombinant NS5Bt *in vitro* (J. Biol. Chem., 273 : 15476, 1998). Wild NS5A weakly stimulated at first (when less than 0.1 molar ratio to NS5B), then inhibited the NS5Bt RdRP activity in a dose dependent manner. The internal-deletion mutants defective in NS5B-binding exhibited no inhibitory effect, indicating that the NS5B-binding was necessary for the inhibition. Taken together, our results support the idea that NS5A modulates HCV replication as a component of replication complex.

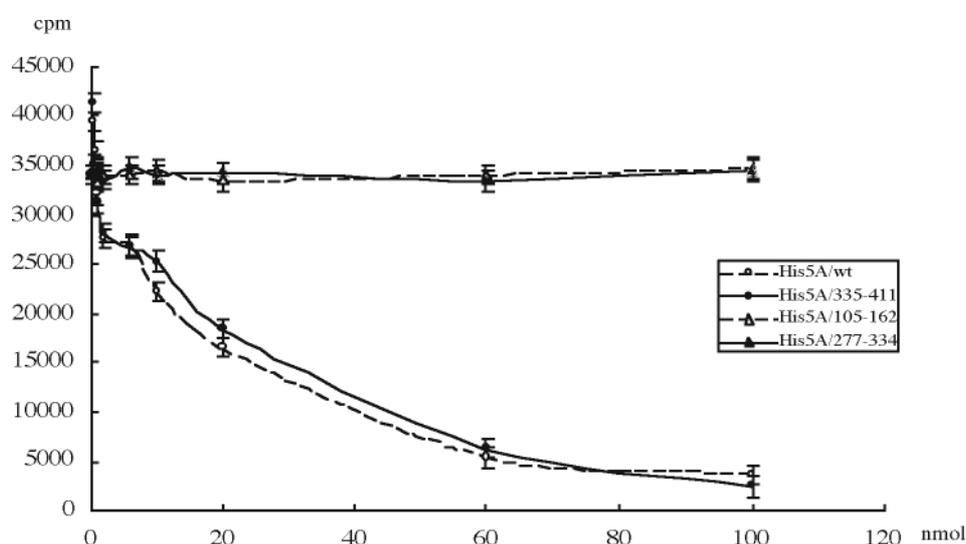


Figure NS5A modulates RdRP activity of NS5B

Ten nmol of purified GST-NS5Bt in the presence of 0, 0.2, 0.6, 1, 2, 6, 10, 20, 60, and 100 nmol of wild or internal-deletion His-NS5A were examined for poly (A)-dependent UMP incorporation assay as described.

Effect of His-NS5A/d163-221 was similar to that of the wild His-NS5A. Weak stimulation effects of wild and mutant NS5A proteins were observed when the molar ratio of His-NS5A to GST-NS5Bt is less than 0.1.

Direct interaction between nucleolin and HCV NS5B

Masaaki Hirano, Shuichi Kaneko, Tatsuya Yamashita, Hong Luo, Weiping Qin, Yukihiro Shirota, Takahiro Nomura, Kenichi Kobayashi, and Seishi Murakami

Hepatitis C virus (HCV) is one of the major causative agents of hepatitis. Two envelope proteins Hepatitis C virus (HCV) NS5B is an RNA-dependent RNA polymerase (RdRP), a central catalytic enzyme in HCV replication. Previously, we identified the C-terminal 21 amino acid sequence as a membrane attachment domain. It is dispensable for RdRP activity but important for subcellular localization of NS5N. While studying the subcellular localization of a NS5B mutant lacking the C-terminal domain, NS5Bt, we found that expression of the GFP-fused form was exclusively nucleolar. Interestingly, the distribution of endogenous nucleolin changed greatly in the cells expressing GFP-NS5B, with nucleolin colocalized with GFP-NS5B in perinuclear regions in addition to the nucleolus, suggesting that NS5B retained the ability to bind nucleolin.

The interaction between nucleolin and NS5B was demonstrated by GST pull-down assay. GST pull-down assay results indicated that C-terminal region of nucleolin was important for its binding to NS5B. Scanning clustered alanine substitution mutants library of NS5B revealed two sites on NS5B that bound nucleolin. NS5B aa 208-214 and aa 500-506 were both found to be indispensable for the nucleolin binding. We reported that the latter sequence is essential for oligomerization of NS5B, which is a prerequisite for the RdRP activity. C-terminal nucleolin inhibited the NS5B RdRP activity *in vitro* in a dose-dependent manner. The inhibition seemed to be dependent upon the ability to interact with NS5B since truncated forms of nucleolin that could not bind NS5B did not affect RdRP activity of NS5B. Taken together, the binding ability of nucleolin may be involved in NS5B functions.

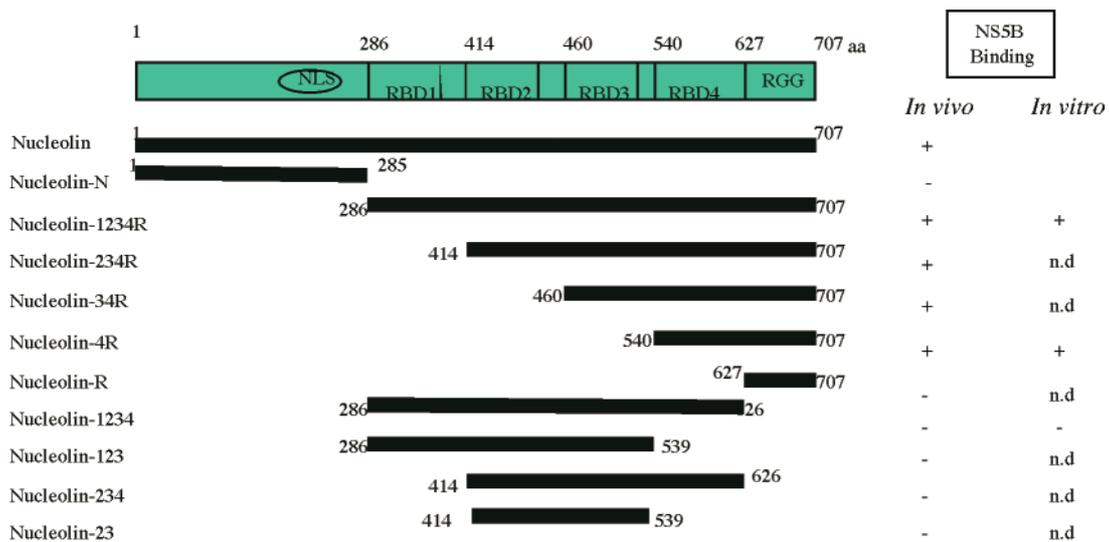


Figure Structures of wild-type and deletion mutant forms of human nucleolin and characteristics of NS5B binding. Boxes represent the structure of different constructs of human nucleolin protein with the boundaries of amino acid residues indicated above. The activity of each nucleolin mutant to interact with NS5B is indicated by a plus or minus sign. NLS; nuclear localization signal. RBD; RNA binding domain.

Telomerase activity reconstituted in vitro with purified human telomerase reverse transcriptase and human telomerase RNA component.

Kenichi Masutomi, Shuichi Kaneko, Naoyuki Hayashi, Tatsuya Yamashita, Yukihiro Shirota, Kenichi Kobayashi, and Seishi Murakami.

Telomerase is a specialized reverse transcriptase that catalyzes elongation of the telomeric tandem repeat, TTAGGG, by addition of this sequence to the ends of existing telomeres. Human telomerase reverse transcriptase (hTERT) has been identified as a catalytic enzyme involved in telomere elongation that requires telomerase RNA, human telomerase RNA component (hTR), as an RNA template. We established a new method to express and purify soluble insect-expressed recombinant hTERT. The partially purified FLAG-hTERT retained the catalytic activity of telomerase in a complementation assay in vitro to exhibit telomerase activity in telomerase-negative TIG3 cell extract and in a reconstitution assay with FLAG-hTERT and purified hTR in vitro. FLAG-hTERT (D712A) with a mutation in the VDV motif exhibited no telomerase activity, confirming the authentic catalytic activity of FLAG-hTERT. The reconstituted complex of FLAG-hTERT and hTR in vitro was detected by electrophoretic mobility shift assay, and its activity was stimulated by more than 30-fold by TIG3 cell extract. This suggested that some cellular component(s) in the extract facilitated the reconstituted telomerase activity in vitro. Geldanamycin had no effect on the reconstituted activity but partially reduced the stimulated activity of the reconstituted telomerase by the TIG3 cell extract, suggesting that Hsp90 might contribute to the stimulatory effect of the cellular components.

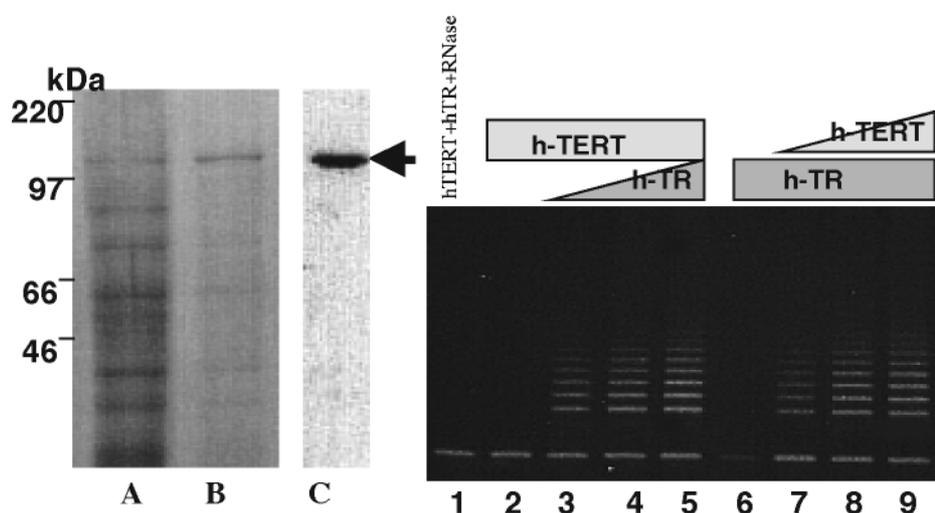


Figure. In vitro reconstitution of telomerase activity with two components.

Solubilized fraction of insect-expressed FLAG-hTERT (A) and partially purified (B) detected by CBB staining and western blotting with anti-FLAG antibody (C). Telomerase activity of FLAG-hTERT with varying amount of template hTR (lanes 2-5) and hTR and varying amount of FLAG-hTERT (lanes 6-9). Equimolar amount of hTR and FLAG-hTERT was pretreated with RNaseA, then subjected to TRAP assay.

Two independent regions of human telomerase reverse transcriptase (hTERT) are important for its oligomerization and telomerase activity

Kuniaki Arai, Kenkichi Masutomi, Shilagardy Khurts, Shuichi Kaneko, Kenichi Kobayashi, and Seishi Murakami

Human telomerase reverse transcriptase (hTERT), the catalytic subunit of telomerase, contains motifs conserved among reverse transcriptases. Several nucleic acid-dependent polymerases (NdnPs) that shared a “fingers, palm and thumb substructure” were shown to oligomerize. Here, we demonstrate that hTERT also has this ability using partially purified recombinant hTERTs and mammalian cells coexpressing different tagged hTERTs. Human-TR, by contrast, has no effect on the structural oligomerization of hTERTs. Therefore, hTERT by itself has an intrinsic ability of oligomerization in the absence of hTR. We identified two separate regions as essential for the oligomerization. The regions, aa 301-538 (amino-terminal region) and aa 914-928 (carboxy-terminal region) outside the fingers and palm substructure covering motif A to D, interact with each other *in vivo*. A dominant negative form of hTERT bound to the wild-type hTERT and inhibited its telomerase activity transiently expressed in telomerase-negative finite normal human fibroblasts. The catalytically inactive truncated forms of hTERT, which contained the binding region to the wild-type hTERT, inhibited the telomerase activity partially, probably by preventing the wild-type hTERT from forming an oligomer. These results imply that the hTERT oligomers having active catalytic centers are critical for telomerase activity.

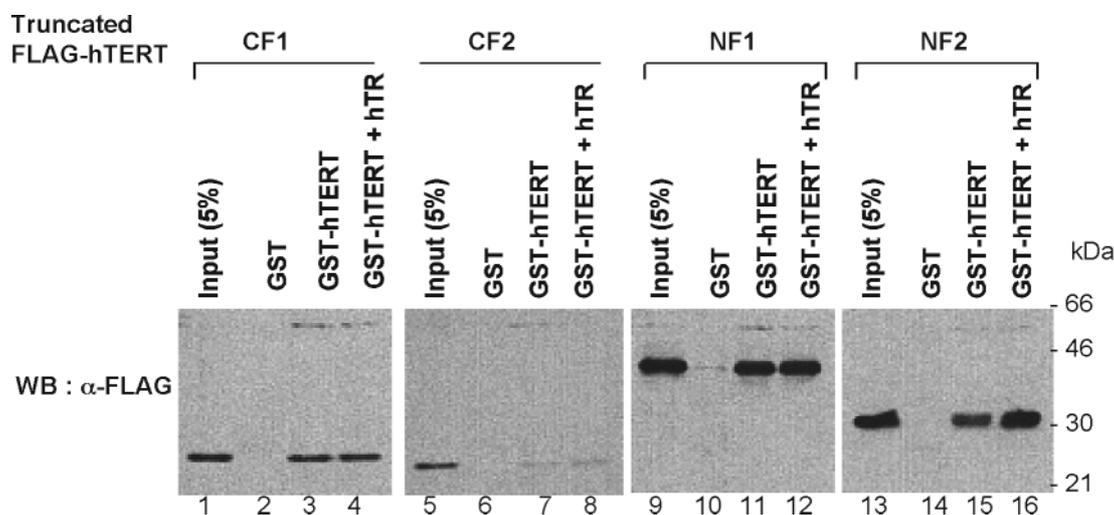


Figure Two independent regions of hTERT exhibited homomeric interaction.

Partially purified truncated forms of FLAG-hTERT were incubated with GST, GST-hTERT, or GST-hTERT and hTR, then subjected to GST pull-down. Recovered FLAG-hTERT was visualized by western blotting with anti-FLAG antibody. CF1, CF2, NF1 and NF2 harbor aa 914-1132, aa 928-1132, aa 201-538, and aa 301-538, respectively.

Stm1 interacts with Cdc13, a regulator of the telomere replication in yeast *Saccharomyces cerevisiae*.

N. Hayashi, and S. Murakami

We tried to isolate and characterize the factors interacting with *CDC13* in order to clarify the molecular aspects of telomere replication. Cdc13 interacts with Est1 and DNA polymerase α , and *cdc13-1*, one of the mutations in *CDC13*, cannot complete the telomere replication at restrictive temperature. This *CDC13* gene, encoding a binding protein with a single G-rich strand at the telomere, is the regulator of telomere replication. In a 2-hybrid screening using *CDC13* as bait, *STM1* cDNA was isolated. Ts^- growth and the altered length of telomeres in *cdc13-1* were restored by introduction of the *STM1* gene on a multi-copy vector, but an out-break of single-stranded telomeres in the *cdc13-1* cell was not restored. On the other hand, we found that a multi-copy of *SGS1*, encoding a helicase to unwind the guanine-quadruplex, inhibited suppression by *STM1* to *cdc13-1*. We found similarity in amino acid sequence between C-termini of Stm1 and β -subunit of telomere binding complex in *Oxytricha*. Telomere binding complex in *Oxytricha* consists of α and β -subunits. The α -subunit binds to single G-rich strand like as Cdc13, and the β -subunit binds to guanine quadruplex like as Stm1. We demonstrated that the fusion of N-terminal interaction region in Cdc13 and C-terminal region in Stm1, which had similarity to β -subunit, could complement the *CDC13* disruptant. Although *STM1* itself was not essential for telomere replication, our findings suggested that *STM1* genetically interacted with *CDC13* and functioned at the telomeres.

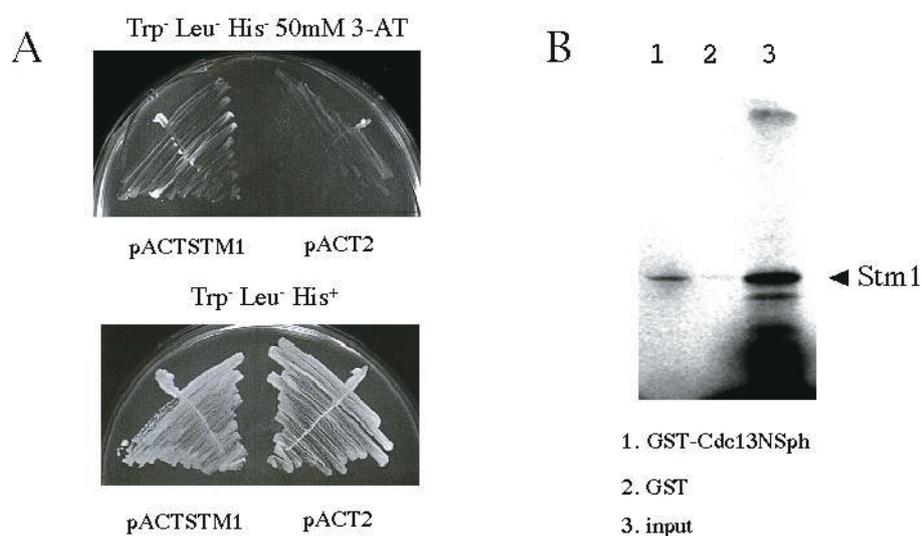


Figure. Stm1 binds to Cdc13. A. 2-hybrid analysis. Full length of *CDC13* was fused with GAL4 DNA binding region, and it was inserted into *CDC13* locus. Plasmid pACTSTM1 expresses fusion protein of Stm1 and GAL4 activation domain, and pACT is vectore alone. B. Pull-down experiment. In vitro synthesized Stm1 precipitated with N-terminal region of Cdc13 fused with glutatione S-transferase (GST).

Screening genes encoding the protein phosphatase, which defend against the cellular senescence.

N. Hayashi, and S. Murakami

Eukaryotic cells must have system to response against ageing stress to keep its life span. A lot of systems, such as replication, cdk-cycline, checkpoint system, metabolic pathway and so on, are controlled for that. Especially, protein phosphorylation should be an important protein-modification to transmit signals. We screened the knocked out series of protein phosphatase genes, whose mutation appeared phenotypes of cellular senescence. We focused telomere length to examine 30 disruptants and 2 mutants encoding protein phosphatases, which were found in the yeast genome project, and examined them by Southern analysis using Y' DNA as probes to detect telomere. In this screening, we found approximately 130 bp short telomere in *sit4* disruptant. *SIT4* gene encodes PP2A, and has been already reported about its functions in G1 cycline pathway. Life span of *sit4* disruptant was shortened comparable to *sgs1* disruptant. We introduced *NOPI-GFP* fusion gene to observe nucleoli structure. Alteration and fragmentation of nucleoli in *sit4* disruptant were also found in major population. Similar phenomena were observed in *pph21 pph22* double mutant. *PPH21* and *PPH22* encode PP2A similarly to *SIT4*. In *sit4* disruptant, Rap1 foci were more dispersed and intensity of Rap1 foci was much weaker than that in wild-type cells. Moreover, *sit4* and *pph21 pph22* lost silencing ability at subtelomere region, suggesting deficiency of the heterochromatin formation. We examined process of cellular death in PP2A disruptants, because cellular death like apoptosis was recently reported in aged yeast cells. Annexine V-FITC staining and TUNEL assay were employed to detect appearance of phosphatidyl serine on cell surface and DNA fragmentation, respectively. In both analyses, some of *sit4* and *pph21 pph22* cells fell into cellular death. These findings suggest that PP2A is required to maintain cellular life span.

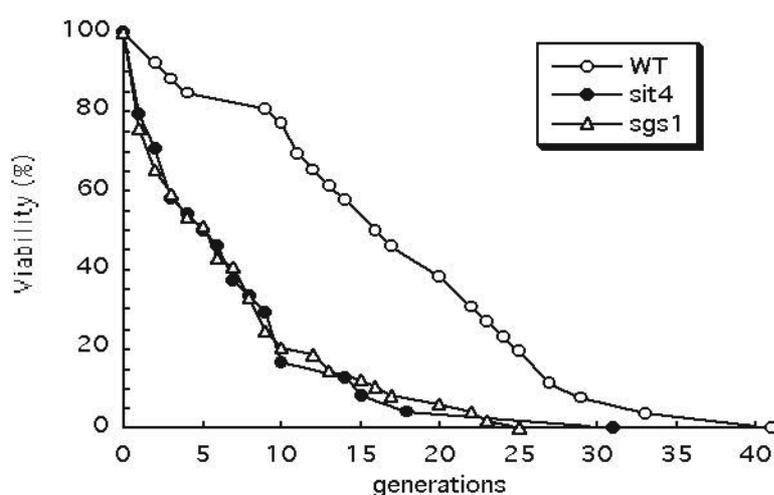


Figure. Life span was shortened in the *sit4* disruptant.

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**Division
of
Molecular
Virology and Oncology**

General Summary of Division of Molecular Virology and Oncology

Malignant tumors are invasive and frequently metastasize to distant organs. Tumor invasion into basement membrane consists of three steps, adhesion, degradation and migration. These steps are not independent but closely related with each other.

The goal of study in Molecular Virology and Oncology Division is to identify genes associated with tumor invasion and metastasis as molecular targets for diagnosis and therapy of malignant tumors.

A) Matrix Metalloproteinases (MMPs):

MMPs are known to play a major role in degradation of basement membrane during the invasion of tumor cells. Among more than 20 MMPs so far known, membrane-type 1 MMP (MT1-MMP), which was identified in this laboratory, is most closely associated with tumor invasion and metastasis. Expression of MT1-MMP was demonstrated to enhance malignancy of tumors in a variety of experimental systems. MT1-MMP was first identified as an activator of pro-MMP-2, and later MT1-MMP was shown to degrade a various extracellular matrix components including collagens. However, the regulatory mechanism of MT1-MMP still remains unknown.

To identify genes associated with the regulation of MT1-MMP activity, we have developed an expression cloning strategy and been screening cDNA libraries of human placenta, fetal brain and fetal kidney in which turnover of extracellular matrix is very active. In addition to several genes which either negatively or positively regulate MT1-MMP activity (1, 2), a several genes the products of which serve as substrates of MT1-MMP were identified (3). These results highlight the multi-functions of MT1-MMP, which may lead to the development of new anti-metastatic agents.

B) Cell Spreading and Migration:

Migration is one of essential steps for tumor invasion, and is closely associated with degradation step. Focal adhesion kinase (FAK) becomes activated and tyrosine-phosphorylated in response to cell adhesion to extracellular matrix proteins in a variety of cell types, and associates a number of signaling molecules, structural proteins, and integrins. Thus, FAK plays a central role in adhesion, spreading and migration of cells. Among various signaling molecules associated with FAK we have focused on c-Jun N-terminal kinase (JNK)/stress activated protein kinase-associated protein 1(JSAP1), a scaffold factor in the mitogen-activated protein kinase (MAPK) cascades (4) and Crk which was originally isolated as an oncogene product of the CT10 chicken retrovirus, and studied their roles in cell spreading, migration and invasion.

1. J. Biol. Chem., 276, 28204-28211, 2001.
2. Cancer Res., 61, 8896-8902, 2001.
3. Oncogene (in press)
4. Oncogene, 21, 6488-6497, 2002.

Claudin Promotes Activation of Pro-MMP-2 Mediated by Membrane-Type Matrix Metalloproteinases.

H. Miyamori, T. Takino, Y. Kobayashi and H. Sato

Genes associated with regulation of membrane-type matrix metalloproteinase-1 (MT1-MMP)-mediated pro-MMP-2 processing was screened in 293T cells by a newly developed expression cloning method. One of the gene products which promoted processing of pro-MMP-2 by MT1-MMP was claudin-5, a major component of endothelial tight junctions. Expression of claudin-5 not only replaced TIMP-2 in pro-MMP-2 activation by MT1-MMP but also promoted activation of pro-MMP-2 mediated by all MT-MMPs and MT1-MMP mutants lacking the transmembrane domain (Δ MT1-MMP). A C-terminal deletion mutant of pro-MMP-2 (pro Δ MMP-2) was processed to an intermediate form by MT1-MMP in 293T cells, and was further converted to an activated form by introduction of claudin-5. In contrast to the stimulatory effect of TIMP-2 on pro-MMP-2 activation by MT1-MMP, activation of pro-MMP-2 by Δ MT1-MMP in the presence of claudin-5 and pro Δ MMP-2 processing by MT1-MMP were both inversely repressed by expression of exogenous TIMP-2. These results suggest that TIMP-2 is not involved in claudin-5-induced pro-MMP-2 activation by MT-MMPs. Stimulation of MT-MMP-mediated pro-MMP-2 activation was also observed with other claudin family members; claudin-1, claudin-2 and claudin-3. Amino acid substitutions or deletions in ectodomain of claudin-1 abolished stimulatory effect. Direct interaction of claudin-1 with MT1-MMP and MMP-2 was demonstrated by immunoprecipitation analysis. MT1-MMP was co-localized with claudin-1 not only at cell-cell borders, but also at other parts of the cells. TIMP-2 enhanced cell-surface localization of MMP-2 mediated by MT1-MMP, and claudin-1 also stimulated it. These results suggest that claudin recruits all MT-MMPs and pro-MMP-2 on the cell surface to achieve elevated focal concentrations, and consequently enhances activation of pro-MMP-2.

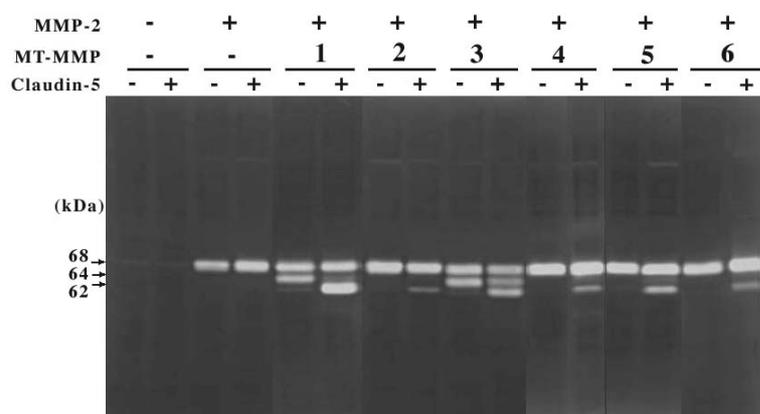


Fig. 1 Claudin-5 Promotes pro-MMP-2 Processing by MT-MMPs. Control (pSG5) plamid or claudin-5 plasmid was co-transfected into 293T cells with plasmids of MMP-2 and each MT-MMPs as indicated. At 48 h post-transfection, the cells were harvested and analyzed by gelatin zymography.

Reference:

Miyamori, H., Takino, T., Kobayashi, Y., Tokai, H., Itoh, Y., Seiki, M., and Sato, H. (2001) Claudin promotes activation of Pro-MMP-2 mediated by Membrane-Type Matrix Metalloproteinases. *J. Biol. Chem.*, 276, 28204-28211.

Expression of Dominant Negative Form of Ets-1 Suppresses Fibronectin-stimulated Cell Adhesion and Migration Through Down-regulation of Integrin $\alpha 5$ Expression in U251 Glioma Cell Line.

D. Kita*, T. Takino, M. Nakada*, H. Miyamori, J. Yamashita* and H. Sato (*Dept. Neurosurgery, Med. Sch., Kanazawa Univ.)

Ets transcription factors are associated with tumor malignancy. We previously reported that the stable transfection of the dominant negative form of Ets-1 (Ets-DN) in the glioma cell line U251 induced down-regulation of urokinase-type plasminogen activator (uPA) mRNA expression and invasiveness (M. Nakada, et al., *J Neuropathol Exp Neurol.*, 58: 329-34, 1999). Here we analyzed effects of Ets-DN-expression on cell adhesion, migration and phosphorylation of focal adhesion kinase (FAK) (1). U251 cells expressing Ets-DN (U251-DN) showed reduced cell adhesion, spreading and extension of actin stress fibers on dishes coated with fibronectin but not on dishes coated with collagen. Migration of U251-DN cells was found to be significantly inhibited compared to that of parental cells when examined by wound-induced migration assay on fibronectin-coated dishes. Phosphorylation levels of FAK in U251-DN cells were also attenuated on dishes coated with fibronectin. Reduced expression level of integrin5 subunit in U251-DN cells was demonstrated by semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis. Furthermore, down-regulation of transcription from the integrin $\alpha 5$ promoter by expression of Ets-DN was shown by luciferase reporter assay. Semi-quantitative RT-PCR of surgical samples of brain tumors revealed that the expression level of Ets-1 mRNA correlated with that of integrin $\alpha 5$ mRNA in glioma. The experimental metastatic ability of U251-DN cells examined in chick embryo was considerably lower than that of parental cells. These results suggest that Ets-1 contributes to glioma malignancy by up-regulating expression of the integrin $\alpha 5$ subunit, which composes integrin $\alpha 5 \beta 1$, mediates intracellular signaling and the subsequent acceleration of the invasive process including cell adhesion and migration.

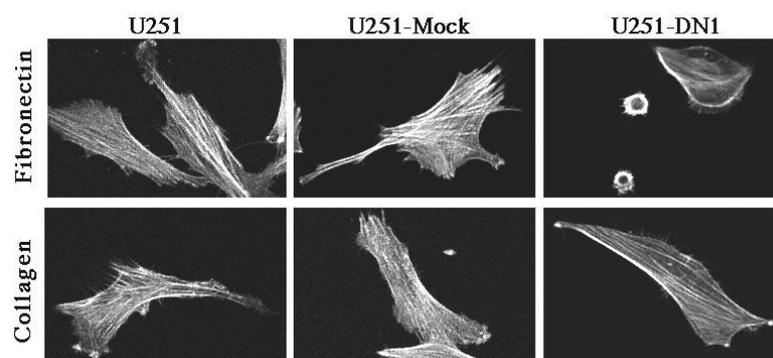


Figure 1. Cell adhesion and cytoskeleton formation. U251, U251-Mock or U251-DN cells were plated onto dishes coated with fibronectin (upper panels) or collagen (bottom panels) and incubated for 2 h at 37°C, and then stained with rhodamine-labeled phalloidin.

Reference: Kita, D., Takino, T., Nakada, M., Takahashi, T., Yamashita, J., and Sato, H. (2001) Expression of dominant negative form of Ets-1 suppresses fibronectin-stimulated cell adhesion and migration through down-regulation of integrin $\alpha 5$ expression in U251 glioma cell line. *Cancer Res.*, 61, 7985-7991.

Suppression of MT1-MMP-Mediated MMP-2 Activation and Tumor Invasion by Testican 3 and its Splicing Variant Gene Product N-Tes.

M. Nakada*, A. Yamada, T. Takino, H. Miyamori, J. Yamashita* and H. Sato (*Dept. Neurosurgery, Med. Sch., Kanazawa Univ.)

Using expression cloning to screen a human fetal kidney cDNA library for regulator(s) of pro-MMP-2 processing mediated by membrane-type-1 matrix metalloproteinase (MT1-MMP), we isolated a cDNA whose product interfered with pro-MMP-2 activation. It encodes the N-terminal 313 amino acids region of a calcium-binding proteoglycan, testican 3 with a 3 amino acids substitution at the C terminus, and thus was named N-Tes. N-Tes comprises a signal peptide, a unique domain, a follistatin-like domain and a Ca²⁺ binding domain, but lacks C-terminal thyroglobulin domain and two putative glycosaminoglycan attachment sites of testican 3 (1). Pro-MMP-2 activation by MT3-MMP was also inhibited by the co-expression of N-Tes. Immunoprecipitation analysis demonstrated direct interaction of N-Tes with either MT1-MMP or MT3-MMP. Expression of testican 1 or testican 3 but not testican 2 also inhibited pro-MMP-2 activation by either MT1-MMP or MT3-MMP. Deletion and substitution of amino acids residues in N-Tes revealed that the unique N-terminal domain of N-Tes is responsible for the inhibition of pro-MMP-2 activation by MT-MMPs. Expression of N-Tes and testican 3 was detected in normal brain, but was down-regulated in glioma tissues. Transfection of either N-Tes or testican 3 gene into U251 glioma cells or MDCK cells transformed by erbB2 suppressed their invasive growth in collagen gel. These results suggest that both N-Tes and testican 3 would interfere with tumor invasion by inhibiting MT-MMPs.

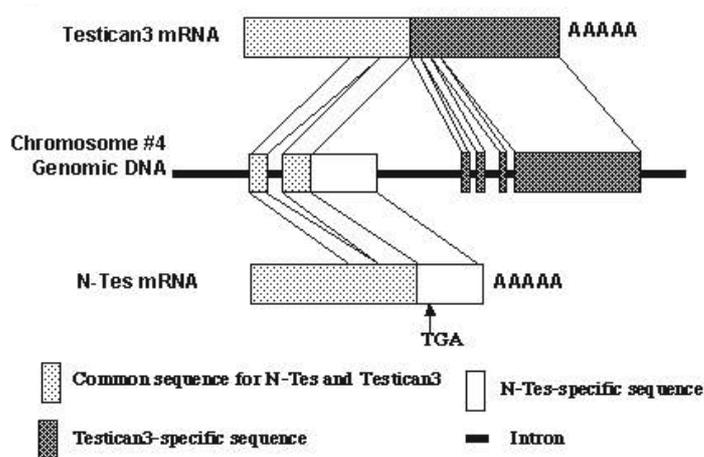


Fig. 1. Genome construction of N-Tes and testican 3 genes.

Reference:

Nakada, M., Yamada, A., Takino, T., Miyamori, H., Takahashi, T., Yamashita, J., and Sato, H. (2001) Suppression of MT1-MMP-mediated MMP-2 activation and tumor invasion by testican 3 and its splicing variant gene product N-Tes. *Cancer Res.*, 61, 8896-8902.

Cleavage of Metastasis Suppressor Gene Product KiSS-1 Protein/Metastin by Matrix Metalloproteinases.

T. Takino, N. Koshikawa¹, H. Miyamori, M. Tanaka², T. Sasaki², Y. Okada³, M. Seiki¹ and H. Sato (²Department of Cancer Cell Research, Institute of Medical Science, University of Tokyo, ²Department of Chemotherapy, Cancer Research Institute, Kanazawa University, ³Department of Pathology, Medical School, Keio University)

A human placenta cDNA library was screened by the expression cloning method for gene products which interact with matrix metalloproteinases (MMPs), and we isolated a cDNA whose product formed a stable complex with pro-MMP-2 and pro-MMP-9. The cDNA encoded the metastasis suppressor gene *KiSS-1*. KiSS-1 protein was shown to form a complex with pro-MMP. KiSS-1 protein is known to be processed to peptide ligand of a G-protein-coupled receptor (hOT7T175) named metastin, and suppresses metastasis of tumors expressing the receptor. Active MMP-2, MMP-9, MT1-MMP, MT3-MMP and MT5-MMP cleaved the Gly¹¹⁸-Leu¹¹⁹ peptide bond of not only full-length KiSS-1 protein but also metastin decapeptide. Metastin decapeptide induced formation of focal adhesion and actin stress fibers in cells expressing the receptor, and digestion of metastin decapeptide by MMP abolished its ligand activity. Migration of HT1080 cells expressing hOT7T175 which harbor a high level MMP activity was only slightly suppressed by either metastin decapeptide or MMP inhibitor BB-94 alone, but the combination of metastin decapeptide and BB-94 showed a synergistic effect in blocking cell migration. We propose that metastin could be used as an anti-metastatic agent in combination with MMP inhibitor, or MMP-resistant forms of metastin could be developed and may also be efficacious.

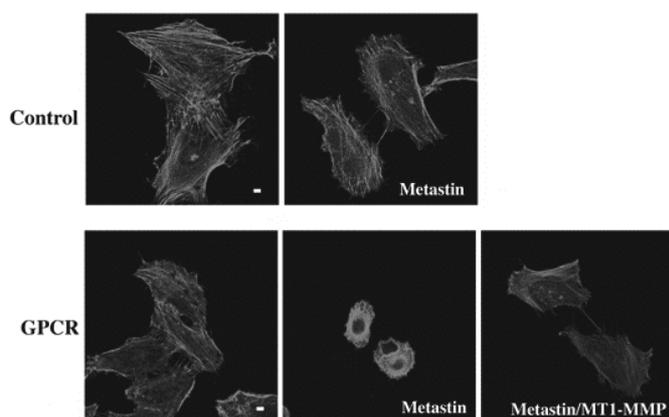


Fig. 1 Inactivation of Metastin by MMP. HeLa cells co-transfected with GFP and control plasmids (panels Control) or hOT7T175 cDNA (GPCR) were treated with vehicle (-), 100 nM metastin 112-121 peptide (Metastin) or metastin 112-121 peptide pre-incubated with MT1-MMP (Metastin/MT1-MMP) at 48 hr after transfection for 1 hr, and then stained with rhodamine-phalloidin.

Reference: Takino, T., Koshikawa, N., Miyamori, H., Tanaka, M., Sasaki, T., Okada, Y., Seiki, M., and Sato, H.: Cleavage of Metastasis Suppressor Gene Product KiSS-1 Protein/Metastin by Matrix Metalloproteinases. *Oncogene* (in press)

A Scaffold Protein in the c-Jun N-terminal Kinase Signaling Pathway is Associated with Focal Adhesion Kinase and Tyrosine Phosphorylated

T. Takino, K. Yoshioka*, H. Miyamori, K.M. Yamada[†] and H. Sato (*Div. of Cell Cycle Regulation, Cancer Res. Inst., Kanazawa Univ., [‡]CDBRB, NIDCR, NIH, Bethesda, USA)

Cell adhesion to the extracellular matrix (ECM) regulates many cellular functions, including differentiation, cell growth, apoptosis, and cell migration. Focal adhesion kinase (FAK) is a widely expressed nonreceptor protein tyrosine kinase localized in focal adhesions, and is critical for integrin-mediated signal transduction pathways. FAK becomes activated and tyrosine phosphorylated in response to cell adhesion to ECM proteins. Activated FAK undergoes autophosphorylation at Tyr-397 and thereby binds to the Src homology 2 (SH2) domain of the Src-family kinase, and SH3 domain of phosphatidylinositol 3-kinase (PI 3-K) p85 regulatory subunit. Src phosphorylates FAK and a number of FAK-associated proteins, including p130 Crk-associate substrate (p130^{cas}) and paxillin, which contain docking sites for CrkII SH2 domain. Integrin-mediated activation of c-Jun N-terminal kinase (JNK) requires the association of FAK with Src and p130^{cas}. FAK is also known to promote cell migration by coupling with p130^{cas} and CrkII. A role of JNK activation in cell migration is still controversial, however, several lines of evidence revealed that MEKK1 and activated JNK are localized in focal adhesion. JNK/stress-activated protein kinase-associated protein 1 (JSAP1), the newly identified scaffolding protein for JNK, binds to JNK, MEKK1 and SEK1. JSAP1 functions as a not only scaffolding factor in the JNK cascade but also suppressor in the ERK cascade by binding to Raf-1 and MEK1.

We have demonstrated that JSAP1 forms complex with N-terminus of FAK. The complex formation was further stimulated by c-Src but not by kinase-deficient Src, in which JSAP1 was tyrosine-phosphorylated and other FAK/Src signaling molecules were recruited. The stimulation of JSAP1 binding to FAK by c-Src required Tyr-397 of FAK. The mutant lacking amino acid residues 343-743 of JSAP1, in which tyrosine residues to be phosphorylated are included, effectively bound to FAK without c-Src. These results suggest that the domain within amino acid residues 343-743 of JSAP1 suppresses association with FAK, and phosphorylation of tyrosine in this region releases the suppression. Fibronectin (FN) stimulation of cells expressing JSAP1 induced its tyrosine phosphorylation concomitant with association with FAK. Expression of JSAP1 in HeLa cells facilitated formation of well-organized focal contacts and actin-stress fibers, and promoted cell spreading onto FN. JSAP1-induced cell spreading on FN was suppressed by expression of dominant negative form of JSAP1. Taken together, these results suggest that JSAP1 is involved in integrin-mediated signaling pathway through FAK/Src by recruiting other signaling molecules, resulting in promotion of cell spreading onto FN.

CrkI Adaptor Protein Modulates Cell Migration and Invasion in Glioblastoma

T. Takino, M. Nakada*, H. Miyamori, J. Yamashita*, K.M. Yamada[‡] and H. Sato (* Dept. Neurosurgery, Med. Sch., Kanazawa Univ., [‡]CDBRB, NIDCR, NIH, Bethesda, USA)

Crk was originally isolated as an oncogene product of the CT10 chicken retrovirus, and it belongs to a group of adaptor proteins that are comprised of SH2 and SH3 domains, which interact with phosphotyrosine and proline-rich regions, respectively. The human *crk* gene is translated into two products, CrkI (28 kDa) and CrkII (42 kDa), by alternative splicing. CrkII, which is predominantly expressed in many cell lines, consists of one SH2 and two SH3 domains with a spacer region between the SH3 domains that includes a tyrosine phosphorylation site (Tyr221). Upon tyrosine phosphorylation, CrkII undergoes intramolecular binding, which results not only in blockade of CrkII SH2-mediated binding to phosphotyrosine residues in other molecules, but also in reduced affinity of the CrkII central SH3 domain. In contrast, CrkI consists of one SH2 and only one SH3 domain, and it lacks this tyrosine phosphorylation site. Since CrkI but not CrkII expression induces transformation in rat 3Y1 fibroblasts, CrkI appears to resemble the *v-crk* oncogene product not only in its structure but also in its function. CrkII has been implicated in FAK-induced cell migration by coupling with p130^{cas}; however, the expression and function of CrkI in tumor cells are poorly understood.

We have demonstrated specific expression of *crkI* in glioblastoma tissues and analyzed the role of CrkI in malignancy of glioblastoma. The *crkII* mRNA was detected both in normal brain and glioblastoma tissues, whereas *crkI* mRNA levels were quite low in normal brain and up-regulated in glioblastoma tissues. Expression of CrkI but not CrkII in glioblastoma U87MG cells induced transformation that stimulated cell migration concomitant with tyrosine phosphorylation of p130^{cas}. When mock and CrkI-transformed U87MG cells were cultured on Matrigel, Akt was phosphorylated to comparable levels. Whereas treatment of control U87MG cells with N-cadherin blocking antibody GC-4 preferentially inhibited PI 3-K/Akt activation, CrkI-transformed cells were resistant to it. Invasion by both mock- and CrkI-transfected cells was inhibited by the PI 3-K inhibitor, indicating that the PI 3-K/Akt pathway is essential for invasion. Consistent with its effects on Akt activation, GC-4 suppressed invasion of mock-transfected but not CrkI-transformed cells. These results indicate that expression of *crkI* but not *crkII* is specifically up-regulated in glioblastoma tissues, which contributes to malignancy of glioblastoma while activating p130^{cas}. CrkI is also involved in promoting the invasive phenotype by activating PI 3-K/Akt signaling without N-cadherin-mediated intercellular interactions. CrkI may be not only a diagnostic marker, but also a molecular target for drug development against glioblastoma.

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**Division
of
Molecular Bioregulation**

General Summary of Division of Molecular Bioregulation

Inflammatory reaction is a host response to various insults including injuries and tumors. In inflammatory reactions, various pathological changes including plasma exudation and leukocyte infiltration ensue, thereby modifying the course of various diseases. The object of the research in our division is to elucidate the molecular mechanism of inflammation, particularly focusing on the endogenous physiological active substances.

A) Molecular mechanism of acute and chronic liver injuries

When dimethylnitrosamine was administered twice a week for 4 weeks, TNF-Rp55-deficient mice developed less fibrotic changes with attenuated Kupffer and stellate cell accumulation, compared with wild-type mice. These results suggest the essential roles of TNF-Rp55 in liver fibrosis, a characteristic feature of chronic inflammation. Overdose of acetaminophen causes frequently severe liver injury characterized by centrilobular hepatocyte necrosis with a massive leukocyte infiltration. We observed that mice deficient in interferon (IFN)- γ were more resistant to acetaminophen than wild-type mice, suggesting its crucial roles. Moreover, we observed that acetaminophen induced IFN- γ expression in liver at the earliest step of liver injury and that IFN- γ promoted the subsequent steps.

B) Molecular mechanism of hepatoma development in Hepatitis B surface antigen (HBs) transgenic (Tg) mice

When myeloablative HBs Tg mice received the bone marrow cells and splenocytes of wild type mice immunized with HBs, Tg mice developed hepatomas about 15 months after the transplantation, through multiple steps including acute and chronic hepatitis, and pre-malignancy. In order to elucidate the molecular mechanism of this process, fluorescence differential display method was applied to identify the genes which were selectively expressed in pre-malignant lesions, compared with control liver. With this method, 25 genes were up-regulated selectively in pre-malignant lesions while 19 were down-regulated. Moreover, among 25 up-regulated genes, 5 were not found in database at the present time.

C) Molecular mechanism of liver metastasis

The interaction between tumor and normal cells including endothelial cells, is presumed to have essential roles in tumor metastasis. TNF-Rp55-deficient mice developed less liver metastasis upon intrasplenic injection of colon carcinoma cells, compared with wild-type mice. Intrasplenic injection of the tumor enhanced vascular endothelial adhesion molecule (VCAM)-1 in wild type but not TNF-Rp55, which may account for the reduced incidence of hepatic metastasis in TNF-Rp55-deficient mice.

D) Development of chemokine gene therapy against tumors

We have previously reported that a mouse adenocarcinoma cell line, colon 26, failed to form tumor upon transduction with interleukin (IL)-4 gene. We observed that the injection of IL-4 gene-transduced cells induced the production of a chemokine, monocyte chemoattractant protein (MCP)-1 in the draining lymph node, which eventually induced the migration of dendritic cells and subsequent tumor rejection. We further observed that MCP-1 gene therapy was effective to prevent hepatoma progression in nude mice, when it was employed in combination with suicide gene therapy using herpes simplex thymidine kinase and ganciclovir.

Molecular mechanism of acute and chronic liver injuries

K. Kitamura, M. Akiyama, Y. Ishida, T. Kondo, and N. Mukaida

We examine the pathophysiological roles of interferon (IFN)- γ in the pathogenesis of acetaminophen-induced acute liver injury. In wild-type mice, intraperitoneal injection of a lethal dose of acetaminophen induced intrahepatic IFN- γ mRNA expression and a marked increase in serum transaminase levels, leading to acute lethality of about 45 %. Histopathological analysis demonstrated centrilobular hepatic necrosis with leukocyte infiltration and a large number of apoptotic hepatocytes later than 10 hr after acetaminophen injection. Concomitantly, mRNA expression of adhesion molecules, pro-inflammatory cytokines, chemokines, Fas, and inducible NO synthetase (iNOS) was enhanced in the liver of wild-type mice injected with a lethal dose of acetaminophen. When IFN- γ -deficient mice were treated in the same manner, all mice survived with reduced serum transaminase elevation and attenuated hepatic necrosis, leukocyte infiltration, and hepatocyte apoptosis. The gene expression of all molecules was significantly attenuated in IFN- γ -deficient mice. Administration of an anti-IFN- γ neutralizing antibody even at 2 and 8 hr after acetaminophen challenge to wild-type mice alleviated acetaminophen-induced liver injury, and all mice survived. Therefore, IFN- γ is responsible for acetaminophen-induced liver injury by mediating leukocyte infiltration, hepatocyte apoptosis, and NO production as well as cytokine and chemokine production.

We also examined the molecular mechanism of chronic liver diseases such as liver fibrosis, particularly focusing on tumor necrosis factor (TNF)- α . TNF- α has pleiotropic functions, but its role in liver fibrosis has not yet been clarified. To understand the pathophysiologic role of the TNF- α /TNF receptor (TNF-R) p55 signals in liver fibrosis, 10 mg/kg of dimethylnitrosamine, a specific hepatotoxicant, was administered twice a week into the peritoneal cavity of both TNF-Rp55 knock-out (KO) and wild-type mice, and the severity of fibrosis was monitored histologically and biochemically. In wild-type mice, histologic analysis demonstrated evident fibrotic changes 1 week after the initiation of dimethylnitrosamine administration, consistent with increased liver collagen contents. Concomitantly, the numbers of Kupffer cells and activated hepatic stellate cells (HSCs) were increased in liver tissue. On the contrary, fibrotic changes were attenuated and the numbers of Kupffer cells and HSCs were decreased in TNF-Rp55-KO mice. Moreover, gene expression of TNF- α and monocyte chemoattractant protein-1, which are involved in Kupffer cell activation or migration, was decreased in the liver of TNF-Rp55-KO mice. Collectively, TNF-Rp55-mediated signals may regulate activation of Kupffer cells and HSCs and eventually enhance fibrotic process.

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Molecular mechanism of hepatoma development in Hepatitis B surface antigen (HBs) transgenic (Tg) mice

C. Fujii, Y. Nakamoto, P. Lu, and N. Mukaida

Hepatocellular carcinoma (HCC) is a common complication of chronic hepatitis B virus (HBV) infection, although HBV does not harbor any oncogenes. Thus, it is presumed that persistent inflammatory reactions are responsible for HCC development. In order to elucidate the molecular and cellular mechanism, one of us (YN) have established a mouse model of HCC by using HBV s antigen (HBs) transgenic mice. In this model, bone marrow cells and splenocytes were obtained from syngeneic wild-type mice, which were immunized with HBs antigen and were transplanted into HBs transgenic mice, which were myeloablated beforehand. At about 15 months after the transplantation, the transgenic mice developed multiple foci of HCC. We obtained samples from non-tumor sites as pre-malignant lesions. Total RNAs were extracted from control liver and pre-malignant lesions for the analysis by fluorescence differential display method (FDD).

FDD using 60 distinct sets of primers demonstrated that 38 bands were up-regulated in pre-malignant lesions while 56 bands were down-regulated in pre-malignant lesions, compared with control liver. The determination of the nucleotide sequence of each band revealed that the expression of 6 unreported genes and 19 known genes was enhanced in the pre-malignant lesions. Moreover, the expression of 19 known genes was diminished in pre-malignant lesions, compared with control liver. A semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) further demonstrated that the mRNA expression of 5 out of 6 unreported genes was enhanced actually in pre-malignant lesions. We are under the way to identify the molecular and functional characteristics of these 5 unreported genes.

Among 19 known genes whose expression was enhanced in pre-malignant lesion, we focused on pim-3. Pim-3 belongs to a family of proto-oncogenes such as pim-1 and -2, which exhibit serine threonine kinase activity, but previous studies reported a selective expression of pim-3 in neuronal system, but not in the course of any types of carcinogenesis. We observed that the expression of pim-3 was also augmented in liver in the course of diethylnitrosamine-induced hepatocarcinogenesis. Moreover, we observed that pim-3 mRNA was constitutively expressed in all human hepatoma cell lines that we examined. These observations suggest the potential involvement of pim-3 in hepatocarcinogenesis.

Molecular mechanism of liver metastasis

H. Kitakata, Y. Nemoto-Sasaki, X. Yang, Y. Takahashi, M. Mai, and N. Mukaida

Intrasplenic administration of a colon adenocarcinoma cell line, colon 26, induced tumor necrosis factor (TNF) α protein expression around the central and portal veins of the liver at 3 days, and liver metastases by 24 days after the tumor injection, in 90% of wild-type (WT) mice. To explore the roles of TNF- α in the process, we administered colon 26 cells into TNF receptor p55 (TNF-Rp55) knockout (KO) mice. Less than 50% of TNF-Rp55 KO mice developed liver metastasis with significantly lower liver weights and the volumes of metastatic foci. These observations suggest the critical roles of TNF-Rp55-mediated signals in this liver metastasis model. The intrasplenic tumor injection induced mRNA expressions of vascular endothelial growth factor, heparin-binding epidermal growth factor, matrix metalloproteinase-9, and tissue inhibitor of matrix metalloproteinase-1 at similar levels in the livers of both WT and TNF-Rp55 KO mice. Immunohistochemical analyses of the livers of WT mice after tumor injection demonstrated the enhanced expression of vascular cell adhesion molecule (VCAM)-1 and E-selectin on sinusoidal endothelial cells. Enhanced E-selectin expression was similarly observed in the liver of TNF-Rp55 KO mice after tumor injection. However, the enhancement in VCAM-1 mRNA expression and its protein production was significantly attenuated in the liver of TNF-Rp55 KO mice when compared with WT mice. Finally, in WT, intrasplenic injection of colon 26 induced TNF- α , IL-1 α , and IL-1 β mRNA expression in liver. On the contrary, in TNF-Rp55 KO, TNF- α but neither IL-1 α nor IL-1 β expression, was attenuated in liver after the intrasplenic injection, compared with WT. Collectively, these observations suggest that TNF-Rp55-mediated signals have a positive feedback loop and can up-regulate VCAM-1 expression in the liver, leading to subsequent liver metastasis after intrasplenic tumor injection.

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Development of chemokine gene therapy against tumors

H. Wang, Y. Sakai, Y. Nemoto-Sasaki, Nakamura, Y. Nakamoto, S. Kaneko, M. Inoue, K. Kobayashi, and N. Mukaida

Immunocompetent BALB/c mice rejected mouse adenocarcinoma cell line, colon 26, genetically engineered to express constitutively IL-4 gene (colon 26/IL-4) but not parental cells or cells transduced with a control gene (colon 26/control). On rechallenge, parental cells and colon 26/control cells were rejected by normal BALB/c mice that had previously rejected colon 26/IL-4. Moreover, several lines of evidence indicate that challenging mice with colon 26/IL-4 tumor cells resulted in the generation of memory cytotoxic T lymphocytes in the draining lymph nodes in an IFN- γ -dependent manner. Furthermore, we provide definitive evidence that the gene expression of a chemokine, monocyte chemoattractant protein (MCP)-1, was enhanced in the draining lymph nodes of the mice injected with colon26/IL-4. Finally, we proved that MCP-1 has essential roles in regulation of dendritic cell trafficking from the tumor sites to the draining lymph nodes and eventually generation of memory T lymphocytes. These observations suggest the potential usefulness of MCP-1 for gene therapy against tumors.

The therapeutic efficacy of herpes simplex virus thymidine kinase/ganciclovir (HSV-tk/GCV) system in many types of tumors is unsatisfactory due to the insufficient spread of gene transfer and insufficient cell killing. Hence, we investigated whether adenovirally delivered MCP-1 potentiates the antitumor effects of the HSV-tk/GCV system in hepatocellular carcinoma (HCC) cells. Subcutaneous tumor foci of the human HCC cell line, HuH7, established in athymic mice were directly transduced with a recombinant adenovirus (rAd) harboring an HSV-tk gene driven by a human α -fetoprotein promoter, followed by GCV administration. Subsequently, another rAd expressing MCP-1 under the universal CAG promoter was injected. The growth of tumors was markedly suppressed by codelivering HSV-tk and MCP-1 genes compared to that by either HSV-tk/GCV or MCP-1 delivery. In the tumor tissues, monocyte/macrophage infiltration was detected immunohistochemically. The antitumor effects of the rAd expressing MCP-1 were markedly reduced by the administration of carrageenan, a compound known to inactivate macrophage. These results indicate that adenovirally delivered MCP-1 enhanced the antitumor effects of the HSV-tk/GCV system synergistically by recruitment/activation of macrophages in tumor tissues, suggesting an effective immunotherapy for HCC and other lineages of tumors when used adjuvantly with a suicide gene.

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**Division
of
Genome Biology**

General summary of Division of Genome Biology

This division was started when Professor Takashi Ito and Assistant Professor Kazuhisa Ota arrived from University of Tokyo in December 1999 and January 2000, respectively. Five graduate students and a technical staff followed them from Tokyo. In October 2001, the Japan Science and Technology Corporation (JST) started to support the group as a part of the Institute for Bioinformatics Research and Development (BIRD), and a postdoctoral fellow and two technical staffs from JST joined the group for the BIRD project in April 2002. Following the retirement of Haruhiko Tokuyama, Keiji Kito was appointed as Assistant Professor in June 2002. The lab is currently composed of one Professor, two Assistant Professors, four postdoctoral fellows, three graduate students, and four technical staffs. The group is concentrating on functional genomics of the budding yeast and epigenetic regulation of mammalian genomes.

A) Functional genomics of the budding yeast *Saccharomyces cerevisiae*

To understand the eukaryotic cell as a system of biomolecules, we are trying to perform a variety of large-scale measurements using the budding yeast as a model cell. We established a comprehensive two-hybrid system to examine all possible binary interactions between the yeast proteins to provide one of the very earliest genome-wide two-hybrid data. We have also developed a system for absolute quantification of yeast transcriptome based on a novel competitive PCR method. Integrative use of these and other technologies is currently planned for systematic analysis of nutritional stress response and cell polarity establishment to provide solid basis for cellular simulation.

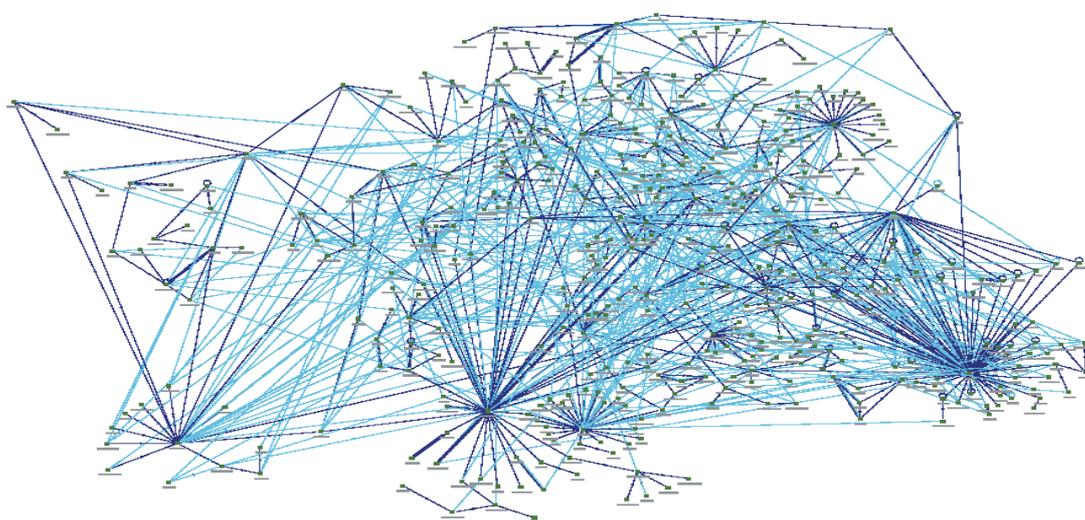
B) Epigenetic control of mammalian genomes

To achieve a more global picture of epigenetic regulation of mammalian genomes, we developed a simple HpaII-McrBC PCR method to examine allelic methylation status of CpG islands. A comprehensive analysis of CpG islands on human chromosome 21 with this method revealed novel methylation imprints and a unique mode of mono-allelic methylation. Efforts are also underway to analyze trans-acting factors for DNA methylation using the budding yeast.

Budding Yeast Protein Interactome

K. Ota, K. Kito, H. Kubota, Y. Yamaguchi, R. Matsuo, T. Chiba, K. Sakuraba, & T. Ito

We established a system for comprehensive two-hybrid analysis to examine all possible binary interactions between the 6,000 open reading frames encoded in the budding yeast genome. Consequently, 4,549 two-hybrid interactions, including 841 with higher reliability, were identified and made available from our web-site (<http://genome.c.kanazawa-u.ac.jp/Y2H/>). This is the very first protein interactome data, which have been providing numerous leads for novel findings in yeast cell biology and have also evoked new bioinformatics on protein interaction networks shown below.

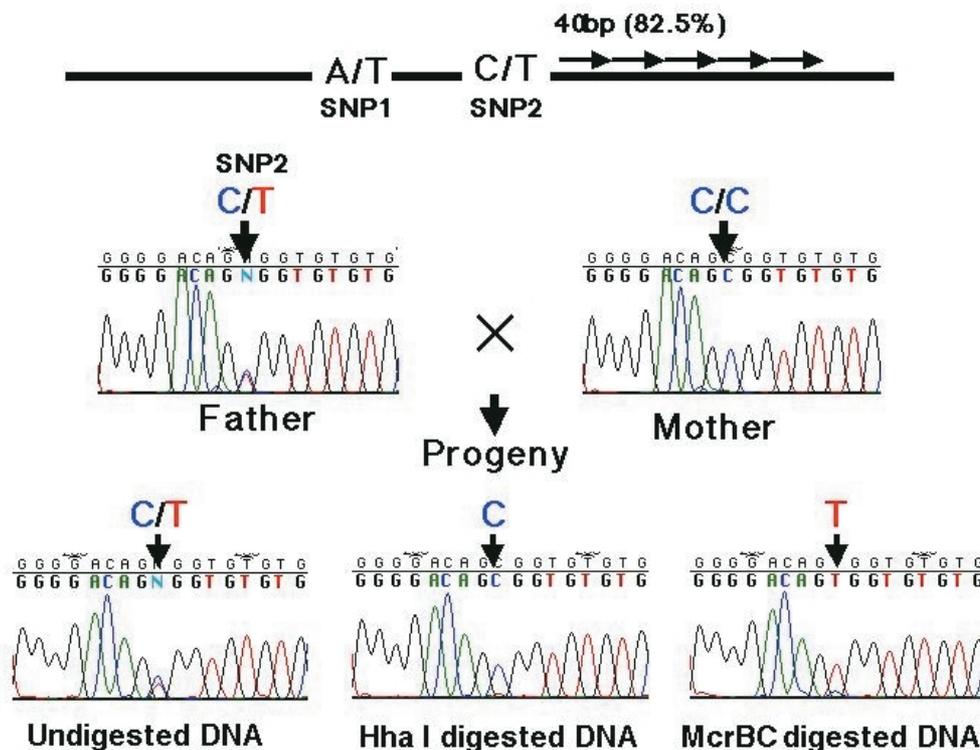


We next intended to assign biological roles to the catalogued interactions. Toward this goal, we are taking two strategies, namely “interaction profiling” and “interaction targeting”. For interaction profiling, we pursue a combinatorial use of quantitative mass spectrometric analysis with isotopic labeling and tandem affinity-tag purification of protein complexes. As a variant of interaction profiling, we are also trying to profile polyubiquitinated proteins by means of a unique parallel affinity-tag purification approach. For interaction targeting, we are developing methods for “two-hybrid footprinting” and “guaranteed reverse two-hybrid screening” to map interaction domains and isolate interaction-defective alleles, respectively. These techniques have been successfully applied to the analysis of protein interactions involved in stress-responsive translational control as well as cell polarity establishment.

Mammalian Epigenomics

Y. Yamada, S.-Y. Feng, K. Ota & T. Ito

To draw a global picture of epigenetic modification of mammalian genome, we conducted a comprehensive methylation analysis of CpG islands on human chromosome 21. For the analysis, we developed a novel versatile screen termed HpaII-McrBC PCR, which notably allows one to evaluate allelic methylation status. Accordingly, we uncovered three regions subject to allele-specific methylation, in addition to CpG islands methylated even in normal tissues. While two of the three are subject to maternal methylation (see below), the other is methylated in an allele-specific but parental-origin-independent manner, thereby representing a novel mode of allele-specific methylation.



To address molecular mechanisms for epigenetic regulation, we develop a system for site-specific DNA methylation in the budding yeast by recruiting M.SssI DNA methylase to the vicinity of target site by means of LexA-fusion. This system may serve as a unique tool for the analysis of trans-acting factors for methylated DNAs.

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**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, novel RNA polymerase II associating proteins and infection mechanism of hepadnaviruses.

A) New functions of small nucleolar RNAs (snoRNAs)

Various low molecular weight RNAs function at many stages in gene expression and concern with maintenance and proliferation of the cells. Although functions of many kinds of low molecular weight RNAs were determined in the last decade, functions of several low molecular weight RNAs are still remained to be elucidated. We are currently interested in the structure, function and biogenesis of low molecular weight RNAs. In this period we focused on the functional characterization of U13 small nucleolar RNA (snoRNA) and U17 snoRNA those functions were not identified until now.

B) Novel proteins associated with phosphorylated RNA polymerase II

The carboxy-terminal domain (CTD) of the largest subunit of RNA polymerase II consists of multiple repeats of a highly conserved seven amino acid sequence YSPTSPS. The CTD can be extensively phosphorylated, especially at the serine 2 and 5 positions in the consensus repeat. CTD phosphorylation has been reported to act as an important signal not only for transcription but also for the recruitment of pre-mRNA processing factors to transcription sites. To approach to the molecular mechanism for coupling transcription with pre-mRNA processing, we tried to identify and characterize novel factors that can directly interact with the phosphorylated CTD.

C) Infection mechanism of hepatitis B viruses

Although much is known about genomic replication and gene expression of hepatitis B viruses (hepadnaviruses), little is known about the mechanisms by which these viruses enter their host cells. We have been attempting to identify components of the hepadnaviral entry pathway, using the duck hepatitis B virus (DHBV) as a model system. Duck gp180, which was identified by us as a 180-kDa host protein binds to the preS envelope protein of DHBV, is now regarded as a host receptor for DHBV. We have characterized gp180 as a novel regulatory carboxypeptidase. Since recent experiments suggest that the second host component may be required for fully reconstitute viral entry, we are searching for other host proteins specifically interact with viral proteins.

he nucleotide sequence and the gene structure of chicken U17 small nucleolar RNA (snoRNA)

K. Satoh, Y. Kido and F. Harada

Two major classes of small nucleolar RNAs (snoRNAs), involved in pre-rRNA processing and modification, have recently been characterized. Members of one group show short conserved sequence motifs, denominated box H (5'-ANANNA-3') and box ACA. Box H/ACA snoRNAs generally function as guide RNAs in site-specific pseudouridylation of pre-rRNA. U17 snoRNA is one of the most abundant H/ACA snoRNAs in the animal cells. However, in spite of the presence of H and ACA boxes, its length is larger than the typical one of H/ACA snoRNAs and it shows a more complex secondary structure. Moreover, its secondary structure does not show any evident pseudouridylation pocket. From these characteristic features, U17 snoRNA may play new role in rRNA biogenesis. To clarify the function of U17 snoRNA, we tried to disrupt the U17 snoRNA gene in a chicken B cell line DT40 by targeted integration.

First of all, we purified U17 snoRNA from total RNA of DT40 cells by the using of gel filtration and polyacrylamide gel electrophoresis. The nucleotide sequence of purified RNA was determined by direct sequencing method using various RNases. The 5'- and the 3'- terminal sequences were determined by oligo-RNA ligation method. Chicken U17 snoRNA consists of 206 nucleotides and 73% identity with human U17 snoRNA.

Then we cloned the U17 snoRNA gene locus using U17 RNA sequence as a probe and sequenced around the RNA gene. U17 snoRNA has been found to be encoded in two (the 1st and the 2nd) introns of the human gene for RCC1 and in each of the six introns of the *Xenopus* and *Fugu* genes for ribosomal protein S7 (rpS7). Like human case, chicken U17 snoRNA was encoded in the RCC1 gene, although only one copy was found in the 2nd intron. However, U17 snoRNA coding sequence was not observed in the introns of the chicken rpS7 gene.

To disrupt the U17 snoRNA gene in DT40 cells, the targeting vectors containing blasticidin and puromycin resistance genes were constructed and then transfected to DT40 cells. Although introduction of blasticidin containing vector gave U17 snoRNA gene +/- clones, U17 snoRNA gene -/- clone was never obtained by successive transfection of puromycin containing vector. Probably U17 snoRNA is essential for maintenance of cells. Further experiments using conditional expression mutant cells for U17 snoRNA might be necessary for determination of the U17 snoRNA function in the cells.

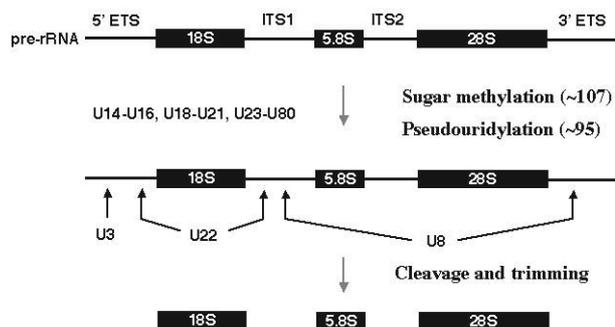
Role of U13 small nucleolar RNA on biogenesis of 18S rRNA

Y. Kido, K. Satoh, Y. Masuda and F. Harada

Since the second half of 1990's, many small nucleolar RNAs (snoRNAs) are shown to be involved in modification (2'-O-methylation or pseudouridylation) of nucleosides on rRNAs. U13 snoRNA was first identified in 1986 as a trimethylguanosine-capped, box C/D class snoRNA isolated from HeLa cells.

We have isolated and characterized the U13 snoRNA in chicken DT40 cells as a trimethylguanosine-capped small RNA associated with larger RNA species. According to the complementarity to the 3'-end of 18S rRNA, U13 RNA has been hypothesized to be involved in maturation of the 3'-end region of 18S rRNA. Cavaille et al. (1996) discussed the involvement of U13 snoRNA in cleavage of pre-rRNA at the 18S rRNA / ITS1 junction. There is, however, no direct evidence supporting the suggested function of U13 snoRNA so far.

In order to elucidate the function of U13 snoRNA we have established U13-knockout (U13-deficient) DT40 cell lines. The U13 snoRNA gene allele has been replaced by a neomycin-resistant gene and a histidinol-resistant gene, respectively. In the U13-knockout cells, the 3'-end position of 18S rRNA was the same as that of wild-type DT40 cells. This observation strongly suggests that U13 snoRNA is not essential for the processing (cleavage and trimming) of pre-rRNA at the 3'-end position of 18S rRNA. To examine the possibility that U13 RNA is involved in nucleoside modification, we have analyzed, with post-labeling method, the 3'-end region of 18S rRNAs isolated from wild type and knockout cells. While there is no difference on modification status of m⁶A1786, m₂A1804 and m₂A1805 respectively, a modified cytidine on position 1796 was not detected in the knockout cells. This observation suggests that U13 snoRNA is responsible for the modification of a cytidine residue in the 3'-end region of 18S rRNA. So far there is no report of any snoRNA responsible for modification of nucleoside except for 2'-O-methylation or pseudouridylation. Therefore U13 snoRNA is the first example of non-methylation-guide and non-pseudouridylation -guide snoRNA.



The molecular mechanism that coordinates transcription and pre-mRNA processing

Y. Hirose, H. Fan, K. Sakuraba, I. Yunokuchi, and F. Harada

Each step of pre-mRNA processing is intimately linked to transcription by RNA polymerase II (RNAP II) mediated by the physical interaction between the carboxy-terminal domain (CTD) of the largest subunit of RNAP II and pre-mRNA processing factors. The CTD consists of multiple repeats of an evolutionary conserved heptapeptide with the consensus sequence Y-S-P-T-S-P-S. The CTD is subject to reversible phosphorylation during the transcription cycle. Phosphorylation of the CTD appears to function as an important regulatory switch for assembly and disassembly of macromolecular complexes carrying out the synthesis and processing of pre-mRNA. To better understand the molecular mechanism that coordinates transcription and pre-mRNA processing, we have identified and characterized novel mammalian factors that can directly interact with the phosphorylated CTD (pCTD).

We screened a human cDNA expression library using ³²P-labeled CTD as a probe and identified several human WW domain-containing proteins as pCTD interacting factors. Among these, we have extensively characterized a novel human protein PCIF1 (Phosphorylated CTD Interacting Factor 1), which consists of 704 amino acids and contains a WW domain near the N-terminus. The PCIF1 WW domain directly and specifically bound to the pCTD. The binding affinity of the PCIF1 WW domain to CTD was significantly increased by phosphorylation of CTD. Co-immunoprecipitation data showed that PCIF1 bound to RNAP II with a hyperphosphorylated CTD (RNAP IIO) *in vivo*. Immunofluorescence confocal microscopy demonstrated that PCIF1 was co-localized with endogenous RNAP IIO in the nucleus. We also observed that the overexpression of PCIF1 in human cultured cells was able to repress the transactivation of luciferase reporter gene driven by various transcription activation domains and that the repression ability was dependent on its WW domain. The WW domain of the PCIF1 exhibits the considerable homology to the WW domain of human cis-trans peptidyl prolyl-isomerase Pin1, which has been shown to bind specifically to a phosphorylated Ser/Thr-Pro motif and the pCTD. *In vitro* data by GST pull-down experiments suggested that PCIF1 shared targets with Pin1, not only RNAP IIO but also other reported Pin1 targets.

Phosphorylation and dephosphorylation of the CTD on Ser 2 and Ser 5 position in the heptapeptide have been suggested to be dynamically and differentially regulated during transcription cycle. We, therefore, examined binding affinities of several WW domains to the differentially phosphorylated CTD peptide. Remarkably, PCIF1 WW domain could preferentially bind to a CTD peptide phosphorylated at Ser 5 compared to a CTD peptide phosphorylated at Ser 2 whereas Pin1 WW exhibited similar affinity to both CTD peptides. We speculate that PCIF1 may play a role in early stage of transcription cycle or in coupling transcription to pre-mRNA processing through the association with RNAP IIO phosphorylated at Ser 5. We are currently investigating cellular functions of PCIF1 by targeted gene disruption in the chicken B-cell line DT40.

Cellular proteins that bind the structural proteins of the avian hepatitis B virus.

K. Kuroki, Y. Yamano, J. Hirano, K. Tanaka, F. Harada

The human hepatitis B virus (HBV) is a small and enveloped DNA virus of the prototype of a family of *Hepadnaviridae* that causes acute and chronic liver disease and increases the risk of developing hepatocellular carcinoma. Despite of considerable understanding of the details of hepadnaviral replication and gene expression, little is known about HBV receptors and about the nature of the entry and the release pathways for this virus.

To understand the nature of the uptake and the maturation pathways for the hepadnaviruses, we have begun the search for the host proteins that interacts to structural proteins, envelope and capsid proteins, of the duck hepatitis B virus (DHBV) as a model of these viruses. After our finding of duck gp180, which is now regarded as a host receptor, recent experiments suggest that second host component may be required with gp180 to fully reconstitute viral entry.

By the biochemical-binding assay, we have identified gp180 and additional three host proteins, 64, 66 and 68-kDa proteins that bind the pre-S region of the DHBV envelope L protein (DHBVpre-S). HBVpreS1-preS2 and HHBVpreS can also bind 64 and 68-kDa proteins in every cell examined. Further experiments are in progress to examine the function of these cellular proteins in the life cycle of hepadnaviruses.

We have also identified two host proteins, 33-kDa and 45-kDa proteins (p33 and p45), that bind the DHBV core protein (DHBVc). DHBVc is the component of the nucleocapsid packaging the DHBV genome. The region of DHBVc associated with nucleocapsid assembly lies within the NH₂-terminal half of the protein. The COOH-terminal half of DHBVc contains arginine clusters that appear to be involved in genome packaging and in nucleocapsid traffic from plasma membrane to nucleus. Both p33 and p45 interact with the COOH-terminal region of DHBVc. This fact suggests that these proteins may be involved in genome packaging or traffic. Interestingly, by the binding assay with recombinant DHBVc, p45 cannot bind intact DHBVc, while it binds short COOH-terminal polypeptide of DHBVc. This may be reflected the dynamic topological change of the core protein caused by the phosphorylation of serine residues located in its COOH-terminal region. Functional p33 and p45 are expressed in a wide variety of tissues in susceptible ducks. The HBV core protein (HBc), like DHBc, binds p33 with high affinity indicating that p33 plays an important role in the life cycle of hepadnaviruses.

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**Division
of
Stem Cell Biology**

General Summary of Division of Stem Cell Biology

The organizer of this laboratory changed from Prof. Takahashi to Prof. Takakura in 2001. Therefore, field of study also changed from immunology to vascular biology and hematology. The issues that we have been addressing are the mechanism of developmental, physiologic and pathologic blood vessel formation and self-renewal of hematopoietic stem cells (HSCs). In the former, we analyzed interaction between endothelial cells (ECs) and hematopoietic cells (HCs) especially focusing on receptors, Flk-1, Tie2, EphB4, and neuropilin-1. In the later, we are establishing an in vitro culture system that promotes expansion of HSCs for the utilization of bone marrow reconstitution and therapeutic angiogenesis. Relating with this Tie2 function, we cloned various genes that might be affected by Tie2 activation. The molecular function of those genes will be determined in the near future. In the blood vessel formation, we would like to compare biological differences between open and close vascular system using ascidians. By this experiment, we know further about blood vessel formation precisely.

A) Role of hematopoietic cells in vascular development

- 1) Angiogenesis is an important event for embryonic organogenesis as well as for tissue repair in the adult. Among various kinds of HCs, HSCs play important roles for angiogenesis during embryogenesis. HSCs, which express angiopoietin-1 (Ang1), directly promoted migration of ECs and capillary formation.
- 2) Neuropilin-1 (NP-1) is a receptor for vascular endothelial growth factor-165 (VEGF165) and acts as a coreceptor that enhances the function of VEGF165 through VEGFR-2 in ECs. We found that NP-1 is expressed on HCs such as B lymphocyte, erythroblast, and monocyte and regulates vascular formation by an exogenous manner.

B) Role of Tie2 for Self-renewal of HSCs

- 1) We found that angiopoietin-1 produced by HSCs stimulates Tie2 on HSCs and ECs in HSC niche and this autocrine and paracrine loop is essential for self-renewal and maintenance of immature phenotype of HSCs (paper in preparation).
- 2) We have been trying to isolate several genes that might be affected by TIE2 activation or inactivation. To isolate such genes, we established constitutive active TIE2 receptor, transduce this mutant cDNA into HC or EC lines and compared the gene expression affected by this constitutive active TIE2. So far, we cloned genes affected by this treatment, have been analyzing one of them by RNAi method and found predominant effect for gastrulation in *C. elegans* (see Dr. Ueno's report). We will further examine the function of such gene in the HSC development.

C) Ancestral cells of endothelium in the open vascular system of ascidians

In the ascidian *Halocynthia roretzi*, the blood vessels of branchial sac are simple tubes within the matrix of connective tissue. Fortunately we found a particular type of cells on the inner surface of the blood vessels. We have been characterizing those cells by electron microscopic analysis. In the future, we will analyze the function of those cells associating with blood vessel formation.

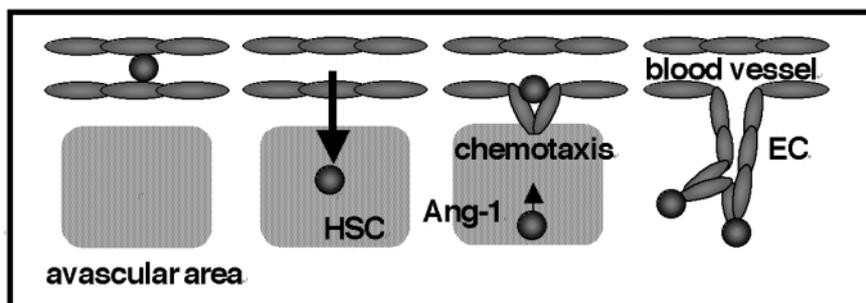
Analysis of Blood Vessel Formation and Self-Renewal of Hematopoietic Stem Cells

N. Takakura, X.-D. Wang, H. Sano, R. Okamoto, W.-L. Li.

Blocking tumor angiogenesis has become a promising approach in managing cancer. In recent years, a large number of anti-angiogenic drugs and recombinant proteins have entered clinical trials, and some of them are already in the final phase of testing. In the future, for remarkable progress in this anti-angiogenic therapy, it is important to know molecular mechanism of blood vessel formation precisely. We have focussed on the relationship between vasculogenesis-angiogenesis and hematopoiesis for better understanding in blood vessel formation. So far we found 1) Hematopoietic stem cells (HSCs) produce angiopoietin-1 and regulate angiogenesis, 2) NP-1 on hematopoietic cells delivers VEGF to Flk-1/VEGFR2 on endothelial cells and regulates vasculogenesis. 3) Moreover, we have analyzing the molecular mechanism how vein and artery are developed separately. On the other hand, we have hoped to translate our research into clinical trial especially in various regeneration therapies focusing on 4) the molecular mechanism of self-renewal of HSC for the in vitro expansion of HSCs, 5) transdifferentiation from mesenchymal stem cells into endothelial cells, and 6) identification of vascular stem cells those can differentiate into endothelial cells and vascular smooth muscle cells (these 5), 6) for regenerative angiogenesis). In this report, we present data that have been already confirmed and published elsewhere.

1) Role of hematopoietic stem cells (HSCs) in promoting angiogenesis.

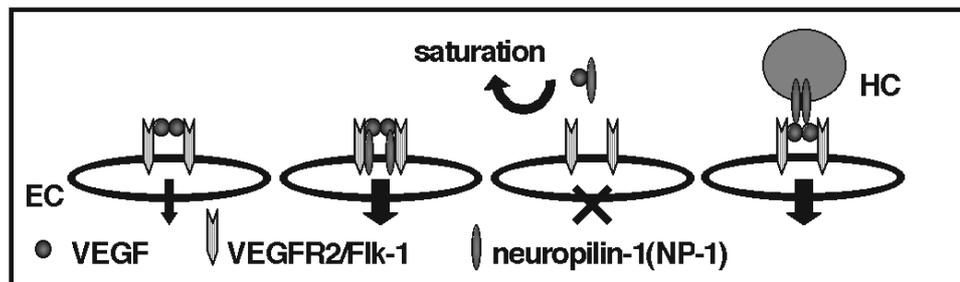
As shown in Figure, when hypoxia and avascular area occur in the location, HSCs migrate in such region at first. Following the migration of HSCs, endothelial cells start to sprout toward HSCs. Finally, endothelial cells form capillary and ischemic region is vasculzied.



This effect of endothelial cell migration is promoted by angiopoietin-1 produced from HSCs.

2) Role of neuropilin-1 on hematopoietic cells (HCs) in enhancing the stimulation of Flk-1/VEGFR2

Neuropilin-1 (NP-1) is co-receptor for Flk-1 on endothelial cell, binds VEGF, and enhance the phosphorylation of Flk-1. Monomer form of soluble NP-1 saturates VEGF



and inhibit VEGF-Flk-1 signal. By contrast, dimer form of soluble NP-1 stimulates FLk-1 exogenously and enhances the FLk-1 signal. We recently found that HCs express NP-1 and work as dimer form of soluble NP-1 and enhance blood vessel formation.

Ancestral cells of endothelium in the open vascular system of ascidians

S. Amano¹, N. Takakura¹ and I. Hori²

¹Cancer Res. Inst., Kanazawa Univ., Kanazawa, ²Dept. of Biol., Kanazawa Medical Univ., Uchinada, Ishikawa

The vertebrates have closed vascular system with an endothelial lining whereas the invertebrates have open vascular system without the lining. In the vertebrates, endothelial cells play principal roles in constructing blood vessels during development. Therefore it is a mystery how the invertebrates' blood vessels are formed without the participation of endothelial cells. We supposed that there are ancestral cells of endothelium in the open vascular system, and tried to study the blood vessels of ascidians.

Blood vessel wall cells of *Halocynthia roretzi*

In the ascidian *Halocynthia roretzi*, the blood vessels of branchial sac are simple tubes within the matrix of connective tissue. Fortunately we found a particular type of cells on the inner surface of the blood vessels. These cells have following conspicuous characters. They are found sparsely on the inner surface of blood vessels, not forming a continuous lining. Electron microscopic studies showed that these cells adhere closely to matrix. In these cells there are a few but very large electron-dense granules. In addition they constantly have well developed rER.

Blood vessel wall cells of other ascidians

Other ascidians also have similar cells on the inner surface of the blood vessels of branchial sac. Among solitary ascidians, we found blood vessel wall cells in *Styela clava* and *Styela plicata*, but not found in *Ciona intestinalis* (Table). All compound ascidians we have studied have no blood vessel wall cells.

Conclusion

The function of the blood vessel wall cells of ascidians is not known, however, they are supposed to be an evolutionary antecedent to the endothelial cells of the vertebrates.

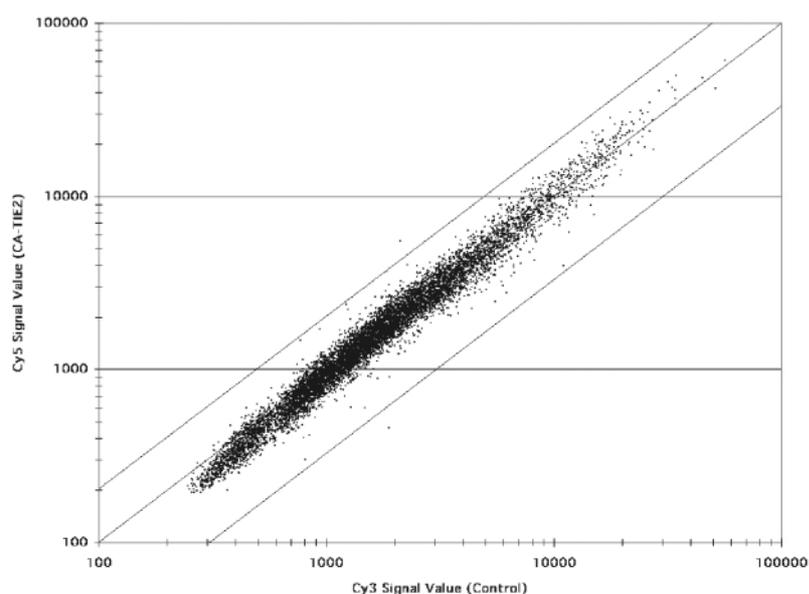
Ascidians with blood vessel wall cells

Species	Blood vessel wall cells	Vessel wall matrix
<i>Styela clava</i>	+	+
<i>Styela plicata</i>	+	+
<i>Ciona intestinalis</i>	—	—
Compound ascidians	—	—

Microarray analysis of TIE-2-activated Endothelial Cells

M. Ueno and N. Takakura

Growth and metastasis of solid tumors depend on the formation of new blood vessels by a process called angiogenesis. These blood vessels grow into the tumor and provide the nutrients and growth factors for tumor progression. Thus, there is considerable interest in understanding the mechanisms of angiogenesis for therapeutic purposes. Angiopoietin-1 (Ang-1) and their tyrosine kinase receptor Tie2 have been shown to play a pivotal role in normal as well as tumor angiogenesis. To understand the downstream of Ang-1/Tie2 signal pathway, we established a constitutively activated (CA)-Tie2 expressed subclone (#41) from an endothelial-like cell line, bEND3, and performed microarray gene expression analysis of 9364 genes between parental bEND3 and #41. Eight genes are up-regulated in #41, and 18 genes are repressed. Genes involved in membrane protein and protease are among those up-regulated, whereas genes involved in tight junction protein are predominantly repressed. We are analyzing each gene, whether gene(s) are participate in tumor angiogenesis.



Cloning and Characteristic of a novel gene, #E11

M. Ueno, S. Amano and N. Takakura

To better understand the molecular regulatory mechanisms of the hematopoietic stem cells, we performed a PCR-based subtraction methods and identified novel genes, #E11. This gene encodes highly conserved protein and its transcripts are found specifically in bone marrow and testis. In *C. elegans*, #E11-deficient embryo arrest with no sign gastrulation and show abnormal chromatin division. We are analyzing the function of #E11 in hematopoietic stem cell.

Molecular cloning of the new fourth allotype of the factor H in the Russian and Kazakhstan wild mouse population

S. Natsuume-Sakai¹, M. Shinmura², K. Tsuchiya³, T. morita², N. miyashita⁴, H. yonekawa⁵, K. moriwaki⁶ and L. Frisman⁷

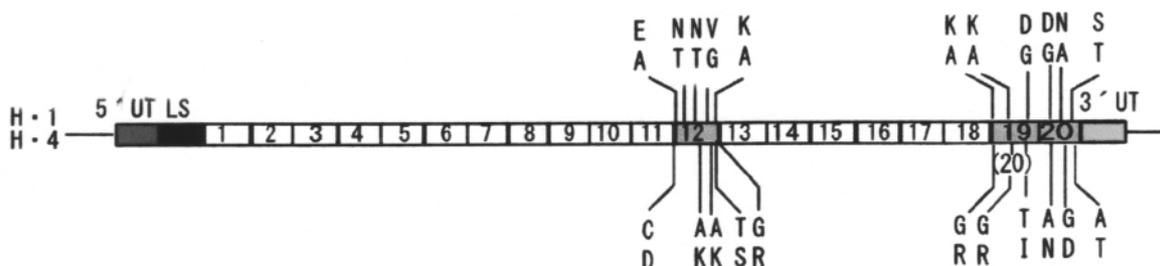
¹Cancer research Institute, Kanazawa University, ²Department of Animal Science, Miyazaki University, ³Experimental Animal Center, Miyazaki Medical College, ⁴Experimental Animal Center, Kagawa Medical University, ⁵Department of Laboratory Animal Science, The Tokyo Metropolitan Institute of Medical Science, ⁶RIKEN BRC, ⁷Institute of Biology and Soil Science, Far East Branch of the Russian Academy of Science

Factor H protein (H), a plasma protein that binds to C3b inactivator, is composed of 20 repeated units called a short consensus repeat (SCR), which is found mostly in the regulatory proteins of the complement system. Three allotypes of murine factor H have been identified in the serological and molecular studies of our previous reports. H. 1 was found in the most laboratory mice and H. 2 was mostly found in Asia including Japan and China, while H. 3 was only observed in France.

cDNA clones coding for the entire length of these three factor H allotypes were isolated from the cDNA libraries constructed from the livers of type strains. cDNA sequences with these three clones showed multiple amino acid replacement / nucleotide substitution at the different position on their coding region.

In the present work, wild mice in Russian and Kazakhstan have a serologically distinguishable new allotype of factor H and molecular cloning coding entire length of the fourth allotype was done. When cDNA sequences were compared between H. 1 and H. 4, amino acid replacement / nucleotide substitution were multiple, characteristic and gathered to the 12th, 19th and 20th SCR units. Furthermore, H. 4 gene (new allotypic gene) deleted the 19th SCR unit was duplicated. The genetic events with H. 4 serum were discussed. Finally alloanti H. 4 allotype was successfully produced in BALB/c mice. Alloanti H. 4 serum developed a single precipitin line against all of H. 4 mouse serum, but did not with other allotype sera.

Amino acid differences between H. 1 and H. 4 allotypic genes



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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) The J domain of Tpr2 regulates its interaction with the proapoptotic and cell-cycle checkpoint protein, Rad9

Human Rad9 is postulated to function in the early phase of cell-cycle checkpoint control. We identified Tetratricopeptide repeat protein 2 (Tpr2) as a novel Rad9-binding protein on two-hybrid screening and found functional interactions between Tpr2 and Rad9.

B) Arg tyrosine kinase is involved in homologous recombinational DNA repair

c-Abl plays important roles in cellular response to DNA damage. However, possible roles for Arg (Abl-related gene) in DNA damage response are unknown. We found that Arg plays a role in homologous recombinational DNA repair by phosphorylating Rad51.

C) Rad17 and Rad9 are essential for DNA replication and S-phase DNA damage checkpoint controls in higher vertebrate cells

Rad9 and Rad17 are thought to function as DNA damage sensors in early phase of DNA damage response. We found that Rad17 and Rad9 are essential for the S-phase DNA damage and DNA replication checkpoint controls in higher vertebrates.

D) Roles for ATM in cellular response to oxidative stress

We prepared antibodies to chicken ATM and ATR to study the functions of these kinases in cellular response to various noxious agents including genotoxic and oxidative stress. We found that ATM is activated not only by genotoxic stress such as X-ray radiation but also by oxidative stress such as hydrogen peroxide.

After Dr. Katsuji Yoshioka left Division of Molecular Pathology to head Division of Cell Cycle Regulation, Dr. Masahiko Kobayashi was newly appointed assistant professor as of January 1, 2000. We are very grateful to investigators outside of this institute for their contributions to our projects; Osamu Matsui, Graduate School of Medicine, Kanazawa University; Shunichi Takeda, Graduate School of Medicine, Kyoto University; Makoto Nakanishi, Graduate School of Medicine, Nagoya City University; Akira Shinohara, Graduate School of Science, Osaka University; Yoshio Maru, Tokyo Women's Medical University; Yosef Shiloh, Sackler School of Medicine, Tel Aviv University, Steve Jackson, Wellcome/CRC Institute, Cambridge University.

The J domain of Tpr2 regulates its interaction with the proapoptotic and cell-cycle checkpoint protein, Rad9

Shuang-Lin Xiang, Tomoyasu Kumano, Shu-ichi Iwasaki, Xiangao Sun, Kastuji Yoshioka, and Ken-chi Yamamoto

Human Rad9 is a key cell-cycle checkpoint protein that is postulated to function in the early phase of cell-cycle checkpoint control through complex formation with Rad1 and Hus1. Rad9 is also thought to be involved in controlling apoptosis through its interaction with Bcl-2. To study the biochemical functions of mammalian Rad9 as both a checkpoint and a possible apoptotic molecule, we used a yeast two-hybrid method with full-length human Rad9 as bait, and identified Tpr2 (Tetratricopeptide repeat protein 2) as a novel Rad9-binding factor. Tpr2 was first identified as a factor interacting with the GAP-related domain of neurofibromin in two-hybrid screening, and is a member of the growing Tpr family. All members of this family contain various numbers of a degenerate 34-amino-acid repeated motif called a tetratricopeptide repeat (tpr). The tpr's are protein-protein interacting motifs, and function in diverse cellular processes such as cell-cycle control, transcription repression, stress response, protein kinase inhibition, mitochondrial and peroxisomal protein transport, and neurogenesis. Tpr2 contains seven tpr motifs in its N-terminus and has been reported to bind Hsc70 through these tpr motifs. In addition, in its C-terminus, Tpr2 has a typical J domain found among members of the Hsp40/DnaJ co-chaperone family. The Hsp40/DnaJ family members regulate the chaperone activity of Hsp70/Hsc70 by stimulating their intrinsic ATPase activity; therefore, it is plausible that Tpr2 also functions as a co-chaperone in various cellular processes. However, the precise cellular functions of Tpr2 remain to be clarified. In the present study, we show that Tpr2 binds not only to Rad9, but also to Rad1 and Hus1, *in vivo*. However, unlike the interactions of Tpr2 with Rad1 or Hus1, both the *in vivo* Tpr2/Rad9 interaction and the cellular localization of Rad9 are influenced by the J domain of Tpr2. These results indicate a critical role for the J-domain of Tpr2 in functional interactions between Tpr2 and Rad9.

Arg tyrosine kinase is involved in homologous recombinational DNA repair

Yingzhu Li, Hiroko Shimizu, Shuang-Lin Xiang, Yoshiro Maru, Noriaki Takao, and Ken-ichi Yamamoto

Ataxia telangiectasia (A-T) is an autosomal recessive disease characterised by radiosensitivity and chromosomal instability. The 350 kDa product of *ATM*, the gene responsible for A-T, is related to a family of large phosphatidylinositol 3 (PI3)-kinase domain-containing proteins involved in cell cycle control and/ or DNA repair. The other members of this family include ATR and DNA-dependent protein kinase. Recent work has shown ATM to act on a number of important effector proteins involved in the cellular reaction to DNA damage, including c-Abl. c-Abl is an ubiquitously expressed nonreceptor-type tyrosine kinase and is activated by DNA damage in an ATM-dependent manner. It plays important roles in growth arrest and apoptosis, and may also function in DNA repair through the phosphorylation of Rad51, a key molecule in homologous recombinational (HR) DNA repair. Arg (Abl-related gene), the only other known member of the c-Abl family, shares considerable structural and sequence homology with c-Abl in the N-terminal SH3, SH2, and tyrosine kinase domains, and abnormal variants of Arg are implicated in some human lymphoid malignancies. However, the roles played by Arg in the cellular response to DNA damage are unknown. To study possible roles for Arg in cellular response to ionizing radiation (IR), we generated *Arg*^{-/-} cells from a chicken B cell line (DT40) by targeted disruption. We found that, unlike *c-Abl*^{-/-} DT40 cells but similar to *ATM*^{-/-} DT40 cells, ionizing radiation (IR)-induced Rad51 focus formation is reduced in *Arg*^{-/-} DT40 cells. This is consistent with the findings that *Arg*^{-/-} DT40 cells display hypersensitivity to IR, elevated frequencies of IR-induced chromosomal aberrations, and reduced targeted integration frequencies. All of these abnormalities in DNA damage repair are also observed in *ATM*^{-/-} but not in *c-Abl*^{-/-} DT40 cells. Finally, we found that Arg interacts with and phosphorylates Rad51 in 293T cells. These results suggest that Arg plays a role in homologous recombinational DNA repair by phosphorylating Rad51

Rad17 and Rad9 are essential for DNA replication and S-phase DNA damage checkpoint controls in higher vertebrate cells.

Atsushi Hirano, Tomoyasu Kumano, Masahiko Kobayashi, Shuang-Lin Xiang, Hiroto Ono, Osamu Matsui, and Ken-ichi Yamamoto

Cell-cycle checkpoints are surveillance mechanisms that monitor the cell cycle and protect genome integrity by inducing cell-cycle arrest or programmed cell death (apoptosis) in response to DNA damage or DNA replication errors. Because several features of cell cycle checkpoints have been conserved throughout evolution, information about cell cycle checkpoints learned from yeast provides a framework for developing a further understanding of the checkpoint pathways in higher eukaryotes (1, 2). Genetic studies in the fission yeast *S. pombe* have identified four key checkpoint molecules (Rad1, Hus1, Rad9, and Rad17) and mammalian homologs of these molecules have been identified. Rad17 is a protein related to replication factor C (RFC) containing Walker A-type and B-type nucleotide binding sites, and associates with four RFC small subunits, forming a pentameric complex. Rad1, Rad9, and Hus1 are all structurally related to PCNA and form a trimeric hetero-complex. It is therefore postulated that the Rad17-RFC complex recognizes certain DNA damage structures and recruits the Rad1-Rad9-Hus1 complex to DNA lesions, in a manner similar to the loading of the PCNA trimeric homo-complex on DNA by the RFC1-5 complex. To study how Rad17-RFC and Rad9-Rad1-Hus1 complexes function in DNA damage response in higher vertebrate cells, we generated Rad17- and Rad9-deficient DT40 cells by targeted disruption. We found that, while Rad17^{-/-} and Rad9^{-/-} DT40 cells were mildly sensitive to X-ray radiation, these cells were highly sensitive to UV irradiation, DNA alkylating agent MMS, and DNA replication inhibitor hydroxyurea (HU). We then analyzed the cell-cycle distributions by flow cytometry after these genotoxic treatments. After UV irradiation or MMS treatment, the rates of DNA replication became much slower, although not blocked, in wild-type and ATM^{-/-} cells. However, Rad17^{-/-} and Rad9^{-/-} cells were defective in this UV- and MMS-induced DNA replication slowing. We further found that, while wild-type and ATM^{-/-} cells were blocked at early S-phase when treated with HU, Rad17^{-/-} and Rad9^{-/-} cells were defective in the DNA replication checkpoint control. In addition, after HU treatment, most of these Rad17^{-/-} and Rad9^{-/-} cells underwent apoptosis. These results indicate that Rad17 and Rad9 are essential for the S-phase DNA damage and DNA replication checkpoint controls.

Roles for ATM in cellular response to oxidative stress

M. Kobayashi, H. Ono, A. Hirano and K. Yamamoto

ATM (ataxia telangiectasia-mutated) is activated in response to DNA double strand breaks (DSBs) after genotoxic treatments such as X-ray irradiation and regulates homologous recombination repair and cell cycle checkpoints. However, the molecular mechanisms underlying DNA damage recognition and subsequent activation of ATM are still unclear. The apoptosis of post-mitotic cerebellar Purkinje cells in AT patients indicates that ATM has other role than in the response to DSBs. Recent study has shown that ATM is a component of a complex of BRCA1-associated proteins including mismatch repair (MMR) enzymes. BRCA1 and MMR enzymes were revealed to be related to the pathway of transcription-coupled repair (TCR) by which a part of oxidative DNA damages is repaired. In addition, MMR enzymes recognize 8-oxo-guanine in DNA, which is generated by endogenous reactive oxygen species and has highly mutagenicity. Furthermore the elevated oxidative damages and the lower expression level of catalase were observed in AT cells compared with wild type. These evidences suggest that AT cells are unable to response to oxidative stress and repair oxidative damages adequately. Therefore we addressed to the study on the signal transduction pathway from oxidative stress to the activation of ATM.

In this study we used an isogenic set of stable chicken B cell line, DT40, for the sake of knockout availability and high compatibility to human cell. By colony assay the differences of sensitivities to hydrogen peroxide and paraquat, which induce different oxidative stress respectively, were examined among wild type, ATM^{-/-}, BRCA1 and newly constructed MSH6^{-/-} and Rad17^{-/-} strains. ATM^{-/-} and BRCA1^{-/-} cells were highly sensitive to hydrogen peroxide and only Rad17^{-/-} cells were sensitive to paraquat, suggesting that the protective process to hydrogen peroxide is different from that to superoxide radical generated by paraquat. MSH6^{-/-} cells showed similar sensitivities to wild type. Moreover we prepared anti-ATM serum to analyze the responses of ATM to the stimuli directly. Anti-ATM serum was collected from the rabbit immunized with purified His-tagged recombinant partial chicken ATM. The ATM-recognition of the anti-ATM serum was confirmed by Western blotting and the irradiation of X-ray activated ATM kinase in wild type cells but not ATM^{-/-}. At present we are analyzing the activation of ATM by the oxidative stress and other genotoxic reagents in the strains described above by kinase assay.

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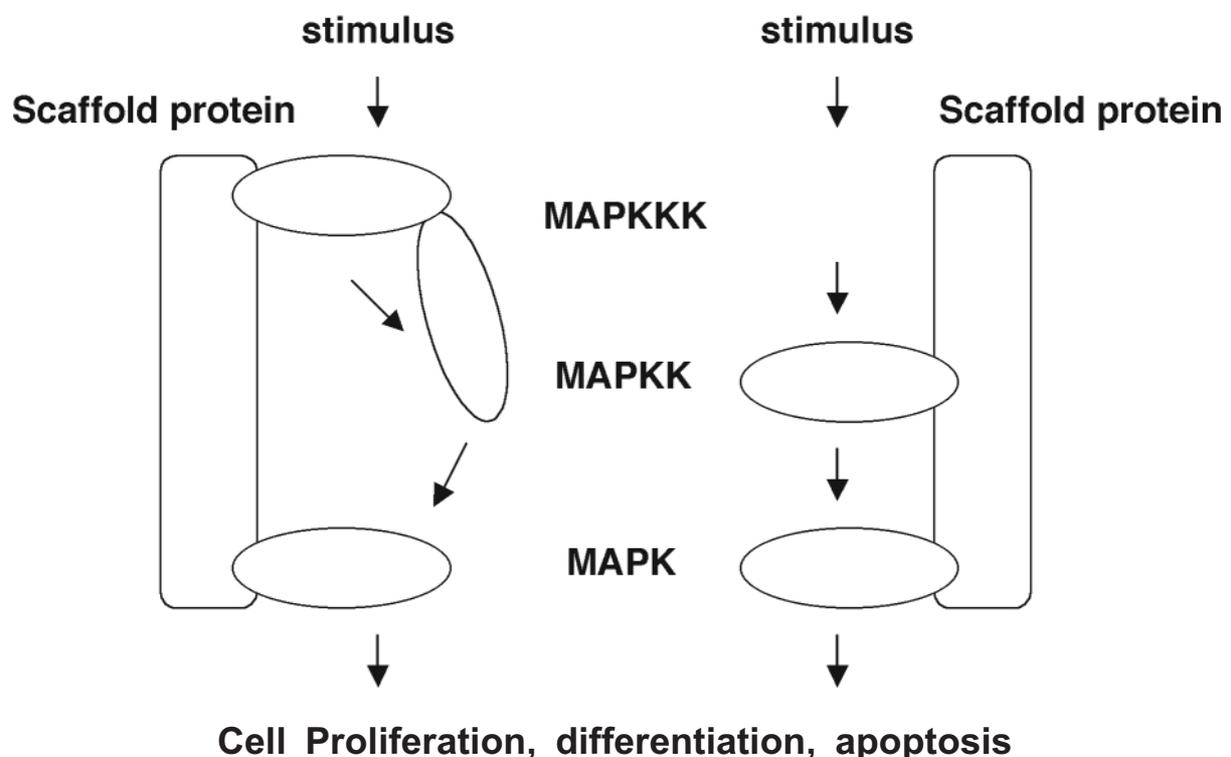
**Division
of
Cell Cycle Regulation**

General Summary of Division of Cell Cycle Regulation

Since 1998 the division has been dedicated to basic researches on MAP kinase (MAPK) signaling pathways, which play key roles in a wide array of cellular processes, including proliferation, differentiation, and apoptosis.

The MAPK pathway is an intracellular cascade consisting of MAPK, MAPK kinase (MAPKK) and MAPKK kinase (MAPKKK). Recent rapid progress in identifying members of MAPK cascades suggests that there are a number of these distinct signaling pathways in cells. However, how the specificity and efficiency of the MAPK cascades is maintained is poorly understood. Furthermore, the function of each MAPK cascade *in vivo* is largely unknown.

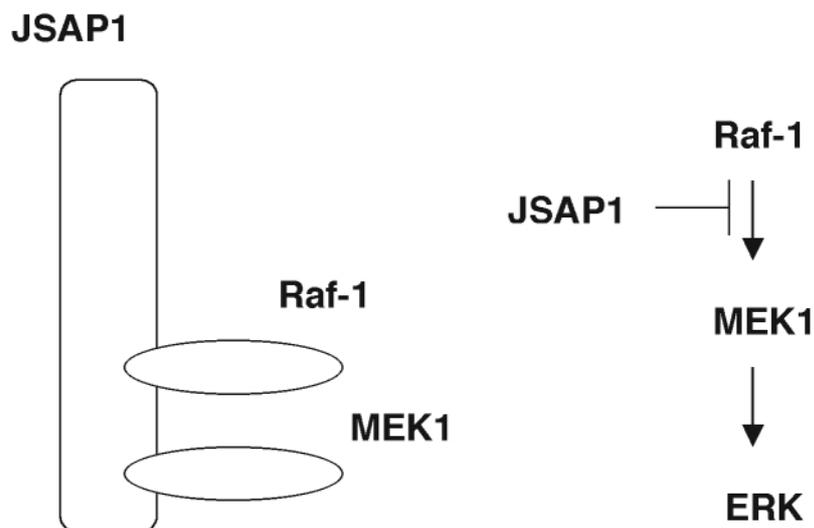
We have identified two novel JNK MAPK-binding proteins, termed JSAP1 and JNKBP1, respectively. The subsequent biochemical analyses revealed that the proteins function as scaffold proteins in the JNK MAPK cascades. We have also identified the family members of JSAP1 and JNKBP1, termed JSAP2 and JNKBP2, respectively. The goal of our projects is to demonstrate the functions of the proteins *in vivo*, and furthermore, to clarify the molecular mechanisms of how these proteins ensure the specific and efficient activation of the MAPK cascades *in vivo*.



JSAP1 Suppresses the ERK MAPK Signaling Pathways

Y. Kuboki, M. Ito, K. Yamamoto, K. Yoshioka

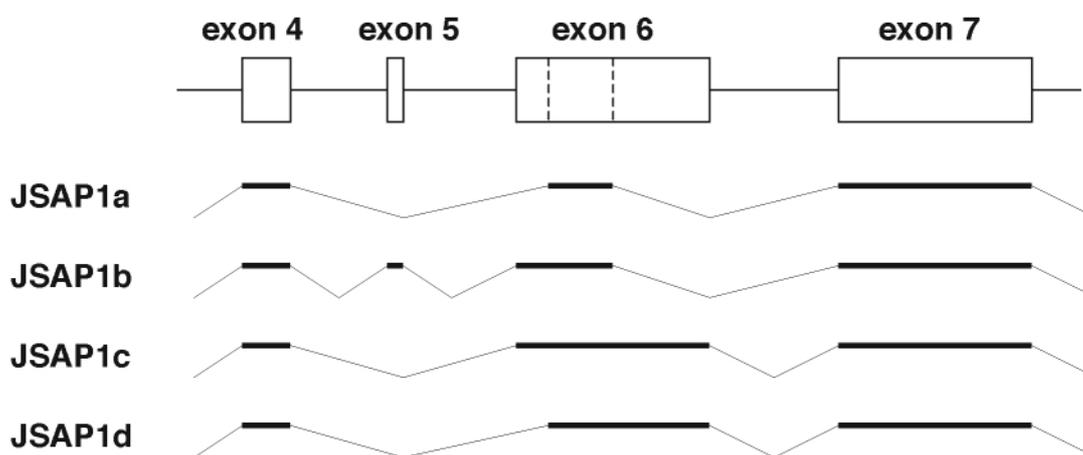
We previously reported that JSAP1 functions as a putative scaffold factor in the JNK MAPK cascades. In that study we also found MEK1 and Raf-1, which are involved in the ERK MAPK cascades, bind to JSAP1. Here we have defined the regions of JSAP1 responsible for the interactions with MEK1 and Raf-1. Both of the binding regions were mapped to the carboxy-terminal region (residues 1054-1305) of JSAP1. We next examined the effect of overexpressing JSAP1 on the activation of ERK by phorbol 12-myristate 13-acetate in transfected COS-7 cells, and found that JSAP1 inhibits ERK's activation and that the carboxy-terminal region of JSAP1 was required for the inhibition. Finally, we investigated the molecular mechanism of JSAP1's inhibitory function, and showed that JSAP1 prevents MEK1 phosphorylation and activation by Raf-1, resulting in the suppression of the activation of ERK. Taken together, these results suggest that JSAP1 is involved both in the JNK MAPK cascades, as a scaffolding factor, and the ERK MAPK cascades, as a suppressor.



Isoforms of JSAP1 Scaffold Protein Generated through Alternative Splicing

M. Ito, T. Shiba, K. Yamamoto, K. Yoshioka

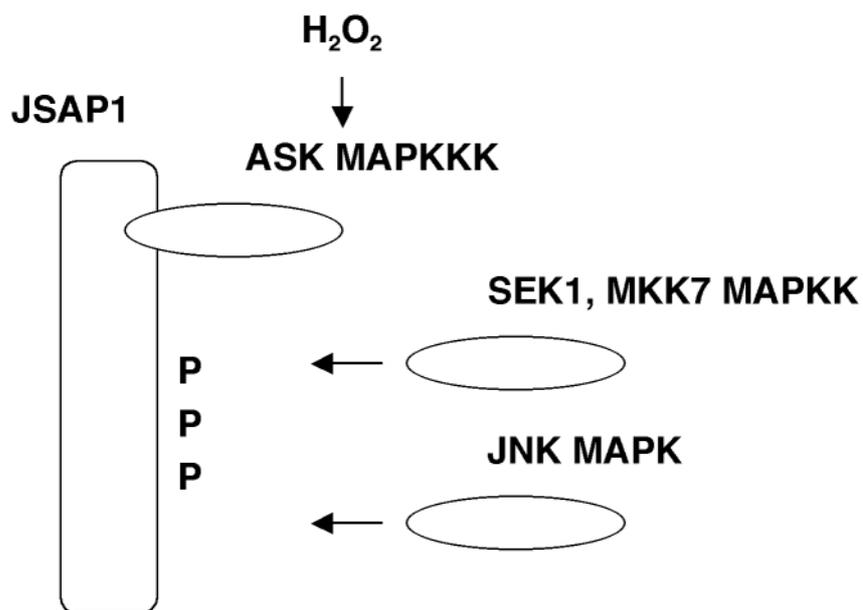
We have identified four isoforms of the scaffold protein JSAP1, termed JSAP1a, JSAP1b, JSAP1c, and JSAP1d. The previously identified JSAP1 was renamed JSAP1a to avoid confusion. Analyses of the exon-intron structure of the JSAP1 gene indicated that the isoforms are generated through alternative splicing involving exons 5 and 6. The mRNA expression levels of the JSAP1 isoforms differed among the mouse tissues examined. We also investigated the region of JSAP1 responsible for its interaction with JNK, and found that the JNK-binding domain is located between amino acid (aa) residues 201-217 in JSAP1a, which is encoded by part of exon 6. As all the JSAP1 isoforms contain this binding domain, we examined the binding affinity of the JSAP1 isoforms for JNK1, JNK2, and JNK3 MAPKs. JSAP1c and JSAP1d, which contain a 31-aa sequence not present in JSAP1a or JSAP1b, had a lower binding affinity for the JNKs, especially JNK3. These results suggest that *in vivo*, JSAP1c and JSAP1d may attenuate the scaffolding activity of JSAP1a and/or JSAP1b in JNK cascades, especially the JNK3 cascade, by interfering with the interaction with JNKs.



Phosphorylation-dependent Scaffolding Role of JSAP1/JIP3 in the ASK1-JNK Signaling Pathway

H. Matsuura, K. Yoshioka, H. Ichijo

JSAP1 (also termed JIP3) is a scaffold protein that interacts with specific components of the JNK MAPK signaling pathway. Apoptosis signal-regulating kinase (ASK) 1 is a MAPKKK that activates the JNK and p38 MAPK cascades in response to environmental stresses such as reactive oxygen species. Here we show that JSAP1 bound ASK1 and enhanced ASK1- and H₂O₂-induced JNK activity. ASK1 phosphorylated JSAP1 *in vitro* and *in vivo*, and the phosphorylation facilitated interactions of JSAP1 with SEK1/MKK4 MAPKK, MKK7 MAPKK and JNK3 MAPK. Furthermore, ASK1-dependent phosphorylation was required for JSAP1 to recruit and thereby activate JNK in response to H₂O₂. We thus conclude that JSAP1 functions not only as a simple scaffold, but it dynamically participates in signal transduction by forming a phosphorylation-dependent signaling complex in the ASK1-JNK signaling module.



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**Division
of
Molecular
Membrane Biology**

General Summary of the Division of Molecular Membrane Biology

In 1999, Dr. H. Ohno was appointed to be the professor and chairman of this division. Since then, the division has been dedicated mainly to basic researches on the mechanisms of membrane trafficking and protein sorting in the secretory and endocytic pathways.

From February 2002, Dr. Ohno has concurrently been running a laboratory at the Research Center for Allergy and Immunology, RIKEN, and has started the research on M cells, a specialized intestinal epithelial cell important for immune surveillance and for the regulation of immune responses, by actively uptaking antigens and microorganisms from the gut lumen and transferring them to the gut-associated lymphoid tissues (GALT) such as Peyer's patches (PPs).

Dr. K. Kuno was promoted to be an associate professor of the Center for Discovery of the Molecular Target of Drugs in this institute in February 2002. Dr. N. Nakamura moved to the Faculty of Pharmaceutical Sciences of this university in October 2002, and Dr. K. Hase joined the division to conduct the M cell project.

A) Functional analysis of Adaptor protein (AP) complexes

Adaptor protein (AP) complexes, along with clathrin, regulate the formation of clathrin-coated vesicles and the signal-mediated sorting of integral membrane proteins in the late secretory and endocytic pathways. AP complexes consist of four subunits including two large chains (α , γ , δ or ε , and β), one medium chain (μ) and one small chain (σ). Four different AP complexes are known to be ubiquitously expressed. In addition, there are two cell type-specific AP complexes: epithelium-specific AP-1B and neuron-specific AP-3B. We showed that AP-1B plays a crucial role in polarized sorting of newly synthesized membrane proteins to the basolateral plasma membrane. We also established AP-3B-deficient mice, which suffered from epileptic seizures. An *in vitro* assay suggested that AP-3B is required for the *de novo* formation of synaptic vesicles from endosomal membranes.

B) Analysis of epithelial differentiation and function

Molecular mechanisms regulating the epithelial differentiation and function are not well understood. F9 mouse teratocarcinoma cells are known to differentiate into epithelial cells in the presence of retinoic acid when cultured as cell aggregate in suspension, but not when cultured on a solid surface such as plastics. We took advantage of the system to develop epithelial differentiation protocol by culturing F9 cells on the filter membrane with retinoic acid. We also isolated a subclone of F9 which could differentiate into epithelial cells cultured on plastics. We are now under the search of genes involved in epithelial differentiation with cDNA microarray approach. We are also studying the differentiation and function of M-cells, a specialized mucosal epithelial cell, which exists in follicle-associated epithelia covering the mucosal lymphoid tissues such as the Peyer's Patch.

C) Golgi matrix proteins and the structural regulation of the Golgi apparatus

Exocytic material is transported through the Golgi apparatus by vesicles shuttling within the Golgi and between the Golgi and other exocytic organelles. In spite of the extensive anterograde and retrograde vesicular flow, the identity and structure of the Golgi apparatus is apparently maintained. It is known that the Golgi matrix proteins help to maintain the identity and structure of the Golgi apparatus. However, the precise mechanism of targeting and organization of Golgi matrix proteins are still obscure. To elucidate the molecular mechanism of the Golgi matrix organization, we aimed to (1) describe the dynamics of Golgi matrix proteins and to (2) identify novel Golgi membrane protein that functions for the anchor of the Golgi matrix proteins.

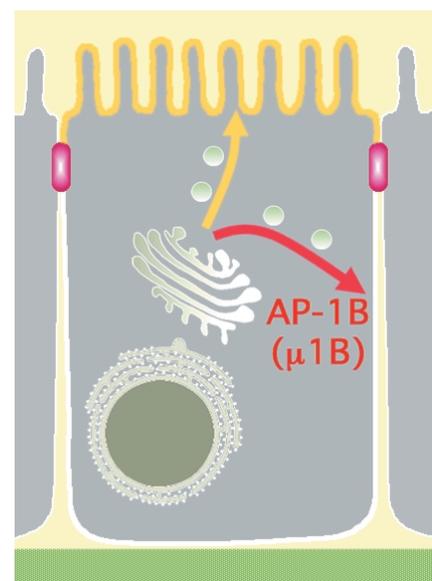
Function of epithelia-specific AP-1B complexes

H. Sugimoto, N. Shioda, T. Mitsunari, F. Nakatsu, M. Kakiuchi, M. Watanabe, N. Nakamura, H. Fölsch, I. Mellman, M. Asano, H. Ohno

The AP complex is a component of coat proteins of the clathrin-coated pit (CCP), which eventually buds off to become the clathrin-coated vesicle, the prototype of transport vesicles delivering the cargo proteins in the secretory/endocytic pathways. We cloned a novel AP μ chain, μ 1B (Ohno et al., FEBS Lett., 1999, 449:215-220). μ 1B is exclusively expressed in epithelial cell types, and makes the epithelium-specific AP complex, AP-1B. Biochemical analyses suggested that the other three subunits of AP-1B are shared with the ubiquitously expressed AP-1A. An epithelial cell line, LLC-PK-1, does not express μ 1B and mistargets many basolateral proteins to the apical surface. Reconstitution of the μ 1B expression in LLC-PK1 selectively restored basolateral targeting (Fölsch et al., 1999, Cell, 99:189-198). Our result, therefore, suggests that μ 1B plays a crucial role in polarized sorting of newly synthesized membrane proteins to the basolateral plasma membrane (figure).

We further analyzed the structural basis of basolateral sorting by making and characterizing a mutant μ 1B. We have previously shown that the μ subunits of AP complexes directly recognize the tyrosine motifs, one of the most commonly appeared among the sorting signals (Ohno et al., Science, 1995, 269: 1872-1875; Boll et al., EMBO J., 1996, 21: 5789-5795; Ohno et al., J. Biol. Chem., 1996, 271:29009-29015; Ohno et al., J. Biol. Chem., 1998, 273:25915-25921). Tyrosine motifs have the consensus sequence of YXX \emptyset (where Y is tyrosine, X is any amino acids, and \emptyset is amino acids with a bulky hydrophobic side chain), in which Y and \emptyset are indispensable for the sorting function *in vivo*. Only the wild-type μ 1B, but not the mutant μ 1B, supported the basolateral sorting of proteins with the tyrosine motifs, as expected. By contrast, both the wild-type and the mutant μ 1Bs were able to target some cargo proteins basolaterally, suggesting that the sorting signal of these proteins interact with a different region of μ 1B than tyrosine motifs. We are now characterizing a series of μ 1B mutants to test the possibility.

Another interesting feature of μ 1B is that it is also required for the monolayer formation of the epithelial cells. The mutant μ 1B can substitute for the wild-type μ 1B in the monolayer formation, suggesting that the basolateral sorting mediated by the sorting signals other than tyrosine motifs is involved in monolayer formation.



Epileptic seizure in neuron-specific AP-3B knockout mice

F. Nakatsu, M. Kakiuchi, M. Watanabe, M. Okada, H. Iwasa, F. Mori, G. Zhu, Y. Kasagi, H. Kamiya, A. Harada, A. Takeuchi, N. Nakamura, K. Nishimura, T. Manabe, S. Yuasa, K. Wakabayashi, S. Kaneko, T. Saito, H. Ohno

There are two isoforms of the AP-3 complex, the ubiquitous AP-3A, and the neuron-specific AP-3B. AP-3A consists of δ , $\beta 3A$, $\mu 3A$ and $\sigma 3$ subunits. AP-3B shares δ and $\sigma 3$ subunits with AP-3A, while the other two subunits of AP-3B, $\beta 3B$ and $\mu 3B$, are neuron-specific. In human, mutations in the $\beta 3A$ subunit of AP-3A were identified in patients suffering from the Hermansky-Pudlak syndrome (HPS), in which function and/or biogenesis of lysosomes and lysosome-related organelles such as melanosomes and platelet dense granules were impaired. *Pearl* mice, one of the HPS model mice, also bear a mutation in the $\beta 3A$ gene and share the same phenotypes with HPS. Another HPS model, *mocha* mice, has mutations in the δ subunit common to AP-3A and AP-3B. In addition to the phenotypes shared with *pearl* mice and HPS patients, *mocha* mice have been reported to suffer from neurological disorders, such as abnormal EEG and inner ear disorders (deafness and balance problems).

To elucidate that these neurological disorders of *mocha* mice result from the impairment in AP-3B, we established $\mu 3B^{-/-}$ mice. The inner ear disorder was not observed in our $\mu 3B^{-/-}$ mice, suggesting that the phenotype is only observed when both AP-3A and AP-3B are deficient in *mocha* mice. However, $\mu 3B^{-/-}$ mice exhibited epileptic seizures (figure). Kindling stimulation and a GABA_A receptor antagonist corroborated the seizure susceptibility of $\mu 3B^{-/-}$ mice. Morphologically, electron microscopy revealed that synaptic vesicles were not homogeneous in size in $\mu 3B^{-/-}$ mice when compared to those in wild-type mice. We also employed an *in vitro* synaptic vesicle formation assay. Brain lysates of wild-type mice, but not of $\mu 3B^{-/-}$ mice, promoted the formation of synaptic-like vesicle formation from isolated endosomal membranes of NGF-treated PC12 pheochromocytoma cells, suggested that AP-3B is required for the *de novo* formation of synaptic vesicles from endosomal membranes. These observations led us to measure the neurotransmitter release. Release of an excitatory glutamate and an inhibitory GABA from the hippocampus was measured in microdialysis experiments. The amount of basal release of the two neurotransmitters was similar between $\mu 3B^{-/-}$ mice and wild-type mice. Of note, however, was that the K⁺-evoked release of GABA was significantly impaired, while that of glutamate was slightly increased, in $\mu 3B^{-/-}$ mice. This difference could not be attributed to the changes in the content of the neurotransmitters themselves in the hippocampus. Taken together, these results suggest that epileptic seizures observed in $\mu 3B^{-/-}$ mice are likely due to impairment in the GABAergic inhibitory neurons. $\mu 3B^{-/-}$ mice may serve as a novel animal model for epilepsy, one of the most common neurological disorders.



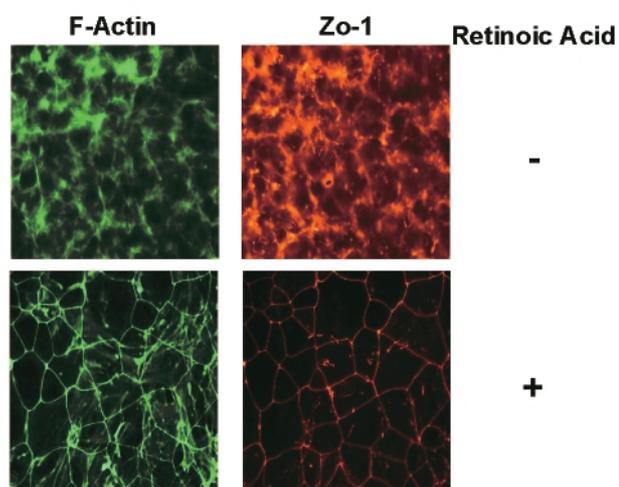
Identification of genes involved in epithelial differentiation

L. Zhong, H. Takatsu, H. Ohno

Molecular mechanisms regulating the epithelial differentiation have not been fully understood. One of the attractive approaches for understanding epithelial differentiation is to enrich the catalog of genes whose expression is specifically changed during epithelial expression. To this end, we decided to employ cDNA microarray analysis to trace the expression profile of the genes during the time course of differentiation. We first tried to set up an *in vitro* epithelial differentiation model using F9 mouse teratocarcinoma cells. F9 cells are known to differentiate into epithelial cells in the presence of retinoic acid. This is observed only when they are cultured as aggregates in suspension, but not when cultured on a solid surface such as a plastic ware. Even in the aggregate culture system, however, only the cells in the outermost single layer of aggregates can differentiate into epithelial cells, remaining the larger number of cells inside the aggregates undifferentiated. This is a disadvantage for the microarray analysis by compromising the detection of specific gene expression with epithelial differentiation by the undifferentiated population.

Epithelial cells generally face the extracellular milieu through their basolateral plasma membrane, where they exchange metabolites. To mimic the situation, we cultured F9 cells on a filter membrane. Interestingly, virtually all the cells on the membrane differentiated into epithelial cells, measured by the formation of tight junction with immunofluorescence microscopy, in the presence, but not in the absence, of retinoic acid.

In the course of the experiments, we found that retinoic acid induced differentiation of a small fraction of F9 cells into epithelial cells on a plastic ware. This prompted us to search for a subclone of F9 cells in which all the cells could differentiate into epithelial cells. As expected, limiting dilution of F9 cells gave rise to a subclone, F9.3, which differentiated into epithelial cells on a plastic ware in the presence of retinoic acid (figure). F9 and F9.3 cells were cultured on a filter membrane or on a plastic ware in the



F9 subclone cultured on plastics

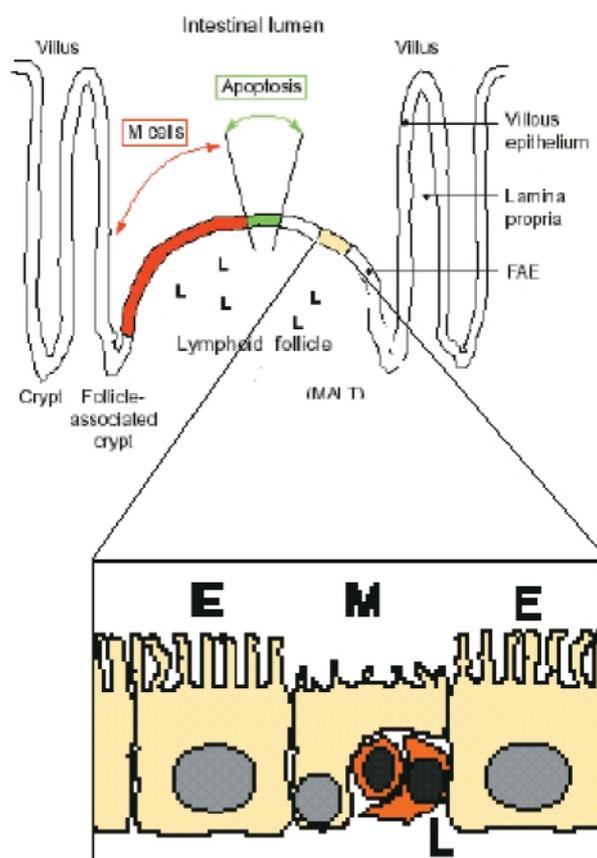
presence or absence of retinoic acid, and RNA was extracted at a various time points during the culture. Amount of the transcript of occludin, a tight junction component, and cytokeratin-18 was quantified with a Taqman real-time PCR method. In F9 cells, both transcripts were detected only when cultured on a filter membrane in the presence of retinoic acid. By contrast, retinoic acid induced the expression of both transcripts in F9.3 cells cultured on either a filter membrane or on a plastic ware. These changes were in good accordance with morphological changes by these cells upon epithelial differentiation. Microarray analysis is now underway using the RNA samples extracted as above.

Study on molecular mechanisms of M-cell differentiation and function

K. Hase, H. Takatsu, Y. Koide, M. Maruyama-Takano, H. Takenouchi, N. Taniura, K. Hashimoto, R. Chihara, H. Ohno

The mucosal epithelium lining the inner surfaces of the body is exposed to various macromolecules and microorganisms. Mucosal membranes are usually a monolayer of epithelial cells, and relatively susceptible to the invasion of microorganisms. Indeed, the mucosal epithelium is the site of invasion for most microorganisms. Therefore, the mucosal membranes are one of the most important places for the immune system to defend the organism from those pathogens. Intestinal epithelial cells are always in contact with enormous numbers of macromolecules and microorganisms through ingestion, and the gut-associated lymphoid tissues (GALT) such as Peyer's patches (PPs) play crucial roles in immunological surveillance and defense at the initial contact site to those pathogens.

PPs are the major site of antigenic macromolecules and microorganisms sampling, which leads to immune responses and/or tolerance. PPs are separated from intestinal lumen by the follicle-associated epithelium (FAE), which contains M-cells (figure). M-cells are specially differentiated epithelial cells. M-cells actively uptake most antigens and microorganisms, and transfer them to PPs for immune response. Thus, passage of those pathogens through M-cells is an essential step for the development of mucosal immunity and the pathology of many infectious diseases. Although the importance of M cells in mucosal and systemic immune responses is obvious, analysis of M cells has been hampered mainly by the limitation in number of M cells ($< 1/10^7$ intestinal epithelial cells) and the lack of specific markers for M cells and of *in vitro* M cell models. Recently, an *in vitro* M cell model has been established, in which the differentiation of M-cells is induced from Caco-2 human colon epithelial carcinoma cells when they are co-cultured with lymphocytes of PPs or Raji B lymphoma cells. We set up the *in vitro* M cell induction protocol to identify molecules involved in M cell differentiation and to understand the cell biology of M cells. We also employ the laser-capture microdissection system to collect M cell-enriched intestinal mucosa for expression profiling analysis using cDNA microarray techniques in search of M cell-specific markers.



Golgi matrix proteins and the structural regulation of the Golgi apparatus

N. Nakamura, S. Yoshimura, A. Shakoori, G. Fujii, T. Ito, H. Ohno

The Golgi apparatus is an organelle situated at the center of the exocytic pathway. Newly synthesized proteins, oligosaccharides and lipids are transported to the Golgi apparatus from the endoplasmic reticulum (ER) and there they are processed, sorted and sent out for their final destinations. In mammalian cells, the Golgi apparatus has typical stacked cisternal structures and these are gathered around the perinuclear region. Exocytic material is transported through the Golgi apparatus by vesicles shuttling within the Golgi and between the Golgi and other exocytic organelles including the ER, endosomes, lysosomes and the plasma membrane. In spite of the extensive anterograde and retrograde vesicular flow, the identity and structure of the Golgi apparatus is apparently maintained. There are structural proteins, namely Golgi matrix proteins, that help to maintain the identity and structure of the Golgi apparatus including golgins, golgin binding proteins and subtypes of ankyrin and spectrin that function for the organization of actin-based membrane skeleton. We have shown that cis-Golgi matrix proteins, GM130 and GASP65 are directly incorporated into the preexisting Golgi matrix (Yoshimura et al., *J. Cell Sci.* 114 p4105, 2001). However, the precise mechanism of targeting and organization of Golgi matrix proteins are still obscure. To elucidate the molecular mechanism of the Golgi matrix organization, we aimed to (1) describe the dynamics of Golgi matrix proteins and to (2) identify novel Golgi membrane protein that functions for the anchor of the Golgi matrix proteins.

- (1) When ER to Golgi transport was blocked, most of Golgi resident proteins were transported back into the ER. In contrast, the cis-Golgi matrix proteins were retained in punctate cytoplasmic structures, namely Golgi remnants. The medial-Golgi matrix proteins were partly retained in the Golgi remnants. These results suggested that cis-Golgi matrix proteins resisted retrograde transport flow and stayed as true residents in Golgi remnants after the inhibition of ER to Golgi transport (Yoshimura et al., submitted).
- (2) A novel family of proteins with multiple transmembrane domains was identified from yeast interactome analysis database and 6 mammalian homologues were isolated. They were localized between the ER and Golgi apparatus and therefore named as Multispan membrane protein localized between the ER and the Golgi apparatus (MERG). Their over expression in the cells disrupted the Golgi apparatus suggesting their role in maintaining the Golgi structure (Shakoori et al., manuscript in preparation).

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**Division
of
Experimental Therapeutics**

General Summary of Division of Experimental Therapeutics

Development of the effective anticancer drugs with excellent specificity, safety, and quality of life is essential for realizing breakthrough in cancer chemotherapy. Recent progression of human genome science has enabled the development of molecular target drugs possessing reasonable mechanisms and high specificity. In this division, design of lead compounds and drug screening, based on molecular mechanisms of pharmacological action, are done.

A) Development of antitumor nucleosides

Development of nucleosides which have unique molecular structure and novel mechanisms of antitumor action, is our main project. We generated the novel synthetic nucleosides (DMDC, CNDAC, ECyd, and EUrd) with strong antitumor activity against human solid tumors *in vitro* and *in vivo*. The clinical investigation of these agents is now underway in the USA. Further basic research on determinant of chemosensitivity and biochemical modulation to improve bioavailability of these nucleosides are in progress. Recently we reported that another nucleoside analogue, 4'-thio-FAC, has an excellent antitumor activity against human gastric cancer MKN-45-P cells in a peritoneal dissemination model using nude mice. 4'-Thio-FAC showed a potent antitumor effect by oral administration and is one of promising antimetabolic agents.

B) Development of anti-angiogenic agents

As urokinase-type plasminogen activator (u-PA) system has been implicated in cancer malignancy, the u-PA system is a considerable target for inhibition of angiogenesis and metastasis. We found out the promising new triazine and ozonide compounds as a result of searching angiogenesis inhibitor targeted for u-PA. Collaborative studies to find new molecular target for prevention of metastasis have been conducted, focused on metastasis-regulating factors including MMP-7, E-cadherin, and S100A4. Furthermore, lymphangiogenesis induced by VEGF-C or VEGF-D is an interesting theme of our research.

Development of antitumor cytosine nucleosides

T. Obata, Y. Endo, M. Tanaka, D. Murata and T. Sasaki

2'-Deoxy-2'-methylidene cytidine (DMDC) and 2'-C-cyano-2'-deoxy-1- β -D-arabinofuranosylcytosine (CNDAC) are novel antitumor cytosine nucleosides with unique mechanisms of action similar in part to those of gemcitabine (dFdC) which has been shown to be an effective therapy against solid tumors. These antimetabolites are incorporated into the cells and phosphorylated by enzymes including deoxycytidine (dCyd) kinase (EC 2. 7. 1. 74). We studied mutational events in dCyd kinase mRNA expression, focusing on aberrant dCyd kinase mRNA which has been frequently observed in established cell lines resistant to antitumor dCyd nucleoside analogues such as Ara-C, dFdC and CNDAC. We reported aberrant dCyd kinase mRNA expression identified as splicing mutants including deletion of the fifth exon, the third exon or the forth exon. The various mutations in the dCyd kinase gene may be caused by acquisition of resistance against several antitumor cytosine nucleosides and dCyd kinase may also be the most important molecule for the activation of the antitumor cytosine nucleoside and their acquisition of resistance.

1-(3-C-ethynyl- β -D-ribo-pentofuralnosyl)-cytosine (ECyd) is a new cytidine analogue showing significant cytotoxicity and antitumor activity in preclinical therapeutic models. The results of several studies have shown that ECyd acts by interfering with RNA synthesis. We examined the effects of dosage schedule on antitumor activity *in vitro* and *in vivo* to determine the optimal administration schedule for ECyd. Furthermore, when the metabolism of ECyd in tumor cells was examined, it was found that ECyd were retained at high concentration for prolonged periods. To elucidate more detail mechanisms, we have established resistant cells to ECyd. ECyd sensitivity of cells was well correlated with the intracellular accumulation of ECTP, which may be affected by both the cellular membrane transport mechanism and uridine/cytidine kinase (UCK; EC 2. 7. 1. 48) activity. UCK is thought to be a rate-limiting enzyme in the pyrimidine salvage pathway for DNA biosynthesis in growing cells. We isolated cDNA encoding the enzyme from human fibrosarcoma cells, then determined its nucleotide sequence by the 5'-RACE method followed by confirmation employing the human genome DNA library. As a result, UCK has two isoforms (UCK1 and UCK2). We investigated the relation between expression of UCK1 and UCK2 at both the mRNA and protein levels and UCK activity in a panel of 10 human cancer cell lines. Expression of UCK2 appeared to be correlated with cellular sensitivity to ECyd, and it may contribute to the tumor-selective cytotoxicity of ECyd.

Moreover, we investigated the antitumor activity of other antitumor nucleoside, 1-2-(deoxy-2-fluoro-4-thio- β -D-arabinofuranosyl)cytosine (4'-thio-FAC) in peritoneal dissemination models of gastrointestinal cancers. Oral administration of 4'-thio-FAC showed a marked effect on the development of ascites and on the survival of nude mice implanted with the highly metastatic MKN-45-P and HCT-15-P. 4'-Thio-FAC is a promising therapeutic agent for peritoneal dissemination of gastrointestinal cancers.

Drug design and synthesis of novel antitumor agents

M. Tanaka, M. Sekine* , F. Yoneda** and T. Sasaki

Phosmidosine was found as a new type of antifungal antibiotic isolated from a culture filtrate of *Streptomyces durhameusis* by Uramoto and Isono *et al.* Later, it was found by mass spectrometry and NMR spectroscopy that phosmidosine is a novel nucleotide-type antibiotic having a N-acyl phosphoramidite linkage which connects a nucleoside analogue, 8-oxoadenosine, with a L-proline residue. A new antitumor active phosmidosine A was successfully synthesized by a series of reactions involving construction of the N-acyl phosphoramidate linkage which was achieved by the reaction of the 5'-O-phosphoramidate derivative in the presence of 5-(3, 5-dinitrophenyl)-1*H*-tetrazole. The growth inhibitory effect of phosmidosine A and its N-acetyl analogue on the various human cell lines was examined. These results showed that the compounds have a significant growth inhibitory activity and that the 6-amino group is not required for the growth inhibitory activity of phosmidosine A. Phosmidosine B is a demethylated phosmidosine derivative with no chirality on the phosphorus. Phosmidosine B was successfully synthesized by the reaction of an N-acetyl-8-oxoadenosine 5'-O-phosphoramidite derivative with an N-protected prolinamide in the presence of 5-(3, 5-dinitrophenyl)-1*H*-tetrazole. The growth inhibitory activity of phosmidosine B and its derivatives in various tumor cell lines was evaluated by the MTT assay. As the result, phosmidosine B showed high antitumor activities and both the diastereomers of phosmidosine were found to have similar but approximately 10 times higher antitumor activity than phosmidosine B. Moreover, it turned out that these phosmidosine derivatives showed characteristic inhibitory activity against tumor cells independent of their p53 phenotypes. These results suggest that phosmidosine and its related compounds would be promising as a new type of antitumor agents having a wide range of inhibitory activities against tumor cells.

Sialic acids are commonly present at the non-reducing terminal positions of carbohydrate chains of glycoproteins and glycolipids on the cell surface and their crucial roles played in biological processes involving cell to cell recognition and interaction masking effects for cell surface antigen, differentiation of cells, and neoplastic transformation have been well studied. Recently, we have synthesized novel 5-deazaflavin substituted with the sialosylalkyl group at the amino group and their physicochemical properties as well as antitumor effects on KB and L1210 cells have been investigated. It has been found that these conjugate molecules show significant antitumor activity. Combination of an 8-amino-5-deazaflavin with the sialosylalkyl group has been found to give rise to significant increase in antitumor activity of the compound. Antitumor effects of 6-nitro-5-deazaflavin-sialic acid conjugate molecules were similar or rather weak in comparison with those of the 6-nitro-5-deazaflavin derivatives without sialosylalkyl group.

(*Tokyo Institute of Technology, **Kyoto University)

Multifunctional anti-angiogenic activity of the novel compounds with antitumor activity

Y. Endo, M. Tanaka, T. Obata, M. Nojima* and T. Sasaki

Angiogenesis is often the primary physiologic response that leads to the development of chronic, sometimes fatal diseases. Indeed, angiogenesis is an essential step that allows tumors to survive initially and to grow at the primary site. Moreover, tumor cells that break away from the primary tumor form metastatic tumors by causing new blood vessels to grow from existing ones. In this way, angiogenesis plays a significant role in both initial tumor development and tumor metastasis. Consequently, compounds that inhibit angiogenesis would be useful in treating not only cancer but also rheumatoid arthritis, diabetic retinopathy and other chronic diseases that depend upon angiogenesis for initial development.

The system that comprises urokinase-type plasminogen activator (u-PA) and its specific receptor (u-PAR) has been a target for anti-angiogenic agent research, since many reports have shown that either the inhibition of u-PA enzymatic activity or the disruption of the u-PA/u-PAR system by small molecules results in decreased metastasis and angiogenesis *in vivo*. However, these u-PA-targeting compounds have not yet been used clinically. Therefore, we aimed to develop a novel anti-angiogenic agent that inhibits u-PA production and also other protease cascades in both endothelial and tumor cells.

During preliminary screening, the effects of 13 ozonides on the inhibition of u-PA production in human fibrosarcoma HT-1080 cells and on the inhibition of angiogenesis on chicken embryonic chorioallantoic membranes were determined. Of the ozonides tested, 9 inhibited *in vitro* u-PA production of HT-1080 cells and 7 of these 9 exhibited strong anti-angiogenic activity. Interestingly, 6 of the 13 ozonides also inhibited cathepsin B activity. 1-Phenyl-1, 4-epoxy-1*H*, 4*H*-naphtho[1, 8-*de*][1, 2] dioxepin (ANO-2) potently inhibited cathepsin B ($IC_{50} = 0.47 \mu M$) as well as u-PA production. Consequently, ANO-2 was selected for further study. ANO-2 inhibited tube formation by human umbilical vein endothelial cells cultured on Matrigel while exhibiting no cytotoxicity. Additionally, *in vivo* administration of ANO-2 inhibited angiogenesis induced by Sarcoma-180 cells tested using the mouse dorsal air sac assay. Moreover, ANO-2 also suppressed primary tumor growth and reduced the number of pulmonary metastases caused by Lewis lung carcinoma cells in mice. These *in vitro* and *in vivo* activities indicate that ANO-2 has considerable potential as a new and potent anti-angiogenic drug that inhibits both u-PA production and enzymatic activity of cathepsins, indicating that ANO-2 may be a multifunctional inhibitor of angiogenesis.

Moreover, the therapeutic potential of a diaminotriazine, 2, 4-diamino-6-(pyridine-4-yl)-1, 3, 5-triazine (4PyDAT), was investigated in a metastatic model using the mouse colon 26 carcinoma variant. The antimetastatic and antitumor activities of 4PyDAT are due in part to inhibition of angiogenesis, rather than direct antiproliferative action on the tumor cells. 4PyDAT may become a lead compound to develop antitumor triazine derivatives based on antiangiogenic action.

(*Osaka University)

Analysis of angiogenesis factors in cancer cells

Y. Endo, Y. Yonemura*, Y. Ohta** and T. Sasaki

Recent advances in molecular biology have clarified the mechanisms of the vasculogenesis, and these have been helped by the isolation of endothelial cell-specific growth factors and their signaling receptors. Vascular endothelial growth factor (VEGF) is well known as an important stimulator of vascular endothelial cell proliferation, migration and permeability and is up-regulated in response to hypoxia. We studied the correlation between VEGF-C and vascular endothelial growth factor receptor-3 (VEGFR-3) expression of 85 primary gastric cancers by RT-PCR and immunohistochemistry, and the results were correlated with the number of lymphatic vessels, stained with anti-VEGFR-3 antibody. RT-PCR and immunohistology demonstrated that VEGF-C was mainly produced from cancer cells, but not from stromal elements. These results strongly indicate that VEGF-C may induce the proliferation of lymphatic vessels in the stroma of primary gastric cancer via activation of VEGFR-3, expressed on the endothelial cells of lymphatic vessels. In these circumstances, cancer cells can easily invade the lymphatic vessel, because of the increase of the contact points of cancer cells with the lymphatic vessels

Expression of VEGF-C and that of its receptors were assessed in non-small cell lung cancer. Immunohistochemistry revealed positive VEGF-C expression in 38.7%(24/62) of the patients studied. A significant positive correlation was found between VEGF-C in cancer cells and VEGFR-3 in vascular endothelial cells, but not between VEGF-C in cancer cells and VEGFR-2 in endothelial cells. We conclude that VEGF-C plays an important role in lymphatic invasion/metastasis and tumor progression in non-small cell lung cancer.

We assessed the association of VEGF on the formation of carcinomatosa pleuritis in orthotopic model systems. Immune-deficient rats were inoculated with PC-14 cells into i) a sub-pleural space of the parietal pleura after pneumonectomy or ii) into the thoracic cavity directly. In the first model, despite no significant difference in the mean volume of the subpleural tumors between the groups, the degree of dissemination was suppressed in the treatment group with less tumor vasculature and with reduced expression of autocrine motility factor receptor in tumor cells. In the second model, although the inhibitory effect on dissemination was not clear, the formation of pleural effusion was inhibited in the treatment group. In addition to the ability of vascular permeability, the results demonstrated here showed the possible association of VEGF with the development of pleural dissemination/metastasis in the context of blood/lymphatic routes and cancer cell motility affected by autocrine motility factor receptor.

(*Shizuoka Cancer Center, **General & Cardiothoracic Surgery of Kanazawa University)

Clinical significance of S100A4 and E-cadherin related adhesion molecules

Y. Endo, K. Kimura*, Y. Yonemura** and T. Sasaki

S100A4, a member of the S100 calcium-binding protein family, has been suggested to be a metastasis-associated molecule. Its overexpression is observed not only in primary and metastatic tumors but also in normal cells with enhanced invasiveness such as macrophages, neutrophils, and T-lymphocytes. E-cadherin has an important role in the homophilic cell-cell adhesion and is an invasion suppressor gene. In the current study, we investigated the histological type and metastatic potential of gastric, non-small cell lung cancer NSCLC and esophageal squamous cell carcinoma from the aspect of the interrelationship of E-cadherin and S100A4 expression. S100A4 protein and E-cadherin were expressed in five of eight gastric cancer cell lines, and inverse expression of the two proteins was found in four cell lines. In the clinical specimens, E-cadherin mRNA expression in differentiated adenocarcinomas (88%, 14 of 16) was significantly more frequent than that in poorly differentiated adenocarcinomas (50%, 22 of 44; $p=0.015$). Western blot analysis demonstrated that S100A4 protein expression in poorly differentiated adenocarcinomas was 1.6-fold higher than in well differentiated adenocarcinoma. Immunohistochemically, S100A4 expression was detected in 51 (55%) of 92 primary gastric cancers. Reduced expression of E-cadherin in primary tumor was found in 66 (72%) of 92 tumors. S100A4 expression in the poorly differentiated adenocarcinomas had a strong relation to positive lymph node involvement or peritoneal dissemination. Also, the S100A4 protein level was significantly higher in tumor tissue than in corresponding normal esophageal mucosa ($p<0.05$) in 22 cases of esophageal carcinoma by western blot analysis. Patients with S100A4-positive carcinoma had significantly poorer prognosis than those with S100A4-negative carcinoma, which was also true in the cases with deep invasion of the primary cancer (T3, T4) ($p<0.01$ and $p<0.05$, respectively). Expression of S100A4 was observed in 81 (60%) of 135 NSCLCs and correlated with progression of the pathological T factor ($p<0.001$), Lymph node metastasis ($p<0.005$), and poor survival ($p<0.05$). The expression of E-cadherin closely correlated with differentiation and inversely with that of S100A4. These results indicate that S100A4 plays a role in the progression and metastasis of various cancers and that simultaneous immunohistochemical detection of S100A4 and E-cadherin may be useful to define subpopulation of lung cancer patients with a possible poor prognosis.

(*General & Cardiothoracic Surgery of Kanazawa University, **Shizuoka Cancer Center)

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**Division
of
Medical Oncology**

General Summary of Division of Medical Oncology

Clinically, we treat patients with various digestive diseases at the department of gastroenterology. So, the conditions of hepatic, pancreatico-biliary, and gastrointestinal cancers from the precancerous state to onset have been basically and clinically analyzed to determine the groups at high risk for each kind of cancer. We have also been evaluating the biological characters and clinical usefulness of tumor-related genes such as oncogenes, oncosuppressive genes, apoptosis-related genes, and so on in various digestive cancers, and their products that have been identified with recent advances in the molecular biology of cancer. Through these researches, we aim to develop new tumor markers that are useful for early diagnosis, selection of treatment methods, and prediction and evaluation of outcomes. In addition, pancreatic disease such as pancreatic cancer (PCa) and pancreatitis has been our major target of our research. We have been working on research projects focused on cancer-related substances, and biological factors associated with pancreatitis.

Formerly, we studied chiefly the characteristics and clinical availabilities of tumor markers such as isoenzymes and carbohydrate antigens. However, most of these substances were identified by chance to be specific to cancer in the tumor-bearing host. Recent molecular biological studies on oncogenesis have revealed that cancer cell phenotypes appear when various changes of oncogenes or oncosuppressive genes occur, and influence the basic character of the cancer cells, such as the degree of malignancy or sensitivity to anticancer drugs. This indicates the potential of tumor-related genes or their products as tumor markers not only for the diagnosis of cancers but also for the evaluation of various characters of cancer. We reported that *K-ras* mutations were detectable in more than 90% of endoscopically aspirated pancreatic juice (PJ) from patients with PCa, using a highly sensitive method such as MASA. However, the frequent presence of *K-ras* mutation was shown in PJ from noncancerous patients, raising questions as to the cancer specificity of this marker. Therefore, we developed a hybridization protection assay which can quantitatively measure mutations of *K-ras* and easily determine the mutational types. This method is a promising approach for the diagnosis of PCa, differentiating from chronic pancreatitis based on a suitable cut-off value, as with the use of a serum tumor marker. Furthermore, mutations of *K-ras* have been found relatively frequently in patients with PCa (66%) or biliary tract cancer (55%) even in duodenal aspirate which is easily obtained. We have approached the same studies as for *p53* and *p16* oncosuppressive genes. The prevalence of *p53* mutations in PJ is about 43%, but not as high as that of *K-ras* mutations. However, *p53* mutations are specifically found only in PCa and also in some PCa patients without *K-ras* mutations, suggesting that it has high specificity for PCa. We have found that the prevalence of gene mutations in the supernatant of PJ is no less than that in the sediment, and the mutations are detectable alone in supernatant in some cases and sediment in others. Using a combination assay with both fractions, the mutations are found out up to 100% in *K-ras* mutations and 56% in *p53* mutations, suggesting an enhanced genetic diagnosis of PCa. In the supernatant of bile obtained from patients with biliary tract cancer by PTC or ERBD, *K-ras* and *p53* mutations are detected at 57% and 50%, respectively. In addition, we have investigated the expressions of gene-products belonging to the so-called cell cycle or apoptosis-related genes, *p53*, *p16*, *p21*, *p27* and *Bcl-2* in a series of pancreatic tissues including normal, benign and malignant tissues by an immuno-histochemical assay to elucidate their clinicopathological significances and mutual relationships of their expressions. Recent studies have demonstrated that activation of telomerase is common and specific event in carcinogenesis. We have tried to measure the telomerase activity in the PJ, but encountered many problems using the TRAP method. Thus, we have been attempting the mRNA level of human telomerase reverse transcriptase (h-TERT) which is a critical determinant of this enzyme activity. h-TERT mRNA is detectable in 38% of the PJ from patients with PCa, but not in any patients with chronic pancreatitis. Further development of new molecular markers and devices for analysis is

needed to improve the genetic or qualitative diagnosis of PCa. We are now researching ways to improve the sensitivity of h-TERT, and analyzing hypermethylation of some genes such as *p16* and *ppENK* in PJ. Additionally, we are currently investigating some overexpressed genes, such as mesothelin, S100A4 which have been identified in PCa by SAGE method, as well as midkine.

On the assumption that in pancreatic diseases, substances derived from the pancreas reflect abnormalities more sensitively in the PJ than in the blood, lactoferrin, reg-protein identical to pancreatic stone protein and tumor carbohydrate antigens in the PJ have been measured, and interesting results were already reported. We have been also analyzing oncogenes such as *K-ras* or *p53* in the pancreatic juice as above mentioned. We are studying the pathophysiological and clinical significance of pancreatitis-associated protein (PAP) belonging to *reg*-gene family, which is almost absent in normal pancreas and is markedly induced in pancreatitis, and have found the increased serum levels of PAP in patients with digestive cancers as well as pancreatitis, reflecting an ectopic expression of PAP in cancer cells. Furthermore, the monitoring of serum PAP levels in acute pancreatitis has been shown the most useful for estimating the severity and clinical healing of the disease. In an attempt to elucidate the role of PAP in the pathogenesis of pancreatitis, we have analyzed expression kinetics of PAP, various cytokines/chemokines, and apoptosis-related factors in spontaneous chronic pancreatitis of the WBN/Kob rat *in vivo*, and in arginine-induced rat pancreatic acinar AR4-2J cell injury *in vitro*. TNF-alpha mRNA peaked at 8 weeks prior to the onset of chronic pancreatitis, PAP, IL-6, IL-8, and TGF-beta peaked at 12 weeks, and IFN-gamma peaked at 16 weeks in the pancreas of WBN/Kob rats. Thus, PAP is strongly induced in pancreatic acinar cells at the onset of chronic pancreatitis in the cascade of cytokines/chemokines. Acinar cell apoptosis assessed by the TUNEL method as well as the expressions of pro-apoptotic factors such as Fas/FasL and *c-myc* showed two peaks at 12 and 20 weeks, suggesting different roles of apoptosis in the onset and progression of chronic pancreatitis. The first peak at 12 weeks was both for pro- and anti-apoptotic factors (PAP, *bcl-2*, clusterin), whereas the second peak at 20 weeks was only for pro-apoptosis. The first peak of apoptosis indicates the acute-phase reaction of the pancreas in response to various stimuli such as oxidative stress, and the second peak suggests the remodeling and pancreatic regeneration in the course of chronic pancreatitis. Therapeutic drugs such as camostat mesilate, TJ-10 and IS-741, suppressed the expressions of these factors, suggesting molecular action mechanisms of the drugs.

Since conventional anti-cancer drugs having direct actions on cancer cells have great limitations in their effects, various therapeutic methods have been studied for the multidisciplinary treatment of cancer. We have also performed multidisciplinary treatment including chemotherapy, BRM therapy, physical therapy such as local injection of alcohol or various BRMs, and endoscopic surgery depending on the patient. The use of endoscopy has been extended from diagnosis to treatment. We have been enthusiastically performing endoscopy to treat cancer such as EMR (endoscopic mucosal resection), although the use of this method requires accurate evaluation of the depth of cancerous invasion. In this respect, endoscopic ultrasonography (EUS) that allows three-dimensional evaluation is of diagnostic value, and we have been conducting fundamental and clinical studies on the diagnostic usefulness of this method in gastrointestinal cancers including submucosal tumor. Furthermore, EUS has been expected to detect mild lesions or small tumor in pancreas where other tests including endoscopic retrograde pancreatocholangiography (ERCP) cannot show diagnostic findings, since EUS is capable of providing not only ductal but also parenchymal features in pancreas. We have intended to elucidate the diagnostic ability of EUS in detecting small pancreatic cancer, applying EUS examination before ERCP in certain patients who are suspected of having pancreatic lesions in a prospective trial. Additionally, we are currently attempting to develop the ability of early diagnosis for pancreatic cancer in which biopsy specimens cannot be easily obtained in combination with imaging test by EUS and molecular biological analysis of tumor-related substances in endoscopically aspirated PJ as above mentioned.

Usefulness of *p53* Gene Mutations in Bile juice for Diagnosis of Biliary Tract Carcinoma: Comparison With *K-ras* Mutations

Y. Yamaguchi, Y. Wang, K. Ohtsubo, H. Watanabe, and N. Sawabu

Biliary tract carcinoma (BTCa) is still difficult to detect at an early stage resulting in a poor prognosis despite recent progress in various imaging modalities, such as ERCP, EUS, CT, and MRI. Cytological examinations of bile have been conducted to diagnose BTCa qualitatively. Although some skillful cytologists have reported relatively high positive rates in BTCa, the accuracy of bile cytology for the diagnosis of BTCa has been limited due to cell injury and degradation induced by various proteases present in bile juice. Thus, the analyses of cancer-related genes in bile for the specific diagnosis of BTCa have been anticipated. Only a few researchers have tried to analyze *p53* or *K-ras* mutations in bile from patients with biliary malignancy, but the detective incidence of *p53* or *K-ras* genetic analyses seems to be limited because of insufficient sensitivity or false positivity. To improve the molecular diagnosis for BTCa, we analyzed *p53* and *K-ras* mutations in DNA extracted from not only the sediment but also the supernatant of bile samples.

For analysis of bile about *p53* and *K-ras* mutations, polymerase chain reaction-single strand conformation polymorphism (PCR - SSCP) and direct sequencing were used for analyses of *p53* mutations in exon 5 through 8. *K-ras* mutations at codon 12 were examined by mutant allele specific amplification, in which the mutation sequence could be determined with high sensitivity. In bile supernatant from patients with BTCa, *p53* and *K-ras* mutations were detected in 50.0% (15/30) and 56.7% (17/30) of cases, respectively. The incidence of *p53* and *K-ras* mutations in the sediment was 33.3% and 43.3%, respectively. On the other hand, in 20 patients with cholelithiasis, *p53* mutations were detected in neither supernatant nor sediment, though *K-ras* mutations were found in the sediment alone in 20%, but not in the supernatant. These data indicated that the supernatant is more favorable for genetic analysis than the sediment. When a combination assay with both genes was used, molecular abnormalities were detected in 80.0% of cases including three in which *p53* alone was positive. In addition, for the cases with BTCa in which bile cytology was examined, either *p53* or *K-ras* mutations were detected in 12 of 15 (80.0%) cases with negative cytology and there were no cases which showed cytology positive and genetic analyses negative as shown in Fig.

In conclusion, the incidence of *p53* and *K-ras* mutations is higher in the supernatant than sediment, and simultaneous analyses of *p53* and *K-ras* in the two bile fractions could enhance the genetic diagnosis of BTCa. Notably, the specificity of *p53* mutations for cancer is very high in bile samples, and the sensitivity is also relatively high.

	cytology	
	class I II III (n=15)	class IV V (n=10)
genetic analyses		

Figure: Comparison between genetic analysis and cytological examination in BTCa. ●, *p53* and *K-ras* mutants; ○, *p53* mutant only; ○, *K-ras* mutant only; △, *p53* and *K-ras* wild-type

Expression of mesothelin mRNA in the pancreatic juice from patients with various pancreatic diseases

H. Watanabe, G. Okada, K. Ohtsubo, Y. Yamaguchi, and N. Sawabu

In recent analysis of gene expression in pancreatic carcinoma (PCa) using serial analysis of gene expression (SAGE) by Ryu et al., the tag for the mesothelin mRNA transcript was present in seven of eight SAGE libraries derived from PCa but not in the two SAGE libraries derived from normal pancreatic duct epithelial cells. Mesothelin mRNA expression was confirmed by *in situ* hybridization in 4 of 4 resected primary PCa and by RT-PCR in 18 of 20 PCa cell lines. Moreover, mesothelin protein expression was confirmed by immunohistochemistry in all 60 resected primary PCa tissues by Argani et al. To improve the genetic diagnosis of PCa, we evaluated mesothelin mRNA expression in pure pancreatic juices (PPJ) obtained from patients with PCa, chronic pancreatitis (CP), and intraductal papillary mucinous tumor (IPMT) by reverse-transcription PCR (RT-PCR). Two products of which the size was 308 and 226 bp were obtained by RT-PCR and the 308 bp RT-PCR product was confirmed as that derived from genomic DNA by direct DNA sequencing. Mesothelin mRNA expression was found out by RT-PCR in 7 (47%) of 15 PPJ from PCa, 5 (45%) of 11 PPJ from IPMT, and 1 (5%) of 20 PPJ from CP. Moreover, RT-PCR product (226 bp) of mesothelin mRNA in PPJ samples with PCa was stronger than that in PPJ samples with IPMT as shown in Figure. These results suggest that detection of mesothelin mRNA in the PPJ may have potential diagnostic implication for pancreatic tumor, and its quantitative analysis may be more useful for genetic diagnosis of PCa.

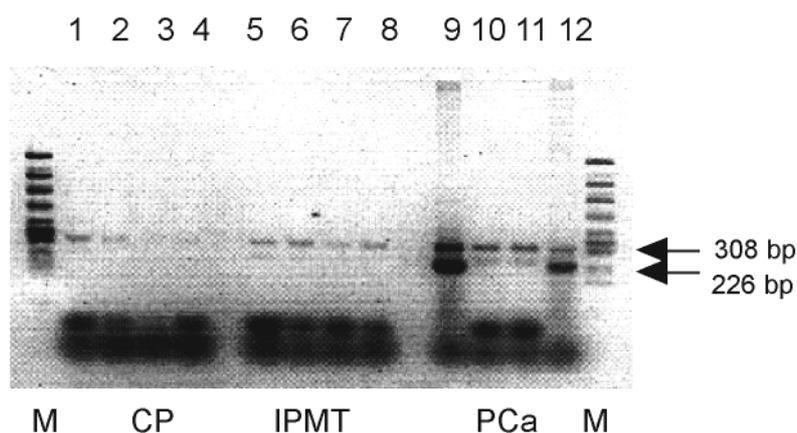


Figure Mesothelin mRNA expression in PPJ by RT-PCR from various pancreatic diseases. M: molecular markers (ϕ X174/Hinc II digest); CP: chronic pancreatitis (1~4); IPMT: intraductal papillary mucinous tumor (5~8) ; PCa: pancreatic carcinoma (9~12)

Usefulness of supernatant of pancreatic juice for genetic analysis of K-*ras* in diagnosis of pancreatic carcinoma

H. Watanabe, A. Ha, Y. Yamaguchi, K. Ohtsubo, Y. Wang, and N. Sawabu

Usefulness of K-*ras* analysis in pure pancreatic juice (PPJ) for the diagnosis of pancreatic carcinoma (PCa) was well known. To ascertain whether analysis of K-*ras* mutation at codon 12 in the supernatant of PPJ is more useful for the diagnosis of PCa than that in sediment, we analyzed K-*ras* mutation at codon 12 in DNA extract from not only the sediment but also the supernatant of PPJ and compared the results. PPJ was endoscopically collected from the 19 patients with PCa and 25 with chronic pancreatitis (CP). DNA was extracted from the supernatant and the sediment of PPJ, respectively. Mutant allele specific amplification (MASA) was performed for K-*ras* mutations analysis with the DNA extracts from these samples. As shown in Table, the incidence of K-*ras* mutations in the supernatant of PPJ was 89% (17 of 19) in patients with PCa and 28% (7 of 25) in patients with CP, whereas that in the sediment was 79% (15 of 19) in patients with PCa and 20% (5 of 25) in patients with CP. Although there was no significant difference in the incidence of K-*ras* mutations between supernatant and sediment, the positive rate of K-*ras* mutations was higher in the former. All of the patients with PCa showed K-*ras* mutations in either the supernatant or sediment of PPJ. Although there was no relation between the incidence of K-*ras* mutations in PPJ and the location and size of tumor, and clinical stage of carcinoma in the patients with PCa, two patients with clinical stage I disease showed K-*ras* mutations in the supernatant. These results suggest that the positive rate of K-*ras* mutations in the supernatant is not less than that in the sediment, and simultaneous analysis of K-*ras* mutations in the supernatant and sediment of PPJ enhances the genetic diagnosis of PCa.

Table Comparison of the incidence of K-*ras* mutations at codon 12 in supernatant or sediment of pure pancreatic juice from patients with pancreatic carcinoma and chronic pancreatitis detected by mutant allele specific amplification

Disease	No. of cases examined	No. (%) of K- <i>ras</i> mutation-positive cases by MASA	
		Supernatant	Sediment
Pancreatic carcinoma	19	17 (89)	15 (79)
Chronic pancreatitis	25	7 (28)	5 (20)

* no significant difference by chi-square test

Abnormalities of Tumor Suppressor Gene *p16* in Pancreatic Carcinoma : Immunohistochemical and Genetic Findings Compared with Clinicopathological Parameters

K. Ohtsubo, H. Watanabe, Y. Yamaguchi, and N. Sawabu

Abnormalities of the tumor suppressor gene *p16* have been reported in a variety of human tumors, but are rare in pancreatic carcinoma (PC) except for cancer cell lines and xenografts. Moreover, their clinicopathological significance remains unknown. The purpose of this study is to examine immunohistochemical and genetic alterations of *p16* in primary PC tissues, and to investigate the relationship between abnormalities of *p16* and clinicopathological parameters in order to elucidate their clinicopathological significance.

We investigated *p16* expression in 60 PC cases by immunohistochemistry using a monoclonal antibody clone G175-405. In addition, we analyzed genetic alterations of the *p16* gene using DNA extracted from microdissected tissue of PC, by PCR-SSCP, DNA sequencing, and hypermethylation analyses using restriction enzymes. We compared the abnormalities of *p16* alterations with clinicopathological parameters in order to elucidate their significance.

On immunohistochemical study, staining for the *p16* protein was strongly positive in 22 (37%) of 60 PC cases, weakly positive in 24 (40%), and negative in 14 (23%). In contrast, *p16* mutations were recognized in 9 (15%) of the 60 PC cases. The incidence of *p16* mutations was 2 (9%) in 22 cases of PC with strongly positive staining, 4 (17%) in 24 with weakly positive staining, and 3 (21%) in 14 with negative staining. Hypermethylation of *p16* was detected in the 2 PC cases with weakly positive staining, although homozygous deletions were not found in any cases. There was no significant correlation between the expression of *p16* protein and any of the clinicopathological parameters. In contrast, for PC with *p16* mutation or hypermethylation, the tumor was significantly larger and the survival period significantly shorter than for PC with an intact *p16* gene ($p < 0.05$) (Table).

These findings suggest that *p16* alterations may participate in the aggressiveness of PC.

Table Correlation between clinicopathological parameters and alterations of the *p16* gene in pancreatic carcinomas.

Parameters	Number	<i>p16</i> alterations (%)	P
Tumor size			
TS1 & TS2	38	4 (11)	0.049
TS3 & TS4	19	6 (32)	
Tumor location			
Head	40	6 (15)	0.30
Body & tail	19	5 (26)	
Pathological findings			
Pap & well	22	3 (14)	0.54
Mod & por	35	7 (20)	
Clinical stage			
I & II	22	2 (9)	0.15
III & IV	37	9 (24)	
Survival (months)			
> 6	25	2 (8)	0.022
≤ 6	23	8 (35)	
LN metastasis			
absent	14	1 (7)	0.067
present	24	8 (33)	
distant metastasis			
absent	35	8 (23)	0.85
present	10	2 (20)	

*pap, papillary adenocarcinoma; well, well-differentiated adenocarcinoma;
mod, moderately differentiated adenocarcinoma;
por, poorly differentiated adenocarcinoma.

Expression of Pancreatitis-associated Protein (PAP) in Human Pancreatic Ductal Adenocarcinoma

Y. Motoo, M.-J. Xie, S.-B. Su, H. Mouri, K. Ohtsubo, and N.Sawabu

Pancreatitis-associated protein (PAP) is a secretory protein of pancreatic acinar cells. It is almost absent in normal pancreas, but is induced in acute and chronic pancreatitis. It functions as an anti-apoptotic factor in acinar cells exposed to oxidative stress. Cytokines increase PAP mRNA expression in the pancreatic acinar cell line AR4-2J. We have reported the expression of PAP mRNA in cancer tissues, and have measured PAP levels in sera and pancreatic juice of patients with gastrointestinal cancers. We found that serum PAP levels were positive (>55 ng/ml) in 40% of patients with pancreatic cancer. Serum PAP levels were significantly higher in pancreatic cancer than in chronic pancreatitis. The aim of the present study was to determine the frequency and pattern of PAP expression in pancreatic cancer at the tissue level, and to evaluate the clinicopathological significance of its expression.

PAP was overexpressed (>30% of total observed area) in 79% (30/38) of pancreatic cancers, in 19% (7/36) of chronic pancreatitis, and in 29% (2/7) of mucinous cystadenomas. PAP was not expressed in normal pancreas. The rate of expression differed significantly between pancreatic cancer and other pancreatic diseases ($P<0.01$). PAP overexpression was found in 50% (4/8) of liver metastasis and 43% (3/7) of lymph node metastasis specimens from patients with pancreatic cancer. Comparison between primary lesions and metastatic lesions showed that PAP was expressed more in metastases than in primary lesions. At the cellular level, PAP was strongly expressed in the cytoplasm of pancreatic cancer cells. PAP overexpression was significantly correlated with nodal involvement ($P<0.05$), distant metastasis ($P<0.05$) and short survival (<12 months) ($P<0.01$). PAP mRNA was expressed in 2 of 4 pancreatic cancers examined, and two pancreatic cancer cell lines, but not in normal pancreas. Multivariate survival analysis revealed that PAP overexpression ($P<0.05$), nutrition ($P<0.005$), and histological type ($P<0.005$) were significantly correlated with survival.

In summary, the present study demonstrates that PAP is overexpressed in human pancreatic ductal adenocarcinoma and would suggest that PAP expression reflects the aggressiveness of pancreatic cancer cells.

Expression of clusterin in pancreatic acinar cell injuries *in vivo* and *in vitro*.

Y. Motoo, M.J. Xie, S.B. Su, H. Mouri and N. Sawabu

Clusterin is a secretory glycoprotein that is highly induced in several tissues in response to injury. Although anti-apoptotic character is reported in clusterin, pathophysiologic significance of clusterin expression in the pancreas remains largely unknown. The aim of this work was to examine whether clusterin is expressed in spontaneous chronic pancreatitis in the WBN/Kob rat and to investigate the relationship between clusterin and apoptosis in rat pancreatic acinar AR4-2J cells. In the *in vivo* study, 4-week-old male WBN/Kob rats were sacrificed at every 4 weeks and the pancreatic alterations were studied with pathological and molecular methods. Only male WBN/Kob rats developed chronic pancreatitis at 12 weeks. Pathologically, the peak of inflammation was seen at 12 weeks and those of fibrosis and acinar cell destruction were at 16 weeks. In RT-PCR analysis, clusterin mRNA was expressed at 12 weeks and then decreased. TNF-alpha mRNA peaked at 8 weeks, PAP, IL-6, IL-8 and TGF-beta mRNAs peaked at 12 weeks, and IFN-gamma mRNA peaked at 16 weeks. Fas and FasL mRNAs as well as acinar cell apoptosis assessed by the TUNEL method peaked at 12 and 20 weeks. Immunohistochemistry showed clusterin expression in the cytoplasm of acinar cells. Thus, clusterin may be expressed in pancreatic acinar cells in response to various stresses and in relation to “cytokine/chemokine cascade” in the pancreas. At the onset of chronic pancreatitis, i.e. at 12 weeks, both pro-, and anti-apoptotic factors are expressed in the pancreas of WBN/Kob rats. Acinar cell death might be determined by the balance between these factors. At 20 weeks, expression of only pro-apoptotic factors and insulin-like growth factor (IGF)-I and c-myc mRNAs increased, suggesting that these factors are involved in acinar regeneration and remodeling during the progression of chronic pancreatitis. In the *in vitro* study, clusterin mRNA and protein were strongly induced in AR4-2J cells treated either with arginine, menadione, TNF-alpha or TGF-beta. In the time course study with arginine or menadione, clusterin mRNA was expressed at 4 hours after the addition of these oxidative stresses and peaked at 24 hours, whereas apoptosis, determined by the DNA fragmentation ELISA assay, peaked at 72 hours. These results show that clusterin is overexpressed in the pancreas at the onset of chronic pancreatitis *in vivo* and in cultured acinar cells in response to various stimuli *in vitro*, suggesting that clusterin is a defense mechanism of the exocrine pancreas.

Endosonographic evaluation of c-kit-positive gastrointestinal stromal tumor

T. Okai, T. Minamoto, K. Ohtsubo, M. Mai, and N. Sawabu

Background: Endoscopic ultrasonography (EUS) is a valuable imaging tool of the gastrointestinal mesenchymal tumor because it allows demonstrating a hypoechoic mass that is contiguous with the fourth hypoechoic layer of the normal gut wall. However, the studies are still a few, and the ability of EUS to distinguish between benign and malignant mesenchymal tumors remained to be confirmed. In the present study we designed to evaluate the endosonographic features of benign and malignant c-kit-positive gastrointestinal stromal tumors (GISTs) in comparison with those of leiomyomas and schwannomas.

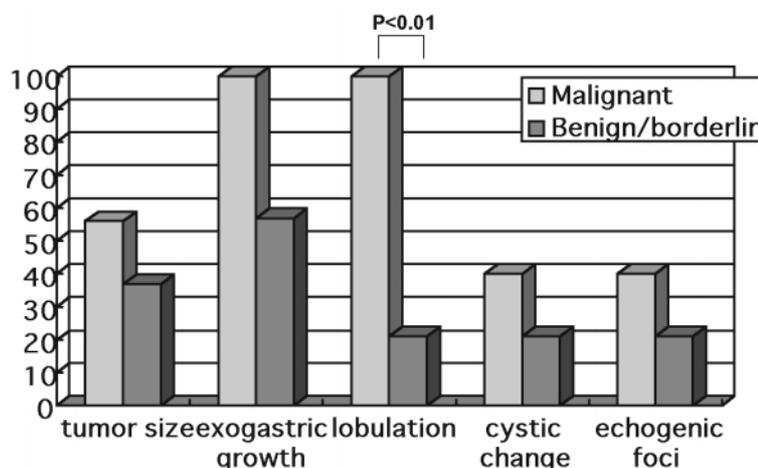
Methods: Twenty-four patients with gastric mesenchymal tumors who underwent EUS and surgical treatment were enrolled. GISTs were defined as c-kit (CD117)-positive tumors, leiomyomas as desmin-positive and c-kit-negative tumors, and schwannomas as S-100-positive and c-kit-negative tumors. Invasion to adjacent organs or more than 20 mitotic counts per 50 high power fields indicated malignancy.

Results: There were 19 GISTs, three leiomyomas, and two schwannomas. All five malignant tumors were GISTs. A marginal halo was found in 12 of 19 GISTs and in both of the schwannomas, but not in any of the three leiomyomas. The echogenicities of GISTs were low but higher than that of the normal proper muscle layer, whereas those of leiomyomas and schwannomas were usually low. Lobulation of the tumor surface was documented only in GISTs, particularly in malignant ones (figure). The tumor doubling time of a malignant GIST was 9.3 months, and that of six benign GISTs was 18.7 months (range = 10.7-28.0 months).

Conclusions: Marginal halo and relatively higher echogenicity on EUS might suggest GIST. Marginal lobulation and a short doubling time may be signs of a malignant GIST.

Figure:

Comparison of EUS features between benign/borderline and malignant GISTs.



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**Division
of
Surgical Oncology**

General Summary of Division of Surgical Oncology

Just one year and half has passed since Cancer Institute Hospital had unified into University Hospital on October 2001. During this period many of doctors have very busily occupied with hospital work and they had no time to spare for research work. Under this circumstance we continued several research works on cancer metastasis, molecular pathology, and tumor immunology. Recent projects may be summarized as follows.

Project on cancer metastasis

To clarify molecular mechanism of cancer metastasis, especially matrix metalloproteinase and angiogenesis using clinical materials, and to inhibit metastasis. We already reported that MT1-MMP expressed in the cancer cell membrane modulates and induces active MMP-2 existed in cancer stroma such as fibroblast or macrophages resulting in degradation of ECM in the process of cancer invasion and metastasis. We also reported that enhanced production of MMP-7 is implicated in the metastasis prognosis of human gastric and colorectal cancer. On the other hand, angiogenesis is essential for tumor growth and metastasis and depends on production of angiogenic factors by tumor cells and/or infiltrating cells. Dr. Takahashi reported clinical significance of these angiogenic factors in various cancer. In order to know metastatic potential before surgery he also investigated mRNA (ISH) analysis of these metastasis related gene products such as MMP-2, VEGF, E-Cadherin, using gastric biopsy specimens. Dr. Yasumoto started to study that metastasis related genes and chemokines have a critical role in determining the metastatic destination of tumor cells to various organs.

Project on chemotherapy including tumor dormancy therapy

The goal of chemotherapy in patients with far advanced cancer should be aimed to prolong the survival of cancer patients, tumor dormancy in other words, rather than tumor shrinkage (killing cancer cells), with fairly good QOL. In order to induce tumor dormancy two approaches are considered: one is a molecular targeting therapy to prolong the dormant period itself and other is to obtain prolonged NC with chemotherapy. We tried a new regimen with continuous administration of low-dose CPT-11 for advanced gastrointestinal cancer patients, resulting in longer TTP (Time to progression) without any adverse effects. As an experimental metastatic models with liver metastasis in nude mice we succeeded to induce tumor regression without overt toxicity by giving anti-VEGF antibody. Tumor dormancy therapy is a promising therapeutic strategy based on cancer biology.

Project on minimally invasive surgery (MIS)

Laparoscopic surgery in patients with early GI cancer allows minimally invasive surgery to be carried out to maintain the patient's quality of life in future. Dr. Omote has conducted this project and extended the indication of MIS in advanced gastric cancer and colorectal cancer. MIS brought less discomfort, less pain and shorter hospital stay, and earlier return to work.

Project on mass screening for GI cancer

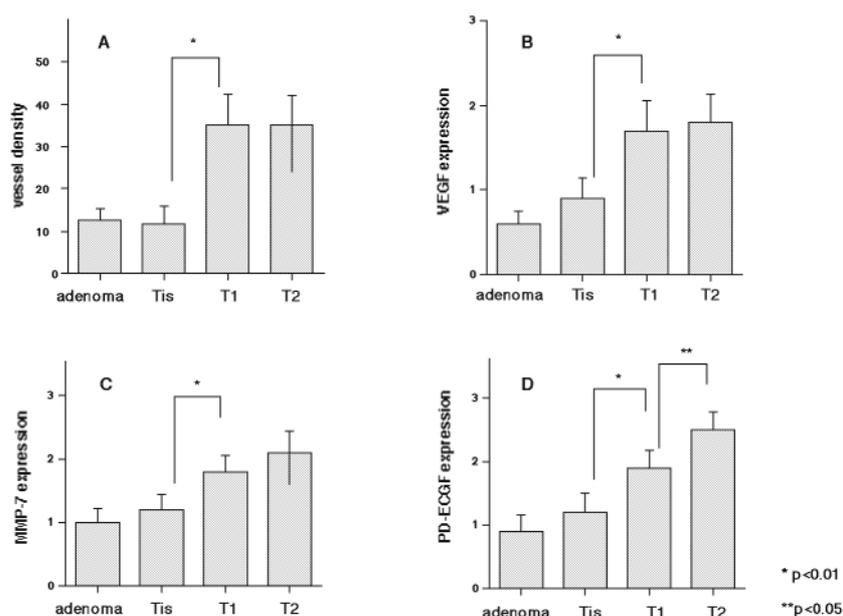
Under the supervisor of Dr. Mai mass screening programs for gastrointestinal cancer were performed in Ishikawa Prefecture. Out of 540,000 persons examined, 947 persons with gastric cancer were detected (detection rate; 0.24-0.17%) during the last 13 years. Kanazawa Medical Association employed a unique screening programs for citizen, i.e, individual screening system which was highly evaluated all over the Japan because of high accuracy of its management. Dr. Mai won an official commendation of two honorable prizes by Japanese Society Against Cancer and by the mayor of Kanazawa City on autumn, 2002.

The Angiogenic Switch of Human Colon Cancer. -It occurs Simultaneous to Initiation of Invasion

Y. Takahashi, M. Mai

We previously reported that vessel count, vascular endothelial growth factor (VEGF) and platelet derived endothelial cell growth factor (PD-ECGF) expression are associated with metastasis formation in human colon cancer. This study was done to determine a stage of colon cancer progression where induction of these factors occurred (i.e. the angiogenic switch). We examined vessel count, VEGF, and matrix metalloproteinase (MMP)-7 expression in cancer cells and PD-ECGF expression in infiltrating cells in 25 adenomas, 35 mucosal cancers (Tis), 29 submucosal invasive cancers (T1) and 33 muscularis propria invasive cancers (T2) by immunostaining. The intensity of staining of VEGF and MMP-7 was evaluated blindly at the invasive edge and was confirmed by image analysis. Intensity of staining for these factors was graded on a scale of 0 to 3+, with 0 representing no detectable stain and 3+ representing the strongest stain. Intensities of PD-ECGF-positive infiltrating cells were similar on a scale 0-3+, as previous studies from our laboratory have demonstrated that PD-ECGF is expressed primarily in tumor infiltrating cells.

There were significant differences in vessel densities, the intensities of VEGF, MMP-7 and PD-ECGF expression between Tis and T1. These results suggest that angiogenic switch may occur between Tis and T1, i.e. simultaneous to initiation of invasion, in the early development of colon cancer.



A new concept of chemotherapy for major solid tumors- A study of low dose, divided MTD

Y. Takahashi, M. Mai

We attempted a new regimen with frequent administration of low dose, divided MTD (maximum tolerated dose) of CPT-11 to reduce its toxicities without any impairment of its efficacy. Twenty-five mg/m² of CPT-11, which was determined by dividing the MTD dose per month by 12, was administered on days 1, 2 and 3 of every week (Fig 1), to 21 consecutive patients of metastatic colon cancers (n=12) and metastatic gastric cancers (n=9). The total delivered dose of CPT-11 per patient in this study was more than 1,000 mg in 17 (80.1%) of 21 cases. Grade-3 marrow depression occurred in 3 (14.3%) patients, but rapidly improved during the washout period. Nausea, vomiting, alopecia, and diarrhea were observed in some cases, but were all categorized as grade 2 or milder (table 2). The antitumor effect was evaluated in 18 patients who had measurable lesions and received CPT-11 according to our regimen for at least 3 weeks. Of these 18 patients, 10, 7 and 1 patients were found to have PR (partial response), SD (stable disease) or PD (progressive disease), respectively, showing a 55.6% efficacy rate [colon 6/10 (60.0%) and stomach 4/8 (50.0%)] (table 1). In addition, time to progression (TTP) was greater than 90 days in 12 (75.0%) of our 18 patients. From these results we suggest that our low-dose, divided MTD of CPT-11 regimen is a promising method with potential for the reduction of toxicities as well as for strengthening the antitumor effect, deserving large-scaled comparative clinical studies to verify such potential.

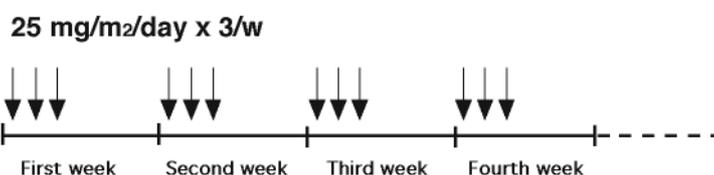


Fig 1. Method of MTD divided low dose CPT-11.

Table 1. Antitumor effects

	Total	Colon	Stomach
CR	0	0	0
PR	10	6	4
NC	7	4	3
PD	1	0	1

Table 2. Grading of toxicities

	Grade			
	0	1	2	3
Leukopenia	6	8	4	3
Diarrhea	8	7	6	0
Nausea/Vomiting	10	6	5	0
Alopecia	11	5	5	0

Laparoscopic Surgery for Early Gastric Cancers

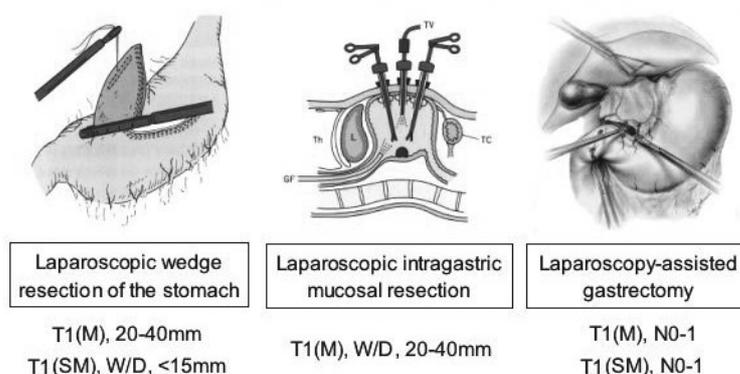
K. Omote, A. Ougolkov, H. Kitakata, K. Yamashita, K. Yasumoto, T. Minamoto, Y. Takahashi and M. Mai

Under the favor of recent advancement of diagnostic endoscopy, gastro-enterologists frequently meet up with early gastric cancers (EGCs) up to 50% of all gastric cancers in usual clinical practice. And recently, endoscopic treatments for EGCs attract the great attention as a minimally invasive therapy for them. Because of good prognosis of EGCs, surgical treatment of them is expected as minimal as possible in order to have a good postoperative quality of life.

Laparoscopic procedures for gastric tumors have been started out in Japan since 1993. And then, they are increasingly and widely accepted in clinical surgery because of their advantages. These procedures cause a small surgical wound, as well as allowing the rapid restoration of peristalsis and a rapid resumption of oral feeding. Thus, compared with conventional open surgery, the hospital stay is shortened and a more rapid return to normal life becomes possible. According to the Practice Guideline of The Japanese Gastric Cancer Association, the Laparoscopic Surgery for gastric cancers should be limited to T1(M or SM) tumors. However, the indication of laparoscopic surgery for gastric cancers has been expanding into the area of indication for more advanced tumors.

The laparoscopic procedures for EGCs are I) Laparoscopic wedge resection of the stomach, “lesion lifting method”, II) Laparoscopic mucosal resection of the stomach, “intra-gastric surgery”, III) Laparoscopy-assisted gastrectomy including laparoscopy assisted distal partial gastrectomy (LAPG) and laparoscopy assisted pylorus preserving partial gastrectomy (LAPPG) (Figure). The last methods have begun to attract attention, because it is employing lymph node dissection being equivalent of a open surgery. Since 1998, we have safely performed LAPG or LAPPG for eight EGC patients associated with good results. Averaged operation time is 296 minutes and averaged postoperative hospital stay is 18 days. For further evaluation of the long time out come and safety of these laparoscopic procedures for gastric cancers, as well as standardization of the surgical technique, many things about these treatments should be well investigated.

The Technique of Laparoscopic Surgery



Analysis of the molecular mechanisms of organ specific metastasis by orthotopic implantation of a human colon cancer cell in nude mice

K. Yasumoto, M. Mai, I. Saiki*

Cancer cell metastasis to distant organs is the major cause of death in cancer patients. However, the molecular mechanisms that control the spread of cancer to distant organs including the lymph nodes are unknown. Tumor metastasis to regional lymph nodes is a crucial step in the progression of cancer. Detection of tumor cells in the lymph nodes is an indication of the spread of the tumor, and is used clinically as a prognostic tool and a guide to therapy. A number of metastasis-related factors have been implicated in the cancer metastasis. Experimental studies with VEGF-C and chemokines have recently shown that they can induce direct metastasis to the lymph nodes.

To clarify the molecular mechanisms regulating the organ specific metastasis, we established an experimental model by orthotopic implantation of a human colon cancer cell in nude mice. This model developed distant metastasis by 6 weeks after tumor injection into the cecum. 100% of nude mice developed primary tumor. 30% of mice with local tumor produced the liver metastasis. 90% of mice produced lymph nodes metastasis.

We now started to investigate the mRNA expression of metastasis related factors (VEGF-isoforms, basic FGF, MMP-2, -7, -9, TIMP 1, MT1-MMP, c-MET) and chemokines (CCR7, CXCR4) using RT-PCR method in the primary site, metastatic sites. In preliminary examination, we observed overexpression of one of VEGF-isoform, VEGF-165, in the metastatic liver tumor. On the other hand, there were no significant differences of the mRNA expression of other VEGF-isoforms (VEGF-121, VEGF-189), basic FGF and VEGF-C among those metastatic sites.

*Dept. of Pathogenic Biochemistry

Research Institute for Wakan-Yaku, Toyama Medical and Pharmaceutical University

Essential roles of tumor necrosis factor receptor p55 in liver metastasis of intrasplenic administration of colon 26 cells

H. Kitakata, Y. Nemoto-Sasaki¹, Y. Takahashi, M. Mai, N. Mukaida¹

¹Division of Molecular Bioregulation

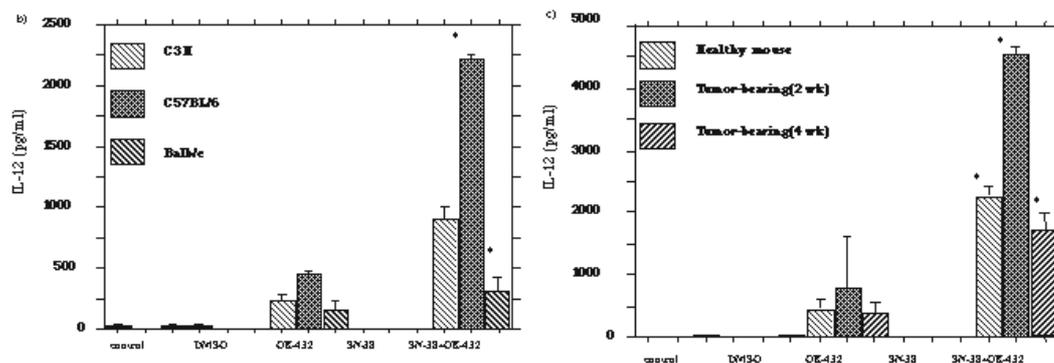
Tumor necrosis factor (TNF)- α was originally identified as a cytokine responsible for endotoxin-induced tumor necrosis. Previous reports demonstrated that therapy with recombinant TNF- α was effective against several types of murine tumor models of hepatic and pulmonary metastasis, particularly when it was administered in combination with interferon- γ or IL-2. Moreover, a Phase I clinical study of TNF- α treatment demonstrated partial efficacy against metastatic spread. However, in several models, the administration of TNF- α or the TNF- α gene transduction into tumor cells, enhanced the incidence of metastasis. These contradictory results may be explained by the differences in the cell types used in each experiment. Consequently, the roles of endogenous TNF- α in the metastatic process remains to be determined. Hence, in order to evaluate the roles of TNF- α , we injected a colon adenocarcinoma cell line into the spleen of wild-type (WT) and tumor necrosis factor receptor p55 (TNF-Rp55)-deficient (KO) mice.

WT mice exhibited enhanced TNF- α protein expression around the central and portal veins of the liver by 3 days after intrasplenic injection of a colon adenocarcinoma cell line, colon 26. Moreover, 90% of WT mice developed liver metastases by 24 days after the tumor injection. In contrast, liver metastasis developed in less than 50 % of TNF-Rp55 KO mice. Liver weights and the volumes of metastatic foci were significantly lower in TNF-Rp55 KO mice. These observations suggest the critical roles of TNF-Rp55-mediated signals in this liver metastasis model. The intrasplenic tumor injection induced mRNA expressions of vascular endothelial growth factor, heparin-binding epidermal growth factor, matrix metalloproteinase-9 and tissue inhibitor of matrix metalloproteinase-1 at similar levels in the livers of both WT and TNF-Rp55 KO mice. Immunohistochemical analyses of the livers of WT mice after tumor injection demonstrated the enhanced expression of vascular cell adhesion molecule (VCAM)-1 and E-selectin on sinusoidal endothelial cells. Enhanced E-selectin expression was similarly observed in the liver of TNF-Rp55 KO mice after tumor injection. However, the enhancement in VCAM-1 mRNA expression and protein production was significantly attenuated in the liver of TNF-Rp55 KO mice, when compared with WT mice. Collectively, these observations suggest that TNF-Rp55-mediated signals can regulate both VCAM-1 expression in the liver and subsequent liver metastasis following intrasplenic tumor injection.

Irinotecan Enhances IL-12 Production by OK-432-Activated Murine Macrophages

B. Zhang, T. Fujimoto, M. Mai

Background: Our previous study showed that the combination of irinotecan (CPT-11) and OK-432 had an additive antitumor effect. The purpose of this study was to analyze the mechanism by which this combined treatment had an effect on immunity. *Materials and Methods:* To investigate the immune effects of murine splenocytes stimulated by SN-38 (the active form of CPT-11) and OK-432, endogenous interleukin (IL)-12 p70 production was assayed by ELISA and flow cytometry. *Results:* Endogenous IL-12 production was increased by SN-38 stimulation of cultures of OK-432-activated splenocytes from C57BL/6, C₃H, and Balb/c mice, which was not observed with LPS-activated splenocytes. IL-12 production by splenocytes was higher at an early stage after tumor inoculation. SN-38 and OK-432 stimulated IL-12 production in cultures of peritoneal exudate macrophages (PEM), and T cell cooperation was essential in cultured splenocytes. *Conclusions:* These results suggest that the interaction of SN-38 and OK-432 may support a type 1 T helper (Th1)-dominant state through increasing endogenous IL-12 production, mainly by macrophages.



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**Division
of
Diagnostic
Molecular Oncology**

General Summary of the Division of Diagnostic Molecular Oncology

To strengthen its capabilities for responding to the rapid pace of advance in basic and clinical cancer research, and to better meet its evolving responsibilities, the Cancer Research Institute of Kanazawa University was restructured in April of 1997. At the time the Division of Diagnostic Molecular Oncology was established as a clinical oncology division. Following a semester of four years for building up research activity and organizing laboratory, the division assumed an active role in July of 2001, when Dr. T. Minamoto was elected Professor and Chief of the division. Through twice movements, the division has been enlarged having more research members and two exclusive laboratories in the Institute.

The mission of the division, broadly, centers on basic and clinical research to improve the molecular diagnosis of cancer. Whereas genetic diagnosis is generally concerned with hereditary (non-neoplastic) diseases, the molecular diagnosis of cancer encompasses not only germ-line diagnosis of hereditary cancers, which are uncommon, comprising less than 1% of all human cancers, but also early detection and molecular assessment of sporadic cancers, which make up the vast majority of malignancies seen in the clinical setting. Molecular diagnosis thus includes assessment of tumor aggressiveness and biological characteristics of individual tumor types relevant to metastatic potential, recurrence and outcome.

Table. Research directions and activities in the Division of Diagnostic Molecular Oncology

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| <p>I. Activities toward molecular diagnosis of hereditary nonpolyposis colorectal cancer (HNPCC)</p> <ol style="list-style-type: none">1. Clinical and genetic analysis of the patients and their families2. Genetic testing for DNA mismatch repair enzymes' genes3. Surveillance and (surgical) intervention <p>II. Molecular diagnosis of pathology and malignant potential of cancers</p> <ol style="list-style-type: none">1. Oncogenic signaling in colorectal cancers, targeting ras, β-catenin and hdm2 (mdm2)2. Post-translational modifications (stability, phosphorylation) and function of p53 tumor suppressor protein3. Molecular pathology of clinical gastrointestinal cancers4. Sensitive detection of ras and β-catenin oncogenes for molecular diagnosis of cancers <p>III. Genome-wide analysis of gene expression in gastrointestinal cancers</p> <ol style="list-style-type: none">1. Genome level: restriction landmark genomic scanning (RLGS)2. Transcriptome level: serial analysis of gene expression (SAGE)3. Epigenetic level: development of a new strategy covering all epigenetic alterations spanning through the genome |
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Current efforts in this division are focused on 1) clinical and basic activities toward molecular diagnosis of hereditary nonpolyposis colorectal cancer (HNPCC); 2) the molecular characterization of gastrointestinal and breast cancers by an interdisciplinary approach bringing together clinical oncology, molecular pathology and biology; and 3) comprehensive (genome-wide) analysis of gene expression and epigenetic alterations in stomach and large bowel cancers by the highly sophisticated methods including RLGS and SAGE, to cover the post-genome research fields (Table). We will intend to translate as much the results of these studies as possible to those responsible for diagnosis and management of cancer patients in clinical setting.

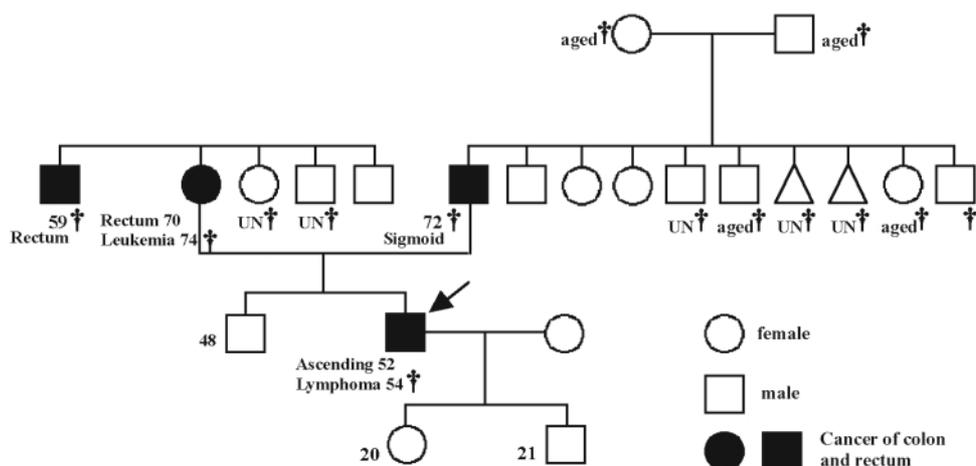
Genetic and clinical study for hereditary nonpolyposis colorectal cancer (HNPCC): toward molecular diagnosis of HNPCC

K. Hirano, Y. Takahashi, M. Mai, T. Minamoto

1. Activities toward molecular diagnosis of hereditary nonpolyposis colorectal cancer

Among the known hereditary cancers, one of the best characterized is the syndrome of hereditary nonpolyposis colorectal cancer (HNPCC), reported to comprise approximately 5-10% of all colorectal cancers. Clinically, this syndrome features multiple instances of colorectal cancer with early onset and a propensity for involvement of the right side of the colon proximal to the splenic flexure. Particularly striking is HNPCC's frequent association with cancers of extracolonic organs—endometrium, ovary, urinary tract, small intestine and stomach. Most HNPCC patients harbor a germ-line mutation in a DNA mismatch repair gene. We have clinically identified HNPCC in 5% of all colorectal cancer patients (459 cases) who underwent surgery in our Institute's hospital. The excitement as a timely topic for basic research has been somewhat diminished, however, one of our ongoing efforts is to establish a program for molecular diagnosis and clinical management of HNPCC patients, addressing ethical, legal, social, and psycho-oncological issues. A preliminary trial of a method for detecting germ-line mutations in *hMSH2* and *hMLH1* is completed here and ready for clinical application.

2. NonHodgkin's Lymphoma in a Patient with colon cancer of HNPCC-Report of A Case



Hematological malignancy has rarely been reported in association with HNPCC. We present here the case of an HNPCC patient in whom nonHodgkin's lymphoma developed after curative resection of colon cancer. Our experience with this rare case encouraged us to determine a possible relationship between the two diseases. A 52-year-old man whose family history was consistent with the criteria of HNPCC had ascending colon cancer. The tumor consisted of adenocarcinoma that was moderately differentiated with mucinous foci. Eight months after surgery, the patient developed nonHodgkin's lymphoma of T-cell origin involving ileum and lungs. Both colon cancer and lymphoma frequently showed microsatellite DNA instability, sharing alteration in a locus of the chromosome 7 (*D7S501*). A possible association of hematological malignancy with HNPCC reported in the literature, together with a report that *MSH2*-deficient mice are susceptible to malignant lymphoma, strongly supports the finding that this patient's lymphoma was HNPCC-related. In all, this case manifested a distinct clinical course similar to that observed in an animal model that is deficient in DNA mismatch repair machinery, thus providing scientific and clinical implications for understanding the molecular basis of these tumors and for critical management of HNPCC patients, respectively.

Oncogenic β -catenin and MMP-7 cosegregate in late-stage clinical colon cancer

A. Ougolkov, K. Yamashita, M. Mai, T. Minamoto

Recent *in vitro* studies demonstrated that β -catenin translocated into the tumor cell nucleus functions as an oncogene by transactivating oncogenes, including MMP-7. We conducted a large-scale analysis of β -catenin and MMP-7 expression in human colon cancer to determine the potential clinical importance of these molecules. In 202 colon cancer patients with known post-operative outcomes, we determined the expression of β -catenin and MMP-7 in the tumors immunohistochemically and correlated the findings with the patients' clinicopathological characteristics and survival. We found two distinct patterns of β -catenin nuclear accumulation (NA) in the colon cancers: diffuse NA (NAd) in 89 cases (44%) and selective NA at the invasion front (NAinv) in 18 cases (9%). The presence of the NAinv pattern was significantly correlated with advanced Dukes' stage ($P=0.0187$) and tumor recurrence ($P=0.0005$) as well as with MMP-7 expression in the tumor invasion front ($P=0.0025$) (Figure 1), resulting in extremely unfavorable clinical outcomes (Figure 2). A multivariate analysis determined that the NAinv expression pattern and Dukes' C stage were independent prognostic factors. Oncogenic activation of β -catenin in the tumor invasion front, as represented by its NAinv pattern of expression, may be an independent and reliable indicator of membership in a subset of colon cancer patients who are highly susceptible to tumor recurrence and have a less favorable survival rate.

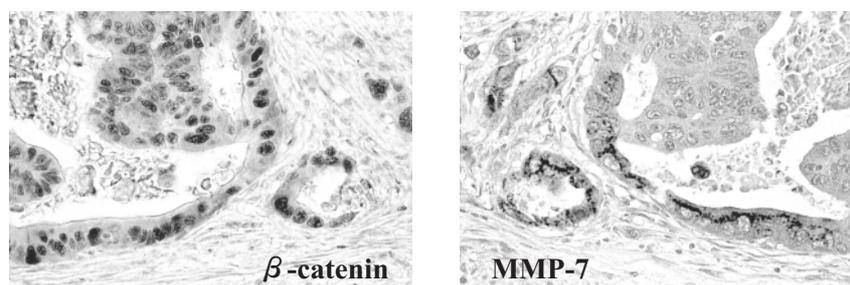


Figure 1. Immunohistochemical staining of a pair of serial sections from the same tumor in a mirror image clearly demonstrated colocalization of nuclear β -catenin and cytoplasmic MMP-7 in the same carcinoma cells in the invasion front.

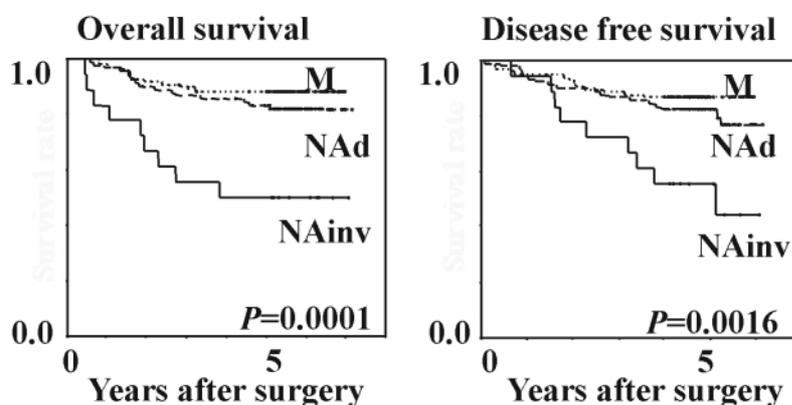


Figure 2. Comparison of survival of the patients with colon cancer according to the different patterns of β -catenin activation in the primary tumors. Kaplan-Meier survival curves showed a statistically significant survival disadvantage in colon cancers with NAinv pattern of β -catenin activation.

Stability of mutant p53 proteins

T. Minamoto, T. Buschmann, M. Mai, Z. Ronai

Identification of Mdm2 (Hdm2) and JNK as proteins that target degradation of wild-type (wt) p53 prompted us to examine their effect on mutant p53, which exhibits a prolonged half-life (Figure). Of five mutant p53 proteins studied for association with the targeting molecules, two no longer bound to Mdm2 and JNK. Three mutant forms, which exhibit high levels of p53 expression, showed lower affinity for association with Mdm2 and JNK in concordance with greater affinity to p14^{ARF}, which is among the molecules stabilizing p53. Monitoring mutant p53 stability *in vitro* confirmed that, while either JNK or Mdm2 no longer affects certain forms of mutant p53, JNK and Mdm2 target others for degradation, albeit at lower efficiency when compared with wt p53 protein. Expression of wt p53 in tumor cells by gene transfection revealed a short half-life, suggesting that the targeting molecules are functional. Forced expression of mutant p53 in p53 null cells confirmed pattern of association with JNK and Mdm2 and prolonged half-life, as found in the tumor cells. Overexpression of Mdm2 in either tumor (which do express endogenous and functional Mdm2) or in p53 null cells decreased the stability of mutant p53, suggesting that, despite its expression, Mdm2 and JNK are insufficient (amount and/or affinity) for targeting mutant p53 degradation. Based on both *in vitro* and *in vivo* analyses, we conclude that the prolonged half-life of mutant p53 depends on the nature of the gene mutation, which either alters association with targeting molecules, ratio between p53 and targeting and/or stabilizing molecules, or their targeting efficacy.

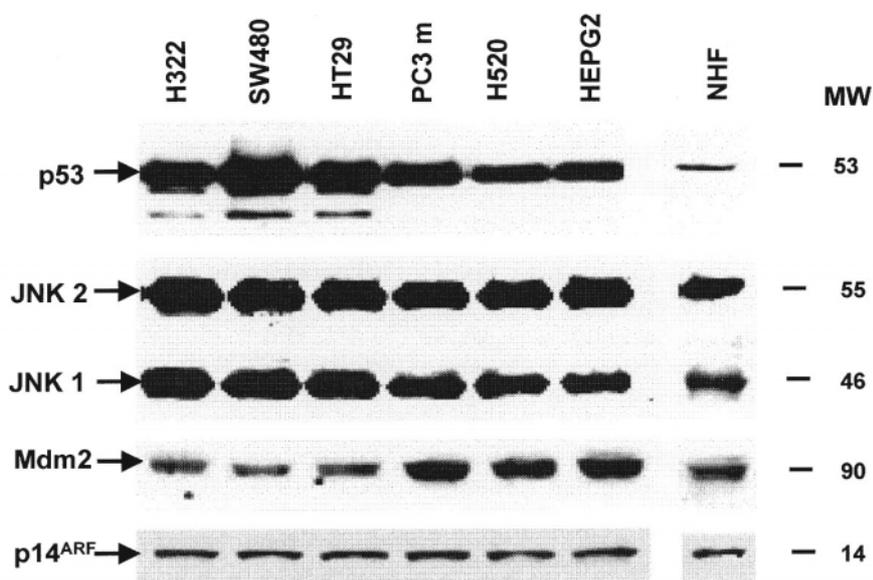


Figure. Expression levels of mutant and wild-type p53 proteins in human tumor cell lines and normal human fibroblast. Whole cell extracts (125 μ g each) of tumor cell lines and normal fibroblast were separated on SDS-PAGE and transferred to nitrocellulose membranes that were probed with the monoclonal antibodies against p53 (DO1), JNK (333), Mdm2/Hdm2 (2A10) and p14ARF (Labvision), respectively. Molecular weight of the protein of interest is indicated on the right to the panel. Origin of cell lines (mutant codon and type of mutation); H322, bronchioalveolar carcinoma (248^{G-T}); H520, squamous cell carcinoma of the lung (146^{G-A}); SW480 and HT29, colon adenocarcinomas (273^{G-A} and 309^{G-T}, both); PC3m, prostate carcinoma (282^{C-T}); HEPG2, hepatocellular carcinoma (wild-type); NHF, normal human fibroblast GM00038A.

Distinct pattern of p53 phosphorylation in human cancers

T. Minamoto, T. Buschmann, Z. Ronai

The protein product of the tumor suppressor gene *p53* is phosphorylated on multiple residues by several protein kinases. The extensive changes seen in *p53* phosphorylation during cell cycle progression led to the hypothesis that tumors may exhibit a different phosphorylation pattern of *p53*, either because of the presence of mutation, or deregulated kinases or phosphatases. Using a battery of 10 antibodies developed against different phosphorylated and acetylated residues of human *p53* protein, we compared the pattern of *p53* phosphorylation (at serines 6, 9, 15, 20, 37, 125, 372 and 392; threonine 81) and acetylation (at lysines 320/373/382) in tumor-derived cell lines, tumor samples, and non-neoplastic cells. Irrespective of tumor types or the presence of *p53* mutation, phosphorylation and acetylation of *p53* was substantially higher in samples obtained from tumor cells and tissues than those found in non-transformed (non-neoplastic) samples (Figure). Among the 10 sites analyzed, phosphorylation of serines 15 and 392, and threonine 81, and acetylation at lysines 320/373/382 were among the more frequent modifications. Analysis of two of the more abundant phosphorylation or acetylation sites on *p53* is sufficient to detect more than 70% of tumor-derived *p53* protein samples. The distinct pattern of *p53* phosphorylation and acetylation in human cancers may offer a new means to monitor the status and activity of *p53* in the course of tumor development and progression.

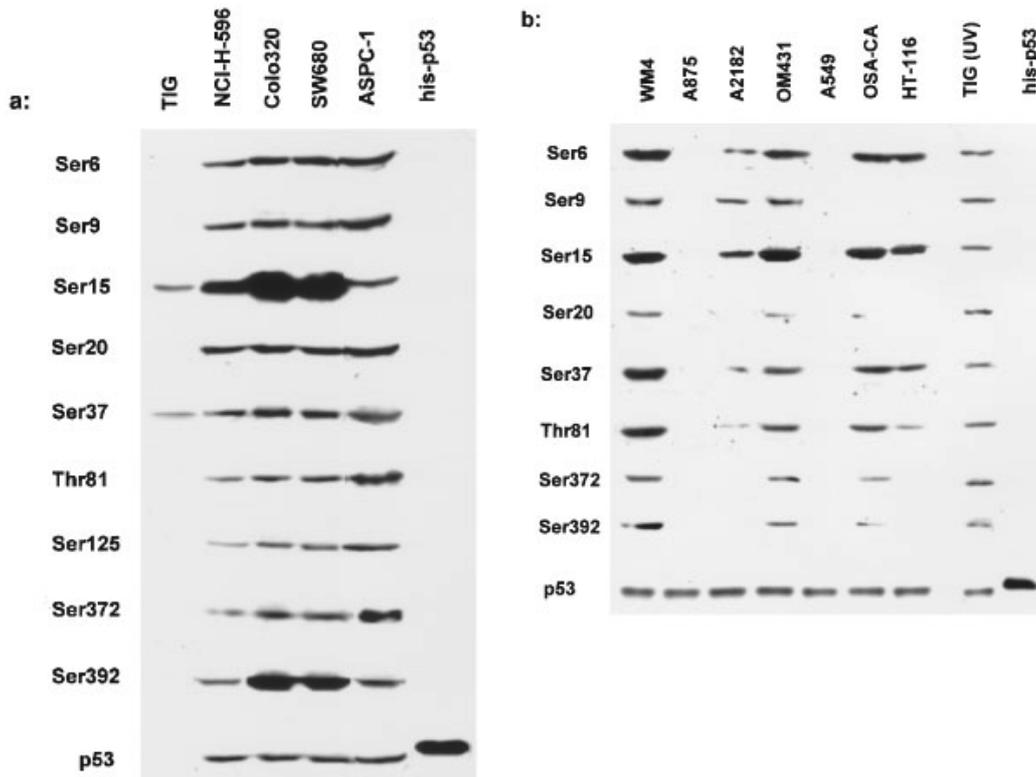


Figure. Phosphorylation of mutant (a) and wt (b) *p53* proteins in tumor-derived cell lines and normal human fibroblast (TIG) without and with UV irradiation, detected by Western blot analysis using antibodies specific to each phosphorylation sites. The amount of *p53* protein in each sample was equal determined by labeling with anti-*p53* antibody, pAb421. NCI-H596 (245^{Gly-Cys}), lung adenosquamous carcinoma; Colo320 (248^{R-W}), SW680 (273^{R-H}), HT-116, colon adenocarcinoma; ASPC-1 (273^{R-H}), pancreas adenocarcinoma; WM4, A875, A2182, OM431, A549, OSA-CA, malignant melanoma; his-*p53*, histidine-tagged recombinant wt *p53* produced in bacteria.

Clinical and molecular analysis of early invasive carcinoma of the colon and rectum: identification of predictor(s) of metastasis

K. Hirano, M. Mai, T. Minamoto

Early colorectal carcinoma is defined as a primary adenocarcinoma confined to the mucosa or submucosa (early invasive carcinoma, EIC), whether it has metastasized or not. Metastasis occurs in 10-15% of all EIC patients. The clinical management of EIC remains controversial, because the decision for endoscopic or local resection vs. radical bowel resection depends on the risk of metastasis. Lymph node metastasis was reported to correlate with such morphological factors as nonpolypoid growth pattern, poorly differentiated histological characteristics, depth of invasion, and lymph vessel involvement. However, prediction of metastasis on the basis of such morphological factors alone is difficult. Reliable risk factors susceptible to more objective determination are needed to assess the presence of metastasis and, in turn, make possible a more reasonable way of identifying the appropriate therapy. In order to place the choice of therapy (endoscopic resection or radical surgery) in early invasive carcinoma of colon and rectum on a more rational basis, this study sought to identify molecular predictor(s) of metastasis. Several morphological risk factors (histological type, degree of tumor invasion, lymphatic and venous invasion) and expression of p53 and p27 proteins in the primary tumor were compared in 80 patients with EIC, including 12 (15%) with metastasis and/or recurrence. Of the factors enumerated, deeper invasion of the submucosal layer, lymphatic/venous invasion, p53 overexpression, and decreased expression of p27 were significantly correlated with metastasis. The results also indicated that altered expression of p53 or p27 is independently relevant to metastasis of EIC. Analysis of these markers together with determination of the morphological risk factors could complement the identification of patients with metastasis on the basis of known morphological risk factors. Since the molecular factors can be assessed more objectively than the morphological parameters, they may strengthen the ability to identify EIC that has undergone, or will undergo, metastasis.

Table 4. Relative influence of the morphological risk factors and altered expression of p53 and p27 proteins on metastasis in the patients with colorectal EIC

Variables	Metastasis		Chi-square analysis
	Present	Absent	
Gross type (n=80)			
Type I (protruding)	6	44	NS
Type II (superficial)	6	24	p=0.33197
Histological type (n=80)			
Well differentiated	4	47	p=0.01744
Moderately differentiated	8	21	
Degree of invasion (n=80)			
Non-massive	1	38	p=0.00238
Massive	11	30	pc=0.00463
Lymphatic/venous invasion (n=80)			
Absent	5	60	p=0.00014
Present	7	8	
p53 overexpression (n=71)			
Absent	3	43	p=0.0046
Present	8	17	
p27 expression (n=66)			
High	2	38	p=0.00161
Low	9	17	pc=0.00486

Abbreviation: n, number of patients analyzed; NS, no significant difference; pc, p value adjusted by Yates' correction

Involvement of erbB-2 and β -catenin oncogenes in stomach cancer

A. Ougolkov, K. Yamashita, Y. Takahashi, M. Mai, T. Minamoto

1. Altered expression of β -catenin and c-erbB-2 in early gastric cancer

To investigate the possible relationship between altered expression (loss of membranous staining or nuclear accumulation) of β -catenin and invasion/metastasis in early gastric cancer (EGC), β -catenin was detected immunohistochemically in 116 cases of EGC, including 86 differentiated and 30 poorly differentiated carcinomas. In parallel, immunohistochemical expression of c-erbB-2 was analyzed in all EGC cases. Regardless of histological types, altered expression of β -catenin was found in 47% of mucosal carcinomas and 89% of carcinomas with submucosal invasion ($p < 0.001$). Of particular interest is that β -catenin alteration was found in most EGCs with lymph node metastasis, even though no significant statistical comparison could be made. These results suggest that molecular changes resulting in abnormal β -catenin expression participate in the process of submucosal invasion and metastasis to the lymph node. While loss of expression was preferentially observed in poorly differentiated EGCs, nuclear accumulation was found exclusively in 24% of differentiated EGCs. c-erbB-2 was overexpressed in only 16% of differentiated EGCs but there was no correlation between this overexpression and presence of invasion or metastasis. However, it is intriguing that 12 out of 14 cases with c-erbB-2 overexpression also showed altered β -catenin expression in the tumors, suggesting that both molecules are involved in the development of a certain set of differentiated EGCs.

2. Abnormal expression of E-cadherin, β -catenin and c-erbB-2 in advanced gastric cancer: its association with liver metastasis

Background and Aims. We investigated expression of E-cadherin, β -catenin and c-erbB-2 in gastric cancer to identify molecular factor(s) relevant to development of liver metastasis, which is a frequent cause of mortality in gastric cancer patients.

Patients and Methods. We analysed by immunohistochemistry and compared expression patterns of E-cadherin, β -catenin and c-erbB-2 in the tumor between 40 cases of gastric cancer (GC) without [GC-H(-)] and 16 with concurrent liver metastasis [GC-H(+)].

Results. Loss of E-cadherin expression in the primary tumor was found in 18% of GC-H(-) and in 19% of GC-H(+). Oncogenic β -catenin activation, represented by its nuclear translocation, was detected in 13% of GC-H(-) and in 31% of GC-H(+). There was no statistical difference in incidence of alteration in these molecules between the two groups of patients. c-erbB-2 overexpression was more frequently observed in GC-H(+) (10/16, 63%) than in GC-H(-) (5/40, 13%) ($p = 0.0001$) while the distribution of histological types of the tumors was similar in the two groups of patients. This overexpression was also detected in metastatic liver tumors and biopsy specimens in the 10 of the former group of patients.

Conclusion. Our results strongly suggest a role of activated c-erbB-2 in the process of liver metastasis, and an importance of detection of this overexpression in biopsy specimens to identify GC patients who are at high risk of developing liver metastasis.

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Other Achievements

1. The 15th Award from the Hokkoku Cancer Research Promotion Foundation, October 2001. Title: Development of germ-line and somatic diagnosis for colorectal cancer
Awardees: Toshinari Minamoto
2. The 34th (2002) Award for Promotion of Science from the Naitoh Foundation, October 2002.
Title: Molecular mechanism(s) that regulates distinct pattern of oncogenic β -catenin signaling in colorectal cancer
Awardees: Toshinari Minamoto
3. The 2002 Award for the Development of New Tchnology in Universities, from the Shibuya Foundation for Promotion of Science, Culture and Sports, October 2002.
Title: Development of a new strategy covering all epigenetic alterations spanning through the genome for application to molecular diagnosis of cancer
Awardees: Yasuo Watanabe (Kanazawa Institute of Technology), Toshinari Minamoto, et al.
4. Preside over the 3rd Annual Meeting of the Kanazawa Society for Research and Practice on Molecular Medicine, March 9, 2002, Kanazawa, Japan.
Co-presidents: Masayoshi Mai and Toshinari Minamoto

**Center for the
Development of
Molecular Target Drugs**

General Summary of Center for the Development of Molecular Target Drugs

This center was established in 1997, and since then Takuma Sasaki, Ph.D., professor of Division of Experimental Therapeutics has held the head position of this center. It substantially started on April 1st, 1998 when Takashi Suda, Ph.D. joined as the full-time professor of this center. Ryu Imamura, M.D., Ph.D. and Takeshi Kinoshita Ph.D. joined us as assistant professors in October 1998, and Yoshio Endo Ph.D. and Kohji Kuno Ph.D. were incorporated as associate professors in January 2002. The purpose of this center is to develop collaborative research projects based on creative research products of our institute and other research groups, aiming the development of clinically truly effective molecular targeted drugs. We have invited and selected 10 collaborative projects in the last two years and spent 1,100,000 JPY. We also perform basic research to be a core of collaborative projects, which is described below. We started to hold an annual symposium since 2001.

Research Projects of Suda's Group

A) Prevention of Hepatocellular Carcinoma Development Associated with Chronic Hepatitis by Anti-Fas Ligand Antibody Therapy

Fas ligand (FasL) is a prototype of death factor that induces apoptosis by binding to its receptor, Fas. We have demonstrated that FasL plays a pathological role in various inflammatory diseases. In this study, using a unique animal model of chronic hepatitis leading to hepatocellular carcinoma, we have demonstrated that administration of neutralizing anti-FasL antibody prevented not only hepatitis, but also hepatic cancer development.

B) Molecular mechanism of inflammatory activity by Fas ligand

Fas ligand (FasL) is a cytokine that induces apoptosis. It has been believed that apoptosis does not induce inflammation. However, recent our studies have indicated that FasL has potential to induce inflammation *in vivo*. We currently focus on the molecular mechanism of inflammatory activity induced by FasL, especially cytokine production and signal transduction pathways by Fas-FasL system for this induction.

C) Molecular mechanism that regulate Bid-induced apoptosis

We searched for proteins that interacted with Bid using the yeast two-hybrid system. One positive clone, termed clone X1, was isolated. Although Clone X1 shares no significant homology with the members of Bcl-2 family proteins, the transient overexpression of clone X1 in Cos 7 cells partially prevented Bid-induced cell death. CloneX1 may represent a new type of apoptosis regulator.

D) Genomic structure and inducible expression of the IL-22R in mice

IL-22 is a newly identified member of the interferon/IL-10 family, and induces the expression of acute phase proteins in the liver. In this study, we identified the gene for mouse IL-22 receptor α chain and investigated its constitutive and inducible expression in vivo. One of our conclusions is that responsiveness of hepatocytes to IL-22 upon LPS stimulation is controlled by inducible expression of IL-22R at the transcriptional level.

Research Projects of Dr. Endo

Multifunctional anti-angiogenic activity of the cyclic peroxide ANO-2 with antitumor activity

The plasminogen activator system has been a considerable target for anti-angiogenic agent research. We developed a novel anti-angiogenic peroxide, ANO-2, that inhibits u-PA production and also cathepsins in both endothelial and tumor cells. ANO-2 may be a promising lead-compound for the development of anti-angiogenic drugs.

Research Projects of Dr. Kuno

Identification of substrate for ADAMTS-1 protease and roles of ADAMTS-1 in organogenesis

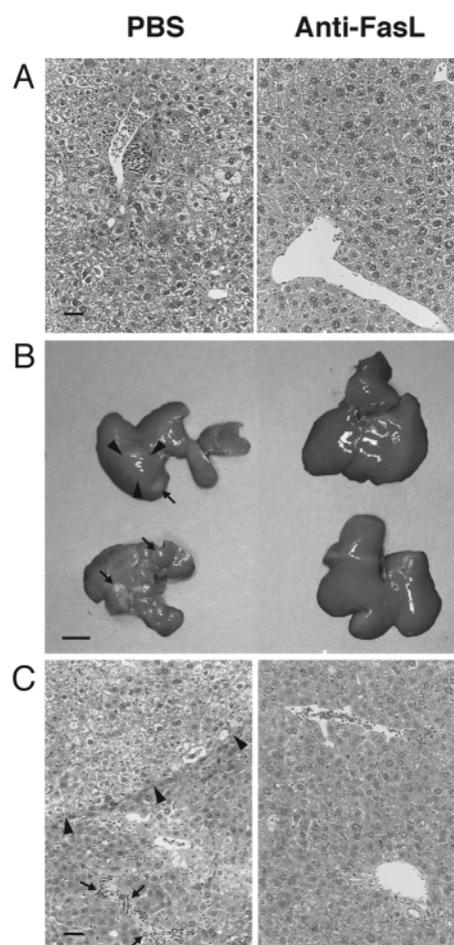
ADAMTS-1 is an active metalloproteinase associated with the extracellular matrix. We showed that ADAMTS-1 is able to cleave a cartilage proteoglycan, aggrecan. ADAMTS-1 gene knockout mice displayed renal anomalies that resemble the ureteropelvic junction obstruction in human. In addition, analyses of ADAMTS-1 (-/-) mice revealed that ADAMTS-1 is necessary for normal development and function of adrenal glands and female genital organs.

Prevention of Hepatocellular Carcinoma Development Associated with Chronic Hepatitis by Anti-Fas Ligand Antibody Therapy

Yasunari Nakamoto, Hong Fan, and Takashi Suda

Hepatitis B virus (HBV) and Hepatitis C virus are widespread pathogens, and cause chronic liver inflammation, leading to hepatocellular carcinoma (HCC). Both clinical and experimental research has suggested that cytotoxic T lymphocytes (CTLs) and its major cytotoxic molecule, Fas ligand play a pivotal role in the pathogenesis of viral hepatitis. However, it has not been directly investigated whether FasL is involved in the development of HCC. In this study, using the unique animal model of chronic hepatitis leading to HCC, we have successfully demonstrated that anti-FasL antibody treatment prevented hepatocyte apoptosis, proliferation, liver inflammation, and the eventual development of hepatocellular carcinoma. The results indicate that FasL is involved not only in direct hepatocyte killing but also in the process of inflammation and hepatocellular carcinogenesis in chronic hepatitis. This is also the first demonstration that amelioration of chronic inflammation by some treatment actually caused reduction of cancer development.

Figure. Prevention of progressive liver dysplasia and HCC development by anti-FasL mAb treatment. The transgenic mice described in the legend to Figure 1 were sacrificed 9 (A) and 15 (B and C) months after the splenocyte transfer. (A) A 9-month liver specimen with PBS (left) or anti-FasL mAb treatment (right). (B and C) Fifteen months after the splenocyte transfer, livers from PBS-injected animals displayed marked atrophy and multiple liver tumors (arrows) up to 11 mm in diameter (arrowheads) (B, left). A representative specimen illustrates the classical histological features of HCC (arrowheads), and the surrounding hepatic parenchyma displays focal lobular inflammatory infiltrates associated with degenerating hepatocytes (arrows) (C, left). Most of livers from anti-FasL mAb-injected animals did not show apparent atrophy or liver tumors (B, right). A representative specimen demonstrates minimal portal infiltrates and very mild lobular disarray (C, right). Liver sections were stained with hematoxylin and eosin. The bars represent 40 μ m in (A and C), and 10 mm in (B).



Reference: J. Exp. Med. (2002) 196:1105-1111

The membrane-bound but not the soluble form of human Fas ligand is responsible for its inflammatory activity

Koyo Shudo, Ryu Imamura, Shigekazu Nagata, and Takashi Suda

The ectopic expression of Fas ligand (FasL/CD95L) in tissues or tumors induces neutrophil infiltration and the destruction of the tissues or the rejection of tumors. It has been suggested that the infiltrated neutrophils are responsible for the latter phenomena. FasL is synthesized as a type II transmembrane protein, and soluble FasL is produced by a proteolytic mechanism from the membrane-bound form. We previously demonstrated that uncleavable membrane-bound FasL of mice induces IL-1 β release from inflammatory cells, and suggested that the IL-1 β enhances neutrophil infiltration. However, recent papers reported that human soluble FasL is directly chemoattractive to neutrophils *in vitro* and proposed that the soluble form of FasL is responsible for its inflammatory activity. Therefore, in this report, we investigated which form is responsible for the inflammatory activities of human FasL. We produced tumor cell lines expressing one or both forms of human FasL. Cells expressing both forms and only the membrane-bound form of FasL induced neutrophil infiltration when transplanted into the peritoneal cavity of syngeneic mice, while cells expressing only the soluble form did not. Purified soluble FasL failed to induce neutrophil infiltration *in vivo*. IL-1 β release from inflammatory peritoneal exudate and acceleration of tumor rejection were also mediated by membrane-bound but not soluble FasL. These results indicate that the membrane-bound form of FasL is primarily responsible for its inflammatory activity.

Fig. 1. Membrane-bound but not soluble FasL induces neutrophil infiltration.

FBL-3 derived transfectants expressing the membrane-bound form (FDC2), the soluble form (FFS) and both forms of FasL (FFL), and control transfectants (FBH) (4×10^6 cells) were injected into the peritoneal cavities of syngeneic mice. Eighteen hours later, peritoneal cells were recovered and analyzed for the proportion of Gr-1 positive cells by flow cytometry.

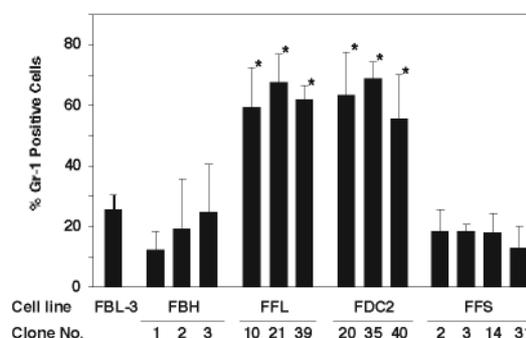
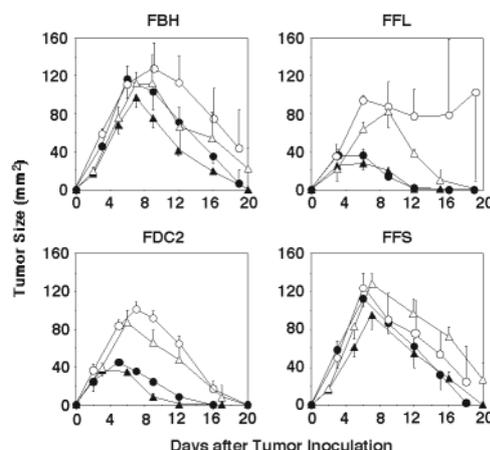


Fig. 2. Membrane-bound but not soluble FasL promotes tumor rejection.

Transfectants (2.5×10^6 cells) were injected into the dorsal skin of 8-12-week-old wild-type (closed symbols) or *lpr/lpr* female C57BL/6 mice (open symbols). Tumor size was measured at intervals of 3 days after tumor inoculation. Each point is a mean of 6 tumors in 3 mice.

Reference: Eur. J. Immunol., (2001) 31:2504-2511

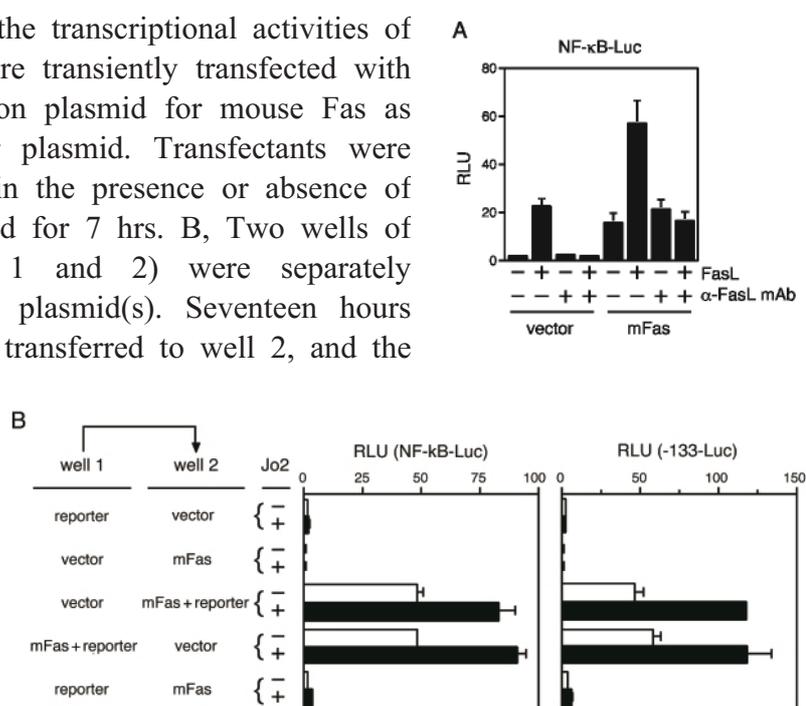


Fas ligand induces cell-autonomous NF- κ B activation and IL-8 production.

Ryu Imamura, Masayuki Fukui, Takaya Kawabe, Masayuki Umemura, and Takashi Suda

Fas ligand (FasL) has been well characterized as a death factor. However, recent studies revealed that FasL possesses inflammatory activity. We here found that FasL induces production of inflammatory chemokine IL-8 without inducing apoptosis in HEK293 cells. Reporter gene assays involving wild-type and mutated IL-8 promoters and NF- κ B- and AP-1 reporter constructs indicated that induction of NF- κ B and AP-1 activity by FasL is required for the maximum promoter activity. FasL induced NF- κ B activation with slower kinetics compared to TNF- α . Yet, this response was cell autonomous and not mediated by secondary paracrine factors. In addition, we demonstrated that between the two physiological forms of FasL, the membrane-bound and soluble forms, the former is mainly responsible for these activities. A proteasome inhibitor or a proteasome resistant mutant of I κ B α completely abrogated both FasL- and TNF- α -induced NF- κ B activation. However, interestingly, FasL did not induce detectable phosphorylation at Ser32 and degradation of I κ B α , while TNF- α rapidly induced these responses. Pancaspase and caspase-8 inhibitors partially inhibited NF- κ B activation but enhanced IL-8 production by FasL stimulation, suggesting that there are both caspase-8 dependent and independent mechanisms, and that caspase-8 delivers both positive and negative signals for IL-8 production. In contrast, caspase inhibitors did not affect these responses to TNF- α . These results revealed that FasL induces NF- κ B activation and IL-8 production by a mechanism distinct from that of TNF- α .

Figure. FasL directly induces the transcriptional activities of NF- κ B. A, HEK293 cells were transiently transfected with pNF- κ B-Luc and an expression plasmid for mouse Fas as indicated, or a control vector plasmid. Transfectants were treated with or without FasL in the presence or absence of an anti-FasL mAb, and cultured for 7 hrs. B, Two wells of HEK293 cell culture (well 1 and 2) were separately transfected with the indicated plasmid(s). Seventeen hours later, the cells in well 1 were transferred to well 2, and the mixed cells were further cultured in the presence or absence of anti-mouse Fas mAb (Jo2) for 7 hrs. The normalized firefly luciferase activity in lane 1 (A, left side bar; B, top bar) in each panel is set as 1 for RLU. Vertical lines indicate standard deviations.



Molecular mechanism that regulate Bid-induced apoptosis

T. Kinoshita, T. Suda

Apoptosis is a highly regulated mechanism of cell death that is required for normal development and maintenance of tissue homeostasis. One of the key events in many types of apoptosis is the release of mitochondrial cytochrome c to the cytosol. Cytochrome c then triggers the formation of a complex containing procaspase-9 and APAF-1, which leads to activation of caspase-9. Caspase-9 is an initiator caspase that activates procaspases, resulting in a cascade of proteolytic events and apoptotic death. Diverse upstream death signals appear to be coupled to downstream events in mitochondria through the activation of members of a subgroup of the Bcl-2 family, BH-3 only proteins, which contain only one of the four domains that define Bcl-2 proteins, the BH-3 domain. Several BH-3 only proteins can be regulated by different posttranslational mechanisms. Signaling pathways activated by certain growth factors induce phosphorylation of Bad, allowing 14-3-3 scaffold proteins to bind and sequester it from mitochondria. Bim is normally sequestered to the microtubular dynein motor complex. Certain apoptotic stimuli free Bim, allowing it to translocate to mitochondria. Bid, another BH-3 only protein, can be cleaved by caspase-8 after Fas/TNF-R1 engagement. The p15 form of truncated Bid (tBid) translocates to mitochondria and induces cytochrome c release, leading to the activation of downstream caspases and apoptosis.

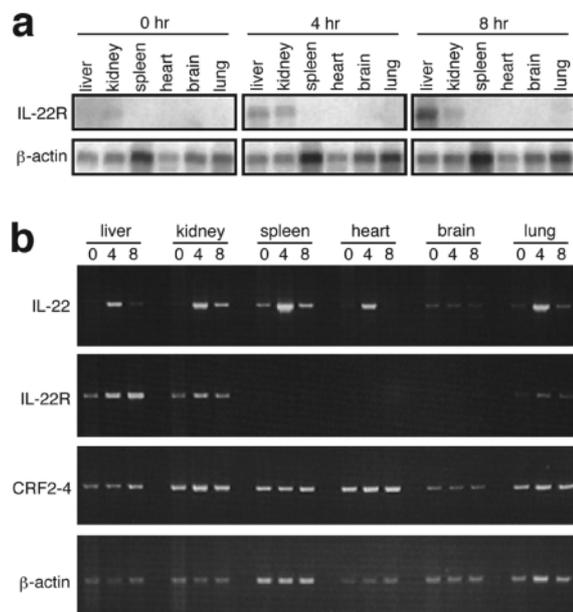
In order to explore the apoptosis regulatory mechanism related to Bcl-2 family member proteins, we searched for proteins that interacted with Bid using the yeast two-hybrid system. One positive clone, termed cloneX1, that specifically interacted with tBid was isolated. CloneX1 cDNA encodes a 218 amino acid protein and contains a carboxyl-terminal hydrophobic tail. Transient transfection of clone X1 into Cos 7 cells followed by indirect immunofluorescence analysis revealed an intracellular membrane-staining pattern. Clone X1 shares no significant homology with the members of Bcl-2 family proteins. The interaction was independent of the BH3 domain of Bid, since it could also interact with BH3-mutated Bid (G94E) in which the amino acid essential for interaction among Bcl-2 family proteins was mutated. Northern blotting analyses showed the clone X1 mRNA was abundant in the brain and liver. Reverse transcriptase-polymerase chain reaction (RT-PCR) analyses showed a number of cell lines (Cos 7, HeLa, HEK293T, HepG2, Jurkat) also expressed clone X1 mRNA. Functionally, the transient overexpression of clone X1 in Cos 7 cells partially prevented Bid-induced cell death. These results suggest that clone X1 could function as a regulator of Bid-induced apoptotic pathway. Clone X1 may represent a new type of regulator of cell survival and apoptosis regulation.

Genomic structure and inducible expression of the IL-22 receptor α chain in mice

Aiko Tachiiri, Ryu Imamura, Yetao Wang, Masayuki Fukui, Masayuki Umemura, and Takashi Suda

IL-22 is a newly identified member of the interferon/IL-10 family. In humans, IL-22 signals through a heteroduplex receptor consisting of IL-22R and CRF2-4/IL-10R β . To investigate the physiological function of IL-22 and IL-22R, we isolated a cDNA encoding the mouse IL-22R, which has been a missing component of the functional receptor complex for mouse IL-22. Subsequently, we identified the genomic sequence of the mouse IL-22R gene by a database search. The gene consists of about 24 kb and is split into 7 exons. Interestingly, intron 2 begins with a GC dinucleotide instead of the consensus GT, although otherwise the overall structure of the mouse IL-22R gene is strikingly similar to its human counterpart. The gene was mapped to mouse chromosome 4 in the region syntenic to the human IL-22R gene locus. In normal mice, IL-22R mRNA is detected at very low levels in restricted organs such as the kidney, liver, and lung. However, upon lipopolysaccharide stimulation, IL-22R mRNA expression is highly upregulated in the liver, in contrast to CRF2-4, which is expressed constitutively in a variety of tissues. Thus, the expression of the functional IL-22 receptor in the liver is regulated at the gene transcription level.

Figure. Constitutive and inducible expression of IL-22R mRNA. a. Mice were left untreated (0 hr) or intraperitoneally injected with 2 μ g of LPS. Four or eight hours later, these mice were sacrificed and poly(A)⁺ RNA was prepared from the indicated organs. IL-22R mRNA (upper panels) and β -actin mRNA as an internal control (lower panels) were then visualized by Northern blotting. b. Poly(A)⁺ RNA was prepared from mice untreated (0 hr) or treated with LPS for 4 or 8hr as described above. Expression of the mRNAs for IL-22, IL-22R, CRF-2-4, and β -actin was visualized by semi-quantitative RT-PCR.



Reference: Genes Immun. (2003) in press.

Multifunctional Anti-angiogenic Activity of the Cyclic Peroxide ANO-2 with Antitumor Activity

Y. Endo, T. Sasaki, M. Tanaka, T. Obata

Angiogenesis plays a significant role in both initial tumor development and tumor metastasis. The system that comprises urokinase-type plasminogen activator (u-PA) and its specific receptor (u-PAR) has been a target for anti-angiogenic agent research, since many reports have shown that either the inhibition of u-PA enzymatic activity or the disruption of the u-PA/u-PAR system by small molecules results in decreased metastasis and angiogenesis *in vivo*. However, these u-PA-targeting compounds have not yet been used clinically. Although promising, this approach is thought to have several drawbacks. Many kinds of solid tumors and their surrounding stromal cells constitutively produce u-PA; therefore, direct, continuous inhibition of u-PA is thought to be difficult *in vivo*. Moreover, tumor cells and stromal cells express several kinds of proteinases including PAs (u-PA and tissue-type PA), matrix metalloproteinases, and cathepsins. Additionally, these proteinases might act on each other's substrates complementarily; consequently, even if a specific enzyme inhibitor successfully blocks the function of the target proteinase, such inhibitors alone might not be enough to inhibit angiogenesis or metastasis. Therefore, the aim of our study was to develop a novel anti-angiogenic agent that inhibits u-PA production and also other protease cascades in both endothelial and tumor cells.

During preliminary screening the effects of 13 ozonides on the inhibition of u-PA production in human fibrosarcoma HT-1080 cells and on the inhibition of angiogenesis on chicken embryonic chorioallantoic membranes were determined. Of the ozonides tested, 9 inhibited *in vitro* u-PA production of HT-1080 cells and 7 of these 9 showed strong anti-angiogenic activity. Interestingly, 6 of the 13 ozonides also inhibited cathepsin B activity. 1-Phenyl-1,4-epoxy-1*H*,4*H*-naphtho[1,8-*de*][1,2]dioxepin (ANO-2) potently inhibited cathepsin B ($IC_{50} = 0.47 \mu M$) as well as u-PA production. ANO-2 inhibited tube formation by human umbilical vein endothelial cells cultured on Matrigel while exhibiting no cytotoxicity. Additionally, *in vivo* administration of ANO-2 inhibited angiogenesis induced by mouse Sarcoma-180 cells tested using the mouse dorsal air sac assay. Moreover, ANO-2 also suppressed primary tumor growth and reduced the number of pulmonary metastases caused by Lewis lung carcinoma cells in mice. These *in vitro* and *in vivo* activities indicate that ANO-2 has considerable potential as a new and potent anti-angiogenic drug that inhibits both u-PA production and enzymatic activity of cathepsins, indicating that ANO-2 may be multifunctional inhibitor of angiogenesis.

Identification of substrate for ADAMTS-1 protease and roles of ADAMTS-1 in organogenesis

K. Kuno

ADAMTS-1 is the first member of the ADAMTS family, which is characterized by having both an ADAM-type metalloproteinase domain and TSP type I motifs. We previously established that ADAMTS-1 is an active metalloproteinase associated with the extracellular matrix (ECM). Because ADAMTS-1 binds to sulfated glycosaminoglycans of the extracellular matrix, we examined whether ADAMTS-1 is able to cleave a cartilage proteoglycan known as aggrecan. Furthermore, to identify the roles of ADAMTS-1 in organogenesis, we disrupted the mouse ADAMTS-1 gene.

1. Aggrecan cleaving activity of ADAMTS-1

We found that ADAMTS-1 is able to cleave a cartilage proteoglycan, aggrecan at the Glu¹⁸⁷¹-Leu¹⁸⁷² bond within the chondroitin sulfate attachment domain. This ADAMTS-1 cleavage site (Glu¹⁸⁷¹-Leu¹⁸⁷²) corresponds to one of the *in vivo* cleavage sites of aggrecan, which have been detected in the synovial fluids from arthritis patients and in cartilage explant culture. It is therefore possible that ADAMTS-1, as well as ADAMTS-4 and 5, play a role in the turnover of aggrecan during physiological processes and/or the degradation of aggrecan under pathological conditions. In addition, deletional analyses demonstrated that the spacer region of ADAMTS-1 is necessary for the degradation of aggrecan.

2. The roles of ADAMTS-1 in organogenesis.

Disruption of the ADAMTS-1 gene leads to renal anomalies involving the enlarged calices and atrophic renal papillae. In addition, dilated calices were visualized by intravenous pyelography of (-/-) mice. The renal abnormalities of these mice resemble the ureteropelvic junction (UPJ) obstruction in humans. In addition to renal anomalies, abnormal adrenal medullary architecture was observed in ADAMTS-1 (-/-) and fertilization was impaired in ADAMTS-1 (-/-) females. These findings demonstrate that ADAMTS-1 is a multifunctional metalloproteinases which is necessary for the structure and function of the UPJ and adrenal glands, and of the female genital organs.

3. Roles of *C.elegans* ADAMTS family gene, *adt-1* in ray morphogenesis.

Disruption of the *adt-1* gene resulted in morphological changes in the male sensory organs, rays, suggesting that *adt-1* plays an important regulatory role in ray morphogenesis of *C.elegans*. We hypothesize that ADT-1 controls the ray extension process via remodeling of the ECM in the cuticle.

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Meetings and Seminars

第21回 がん研究所セミナープログラム

2000 (平成12). 3.16~17

金沢大学医学部記念館 2階ホール

◆細胞情報調節研究分野

- サマリー 原田 文夫
- 新規リン酸化 RNA ポリメラーゼ II 結合蛋白質の同定と機能検索 広瀬 豊
- 哺乳動物テロメラーゼ RNA の二次構造の分析 木戸 敬治

◆細胞分子病態研究分野

- サマリー 山本 健一
- ATM 遺伝子欠損細胞を用いた ATM タンパク質機能ドメインの解析 清水 弘子
- Influence of monoclonal antibody KTC-3 on mouse tumor cell EL4. 佐藤 信生

◆細胞周期制御研究分野

- サマリー 善岡 克次
- JNK カスケードにおけるスカフォールドタンパク質の同定とその解析 善岡 克次
- B型肝炎ウィルスレセプター 黒木 和之

◆細胞分化研究分野

- サマリー 高橋 守信
- 多重変異を有するマウス H 因子 cDNA の蛋白発現と機能解析 坂井俊之助
- 線虫 *C. elegans* の機械的刺激による慣れ学習を形成する部位 天野 重豊
- ヒトのテロメラーゼ hTERT サブユニット遺伝子のプロモーターに存在する転写抑制領域の解析：MZF2 結合配列の役割 藤本幸太郎

◆遺伝子発現研究分野

- サマリー 村上 清史
- RNA ポリメラーゼサブユニット 5 (RPB5) と会合する RMP の機能と細胞内局在 野村 孝弘
- 出芽酵母におけるテロメア複製過程の制御機構 林 直之

◆細胞分子構築研究分野

- サマリー 伊藤 隆司
- 蛋白質間相互作用へのゲノム的アプローチ：酵母をモデルとした網羅的 2 ハイブリッド解析システムの構築 伊藤 隆司
- 新しい蛋白質間相互作用領域マッピング法 2 ハイブリッドフットプリンティング 太田 一寿

- クラススイッチ DNA 組換えの開始機構の解析：マウス μ (+) B 細胞にクラススイッチ誘導刺激を与えるとマウス Spoll が発現される 徳山 春彦
- ◆化学療法研究分野
- サマリー 佐々木琢磨
 - 肺癌に対する動注治療実験モデルの確立 田中 基裕
 - 新規オゾニド化合物の血管新生阻害作用機序の研究 遠藤 良夫
 - 垂ひ酸塩と Taxol との併用による実験癌治療の試み 氏家 俊光
- ◆分子標的薬剤開発センター
- サマリー 須田 貴司
 - 炎症性疾患治療薬としての抗 Fas リガンド中和抗体の意義 須田 貴司
 - 膜型および可溶性 Fas リガンドの *in vivo* での機能 今村 龍
 - アポトーシス時におけるチトクロム C 放出機構の解析 木下 健
- ◆細胞機能統御研究分野
- サマリー 佐藤 博
 - EB ウィルス核蛋白 EBNA-2, EBNA-LP の機能解析及び膜蛋白 LMP1 の上皮系細胞における機能 原田志津子
 - 癌浸潤における細胞運動とマトリックスメタロプロテアーゼ (MMP) 発現の協調的制御機構の解析 滝野 隆久
- ◆分子薬理学研究分野
- サマリー 大野 博司
 - AP 複合体および輸送シグナルによる細胞内蛋白質選別輸送 大野 博司
 - コイルド-コイルタンパク質によるゴルジ体の構造と機能の制御 中村 暢宏
 - 細胞外マトリックス結合性メタロプロテアーゼ, ADAMTS 1 の機能解析 久野 耕嗣
- ◆遺伝子・染色体構築研究分野
- サマリー 向田 直史
 - 肝線維化過程における腫瘍壊死因子の病態生理学的役割の解析 秋山萬里子
- ◆腫瘍外科研究分野
- サマリー 磨伊 正義
 - Tumor dormancy therapy の実践- low dose CPT-11法の開発- 高橋 豊
 - 癌治療における OK-432, とくに IL-12inducer としての役割について 藤本 敏博
 - 早期胃癌に対する Minimally Invasive Surgery の確立 表 和彦
- ◆遺伝子診断研究分野
- サマリー 源 利成

○ヒト大腸癌における β -catenin および MMP-7 の発現

源 利成

◆腫瘍内科研究分野

○サマリー

澤武 紀雄

○3次元超音波内視鏡による胃癌の深達度診断

岡井 高

○膵炎におけるアポトーシスから見た PAP, p8 発現の意義

元雄 良治

○小膵腫瘤性病変の診断—EUS 像と遺伝子診断を中心に—

渡邊 弘之

○膵癌の膵液を用いた遺伝子診断能の向上に対するアプローチ

山口 泰志

第22回 がん研究所セミナープログラム

2001 (平成13). 3.22~23
金沢大学医学部基礎第二講義室

◆分子薬理学研究分野

- サマリー 大野 博司
- コイルドコイル・タンパク質によるゴルジ体の構造と機能の制御 中村 暢宏
- 細胞外マトリックス結合性メタロプロテアーゼ ADAMTS-1 および線虫
C.elegans ADAMTS ファミリー遺伝子の機能解析 久野 耕嗣
- 細胞増殖因子受容体のユビキチン化と内在化・脱感作の制御機構の研究 田中 直孝

◆遺伝子発現研究分野

- サマリー 村上 清史
- ヒトB型肝炎ウィルスX蛋白による細胞形質転換の試み 野村 孝弘
- テロメア複製過程の制御機構 林 直之

◆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

◆組織分子構築研究分野

- サマリー 向田 直史
- 転移巣形成過程初期の癌細胞と正常細胞の相互作用の解析 佐々木洋子

◆遺伝子・染色体構築研究分野

- サマリー 伊藤 隆司
- Spoll Antisense oligonucleotide はクラススイッチ組み換えを抑制する 徳山 春彦
- キメラ転写因子を用いた遺伝子発現制御ネットワーク解析 太田 一寿

◆化学療法研究分野

- サマリー 佐々木琢磨
- 新規核酸代謝拮抗剤3'-エチニルシチジンの感受性規定因子の解析 田中 基裕
- 亜ヒ酸塩の治療効果を増強する併用薬剤の探索 氏家 俊光

◆分子標的薬剤開発センター

- サマリー 須田 貴司
- 1) 膜型および可溶性 Fas リガンドの in vivo での機能
2) デスドメインをもつ核蛋白質 p84N5 の解析 今村 龍
- アポトーシス時におけるチトクロムC放出機構の解析 木下 健

- ◆細胞分化研究分野
- ロシアおよびカザフスタン産野生マウスに新たに発見された補体系制御蛋白H因子の変異機構の解析 坂井俊之助
 - 線虫 *C. elegans* における慣れ学習の個体発生 天野 重豊
- ◆細胞分子病態研究分野
- サマリー 山本 健一
 - ATM タンパク質の機能発現におけるキナーゼ領域の役割について 清水 弘子
 - ATM シグナル伝達経路における DNA 損傷認識因子の解析 小林 昌彦
- ◆細胞情報調整研究分野
- サマリー 原田 文夫
 - 新規リン酸化 RNA ポリメラーゼ II 結合蛋白質の同定と機能検索 廣瀬 豊
 - ニワトリ U13snoRNA の同定と機能解析 木戸 敬治
- ◆細胞周期制御研究分野
- サマリー 善岡 克次
 - B型肝炎ウイルスの感染 黒木 和之
- ◆腫瘍外科研究分野
- サマリー 磨伊 正義
 - 抗 VEGF 抗体による血管新生治療の確立—分子標的治療の治療戦略— 高橋 豊
 - 胃癌に対する免疫化学療法の効果と至適併用方法の検討 藤本 敏博
 - 早期胃癌に対する Minimally Invasive Surgery の確立 表 和彦
- ◆遺伝子診断研究分野
- サマリー 源 利成
 - ヒト大腸癌における β -catenin シグナル伝達系活性化・制御機構の解明 源 利成
- ◆腫瘍内科研究分野
- サマリー 澤武 紀雄
 - 超音波内視鏡による胃非上皮性腫瘍の判断 岡井 高
 - 膵癌における p 8 の発現とその臨床病理学的意義 元雄 良治
 - 膵癌診断における膵液虫 hTERT mRNA 測定の有用性 渡邊 弘之
 - 膵、胆道疾患における胆汁中の遺伝子診断 山口 泰志

第23回 がん研究所セミナープログラム

2002 (平成14). 3.26~27

金沢大学医学部記念館 2階ホール

◆腫瘍外科研究分野

- サマリー 磨伊 正義
- Tumor dormancy therapy としての MTD dividide low dose chemotheapy 高橋 豊
- 腹腔鏡手術における Robotic Assistance 表 和彦

◆遺伝子診断研究分野

- サマリー 源 利成
- ヒト癌における p 53がん抑制蛋白質のリン酸化に関する検討 源 利成

◆腫瘍内科研究分野

- サマリー 澤武 紀雄
- 早期胃癌の深達度診断における 3次元超音波内視鏡検査 (3 DEUS) の意義 岡井 高
- ヒト膵管癌における pancreatitis-associated protein(PAP)の発現 元雄 良治
- 悪性胆道疾患における胆汁中 p 53変異検出の有用性 山口 泰志
- 膵癌組織ならびに膵液における p 16癌抑制遺伝子異常の検討 渡邊 弘之

◆分子薬理学研究分野

- サマリー 大野 博司
- ゴルジ体の構造維持の分子機構の解析 中村 暢宏
- 細胞膜蛋白質のユビキチン化と内在化・脱感作制御機構の研究 田中 直孝

◆遺伝子発現研究分野

- サマリー 村上 清史
- ヒト B型肝炎ウィルス X 蛋白はテロメラーゼの発現を活性化するか 野村 孝弘
- TELOMERE 複製と細胞老化 林 直之

◆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

◆組織分子構築研究分野

- サマリー 向田 直史
- 転移巣形成過程初期のがん細胞と正常細胞の相互作用の解析 佐々木洋子

◆遺伝子・染色体構築研究分野

- サマリー 伊藤 隆司
- クラススイッチ組み換え刺激によりリンパ球特異的 Spo11の発現が誘導され、Spo11 Antisense oligonucleotide はクラススイッチを抑制する 徳山 春彦
- パラレルアフィニティタグによるユビキチン化プロテオーム解析 太田 一寿

◆化学療法研究分野

- サマリー 佐々木琢磨
- 核酸代謝拮抗剤を用いた併用化学療法の基礎的研究 田中 基裕
- 新規血管新生阻害剤の開発研究 遠藤 良夫
- 翻訳制御における GCN 経路と TOR 経路のクロストーク 小幡 徹

◆分子標的薬剤開発センター

- サマリー 須田 貴司
- C.elegans ADAMTS ファミリー遺伝子 adt-1 の雄尾部感覚器形態形成における役割および ADAMTS-1 の腫瘍増殖に及ぼす影響について 久野 耕嗣
- Fas リガンドによる NF-kB 活性化機構の解析 今村 龍
- アポトーシス情報伝達の分子機構 木下 健

◆細胞分化研究分野

- サマリー 高倉 信幸
- ロシアおよびカザフスタン産野生マウスに発見された補体系制御蛋白 H 因子の新たな変異体 cDNA の塩基配列とその特性 坂井俊之助
- 開放血管系における血管形成 天野 重豊
- 血管内皮細胞特異的受容体型チロシンキナーゼ TIE 2 により活性化される遺伝子の同定とその機能解析 上野 将也

◆細胞分子病態研究分野

- サマリー 山本 健一
- DNA 損傷修復経路におけるチロシンキナーゼ Arg の機能の解析 清水 弘子
- DNA 損傷, 酸化ストレスに対する ATM シグナル伝達の解析 小林 昌彦

◆細胞情報調節研究分野

- サマリー 原田 文夫
- B 型肝炎ウィルスの分子生物学 黒木 和之
- リン酸化 RNA ポリメラーゼ II 結合蛋白質の機能検索 廣瀬 豊
- 18SrRNA の成熟機構における U13snoRNA の機能 木戸 敬治

◆細胞周期制御研究分野

- サマリー 善岡 克次

International Symposium on Tumor Biology in Kanazawa 2002

In Commemoration of 100th Anniversary of Dr. Hajime Okamoto's Birth

Program Schedule

March 8 (Friday)

Opening Remarks : **Ken-ichi Yamamoto** (Kanazawa University)

Session A. Dynamic Interaction between Tumor and Host

Chairperson : **Seishi Murakami** (Kanazawa University)

Fuyuki Ishikawa (Tokyo Institute of Technology)

Telomerase, Stress Responses and anti-tumor effects

Takashi Suda (Kanazawa University)

Role of Fas Ligand in Inflammation, Inflammatory Disease, and Cancer

Hiroshi Sato (Kanazawa University)

Role of Matrix Metalloproteinase in Tumor Invasion

Chairperson : **Takashi Suda** (Kanazawa University)

Nobuyuki Takakura (Kanazawa University)

Control of Hematopoietic Stem Cells : A New Approach for Tumor Angiogenesis

Albert Zlotnik (Eos Biotechnology)

A Role for Chemokine Receptors in Cancer Metastasis?

Session B. Novel Strategies in Cancer Diagnosis and Treatment in Post-genomic Era

Chairperson : **Toshinari Minamoto** (Kanazawa University)

Gozoh Tsujimoto (National Children's Medical Research Center)

Functional Genomic Approach for Multi-gene Disorders

Hiroyuki Aburatani (University of Tokyo)

Molecular Profiling of Liver Tumor

Kazuto Nishio (National Cancer Research Institute)

Selection of Surrogate Markers by cDNA Expression Array in Clinical Setting

Chairperson : **Naofumi Mukaida** (Kanazawa University)

Yutaka Kawakami (Keio University)

Human Tumor Antigens Recognized by T Cells and Antibodies

Bingliang Fang (MD Anderson Cancer Center)

Targeted Expression of Proapoptotic Genes for Cancer Therapy

Closing Remarks : **Masayoshi Mai** (Kanazawa University)

International Symposium on Tumor Biology in Kanazawa 2003

Program Schedule

March 12, Wed.

Opening Remarks : **Ken-ichi Arai** (IMS, University of Tokyo)

Session A. Search for Novel Targets for Cancer Treatment

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

Masa-aski Muramatsu (Tokyo Medical and Dental University)

Application of single nucleotide polymorphism (SNP) to clinical and epidemiological studies

Satoshi Inoue (University of Tokyo)

Estrogen responsive genes and breast cancer

John N. Weinstein (NIH, USA)

Integromics for cancer drug discovery : integrating genomics, proteomics, and bioinformatics

Chairperson : **Tadashi Yamamoto** (IMS, University of Tokyo)

Masafumi Shibuya (IMS, University of Tokyo)

A unique signal transduction of VEGF receptors involved in tumor angiogenesis and metastasis

Kyu-Won Kim (Seoul National University)

Novel HIF-1 α interacting proteins and their roles in hypoxia-induced tumor angiogenesis

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

Motoharu Seiki (IMS, University of Tokyo)

Membrane-type 1 matrix metalloproteinase (MT1-MMP) in cancer invasion and angiogenesis

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

Distinct pattern of oncogenic b-catenin activation in colorectal cancer

Makoto Taketo (Kyoto University)

Mouse models for colon cancer : studies on chemoprevention and chemotherapy

March 13, Thu.

Session B. Novel Approaches to Cancer Treatment

Chairperson : **Masafumi Shibuya** (IMS, University of Tokyo)

Michael O'Reilly (MD Anderson Cancer Center, USA)

Angiogenesis : from the laboratory to the Clinic

Yutaka Takahashi (Cancer Res. Inst., Kanazawa Univ.)

Tumor Dormancy Therapy-paradigm shift of cancer therapy

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

Yoshihiro Hayakawa (Peter MacCallum Cancer Institute)

Effectors and regulators of cancer immune surveillance

Akira Yamada (Kurume University)

Development of peptide-based vaccine for epithelial cancer

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

Tatsutoshi Nakahata (Kyoto University)

Ex vivo expansion of human hematopoietic stem cells for a variety of clinical applications

Akiyoshi Takami (Graduate School of Medical Science, Kanazawa Univ.)

Nonmyeloablative transplantation for renal cell carcinoma: New application of allogeneic immunotherapy

Closing Remarks : **Ken-ichi Yamamoto** (Cancer Research Institute, Kanazawa University)

Organization and Personnel

as of February, 2003

List of Members of 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	~	Present

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Research Technician	Mami Yasukawa	(安川 麻美)
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Graduate Student	Yuya Mori	(森 勇也)
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Graduate Student	Pei Long Lu	(陸 培榮)
Graduate Student	Xiaoqin Yang	(羊 曉勤)
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Technician	Miyako Takegami	(竹上美也子)
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Research Fellow	Yoshihiro Yamaguchi	(山口 佳洋)
JSPS Research Fellow	Hiroyuki Kubota	(久保田浩行)
JSPS Research Fellow	Yoichi Yamada	(山田 洋一)
JSPS Research Fellow	Fumihito Miura	(三浦 史仁)
Graduate Student	Miyuki Onda	(恩田 美雪)

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Visiting Professor	Zhigang Tian	(ティアン ツィーガン)
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Takashi Suda

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