

2007

# Annual Report

No.19

for the Promotion of Science

The NOVARTIS Foundation(Japan)

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for the Promotion of Science

# Introduction

## **“NOVARTIS Foundation’s Raison d’etre”**

Akimichi Kaneko, Chairman

This year marks the 20<sup>th</sup> anniversary of the NOVARTIS Foundation (Japan) for the Promotion of Science. Twenty years have passed since it was established as the Ciba-Geigy Science Promotion Foundation in 1987, based on the one billion yen donation from the Ciba-Geigy Group. We sincerely thank all people whose financial, business and spiritual supports have made our present success. I am honored to witness this historical moment as the Chairman.

Recently I found that the 1<sup>st</sup> Annual Report describes the detailed record of how the Foundation was established. On the next page an excerpt from the part of the description is reprinted. I believe it is an important message for those who did not witness the start of the foundation 20 years ago to help understanding the purpose and the background of the establishment of the Foundation. The record shows clearly that the Foundation’s principle has never changed since then.

Since its establishment the Foundation has aided 871 research grantees, 153 research meetings, 94 researchers for Europe-Japan researcher exchange program, 60 researchers for overseas business trips and 14 researchers for other special aids. The total amount of the aids has reached 1.38 billion yen. Many researchers who were supported at that time are now at the forefront in each field, such as being the professors of universities or major institutions to teach younger researchers throughout Japan.

What has the Foundation dedicated to those researchers? Even if the aided amount was small compared to recent large-scale research funds, I imagine that they were greatly encouraged by this, because obtaining high appreciation from this Foundation indicates that the granted researcher is highly evaluated. For example, researchers selected for the competitive Research Grant of this Foundation must first obtain the universities’ recommendation, which is already a quite high hurdle. In order to estimate grant applicants fairly and correctly, the NOVARTIS Foundation is asking leading researchers in the subject field for screening. We thank these researchers for kindly sparing their precious time for us.

At this historical milestone of this Foundation, we reconfirm our resolve to continue the promoting activities to support promising researchers, based on the unchanged fundamental principle.

# Background to Establishment

The NOVARTIS Foundation (Japan) for the Promotion of Science was established on September 3<sup>rd</sup>, 1987 under the authorization of the Ministry of Education by following the process below.

## **Started Preliminary Investigation (Aug. 1985)**

It was decided that the first fundamental research laboratory in Japan shall be established as the part of the Ciba-Geigy Group. Along with the establishment, an exclusive project team was formed to investigate the current situation of Japanese science and what role the established organization must play for the Japanese science by visiting prominent figures in the target fields or other company laboratories. The original purpose of this investigation was to make a plan for the establishment. However, as the investigation progressed, the team members realized the importance of juridical foundations because they were greatly contributing to Japanese science. This is how the Ciba-Geigy Group established a new juridical foundation in Japan. Since then, the team had investigated a number of Japanese research aid foundations for about one year.

## **Started Preparation for Establishing Foundation (Sept. 1986)**

Ciba-Geigy Limited and Nihon Ciba-Geigy K.K. decided to establish a new juridical foundation in Japan to respond anticipated tremendous benefits from Japanese society when the new research laboratory would be completed. At that moment the Ciba-Geigy Group had already established Friedrich Miescher Institute (FMI) in Switzerland and Ciba Foundation in London, so the new foundation would be the third one.

The project team started the preparations for establishing the new foundation and the new research laboratory simultaneously. First they created a charter for the new foundation. The charter was stating three main points; the importance of creative research in Japan, the necessity of international exchange of researchers, and the Group's strong decision to establish a research aid foundation in Japan as the part of the Ciba-Geigy Group. The considerably broad range of target fields including bioscience, chemistry and polymer science was set up. Also the team members asked the selected persons for being the founders, trustees or councilors of the new foundation.

## **Holding Explanatory Meeting for Establishing Foundation (Jan. 1987)**

The team invited major founders to Nihon Ciba-Geigy K.K. to explain them the progress and the purpose of the establishment.

**Visited Scientific Research Aid Division of the Research Promotion Bureau, Ministry of Education (Feb. 1987)**

The team asked the Ministry of Education (present Ministry of Education, Culture, Sports, Science and Technology) in Japan for administration because the new foundation would cover such a wide range of fields and its main purpose was to support mainly fundamental researches. Although there were somewhat exceptional factors as it was the first foreign-financed foundation in Japan, the Ministry of Education gave the team flexible responses and detailed advices appropriately.

**Receiving Donation from Ciba-Geigy Limited as Basic Fund (Mar. 1987)**

Ciba-Geigy Limited proposed for donation to the new foundation; 1,000,000,000 yen as its basic fund and 50,000,000 yen every year as its operating fund.

**Holding Founders' Meeting (June 1987)**

The Founders' Meeting was held on June 3<sup>rd</sup>. The founders were Drs. Yuichi Yamamura, Saburo Fukui, Hitoshi Nozaki, Hiroshi Mikawa, Morio Ikehara, Yoshiro Okami and Ryo Sato, and additionally, Messrs. Paul Dudler, Toshiaki Simizu, Peter Baumann and Max. M. Burger from Ciba-Geigy Limited. In that meeting Dr. Saburo Fukui was elected for the representative of the founders. Other necessary issues such as the content of the charter were also decided in this meeting.

- Quoted from the 1<sup>st</sup> issue of the Annual Report



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Masaki Inada

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# Part I

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Reports from the Recipients of Novartis  
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1) Biological Sciences  
1-1) Molecular Biology

## Oncogenesis mediated by the stabilization of AU-rich element containing mRNA

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### Introduction

E4orf6 is an oncoprotein which can cooperate with adenovirus E1A to transform primary cells. AU-rich element (ARE) is present in a certain mRNA transcribed from the genes such as proto-oncogene. HuR binds to the ARE elements to protect ARE-mRNAs from the rapid degradation. Under the condition of heat shock, HuR makes a protein complex with pp32 to export ARE-mRNAs to the cytoplasm in a CRM1-dependent manner.

We previously identified pp32 as an E4orf6 associated protein and revealed that E4orf6 exports ARE-mRNAs by perturbing CRM1-dependent pathway (1). To explore the purpose of this E4orf6 function, we examined the stabilization of ARE-mRNAs.

### Results

We examined in detail about the effects of E4orf6 on stabilization of ARE-mRNAs, such as *c-fos*, *c-myc* and *COX-2*. The stabilization was estimated by quantitative real-time RT-PCR using transformed baby rat kidney (BRK) cells. Accumulated *c-fos* mRNA in E4orf6 expressing BRK cells (BRK E1+E4) was about 4.0 times more abundant than that in control BRK cells (BRK E1) (Fig. 1a). BRK E1+E4 cells also expressed approximately 5.5- and 4.2-fold greater levels of *c-myc* and *COX-2* mRNAs, respectively, than BRK E1 cells (Fig. 1a).

For elimination of the transcriptional activation of each gene, the cells were treated with actinomycin D (Act. D). After 0, 60 and 120 minutes of Act. D treatment, the quantity of each mRNA species in BRK cells was measured by quantitative real-time RT-PCR. The half-lives of *c-fos*, *c-myc* and *COX-2* mRNAs in BRK E1+E4 cells were about

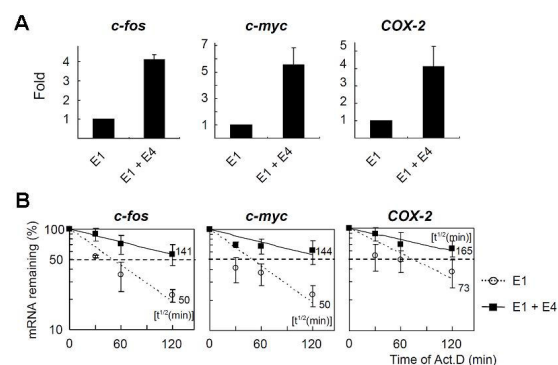


Fig.1

141, 144 and 165 minutes, respectively. These values were significantly longer than those of BRK E1 cells (50, 50 and 73 minutes, respectively) (Fig. 1b).

Since a previous study demonstrated that *c-fos* mRNA was stabilized by Act. D treatment, we therefore examined the stabilization of *c-fos* mRNA by using a luciferase assay system. We constructed pGL3-based luciferase reporter plasmids with and without the 3'-UTR of *c-fos* cDNA including the AREs. In BRK E1+E4 cells, the ARE-containing reporter (pCMVGL-ARE) showed higher luciferase activity (about 6-fold) than in BRK E1 cells (Fig. 1c; *right*). On the other hand, the control reporter (pCMVGL) did not show such activity (data not shown). We concluded that E4orf6 has a potential to stabilize the ARE-containing mRNA.

The fact that E4orf6 can stabilize ARE-mRNAs indicates that E4orf6 should enhance the oncogenic potential mediated by ARE-mRNA. To confirm this hypothesis, we carried out a colony formation assay. The expression plasmid of full-length *c-myc* cDNA was transfected into rat 3Y1 cells with or without the expression constructs for E4orf6 and its mutants (E4orf6 L245P, the mutant in the oncodomain). E4orf6 enhanced the colony formation activity of *c-myc* oncogene. Furthermore, the *c-myc* mRNA was obviously stabilized in transformed cells expressing wild-type E4orf6, whereas it was not observed in E4orf6 L245P-expressing cells, consistent with the results of the colony formation (data not shown). Therefore, the ability of E4orf6 to enhance *c-myc*-mediated oncogenesis was confirmed.

To estimate the requirement of the stabilization of ARE-mRNA for the oncogenic activity of E4orf6, we produced HuR knockdown cells (Fig. 2a), since HuR is the only protein that interacts with ARE-mRNA directly. We expected that the ARE-mRNAs are not able to be exported and stabilized in HuR knockdown cells, even if E4orf6 is expressed in the cells. These cells were treated with Act.D and the amounts of *c-fos* mRNA were estimated as Figure 2b. This data indicate that HuR is necessary for the stabilization of ARE-mRNA mediated by E4orf6. The export of ARE-mRNA was not observed by HuR knockdown, even if E4orf6 is expressed in the cells (data not shown).

The oncogenic activities of these cells were estimated by soft-agar colony formation assay. BRK cells expressing E1 and E4orf6 was failed to make colony by HuR knock down (Fig. 2c).

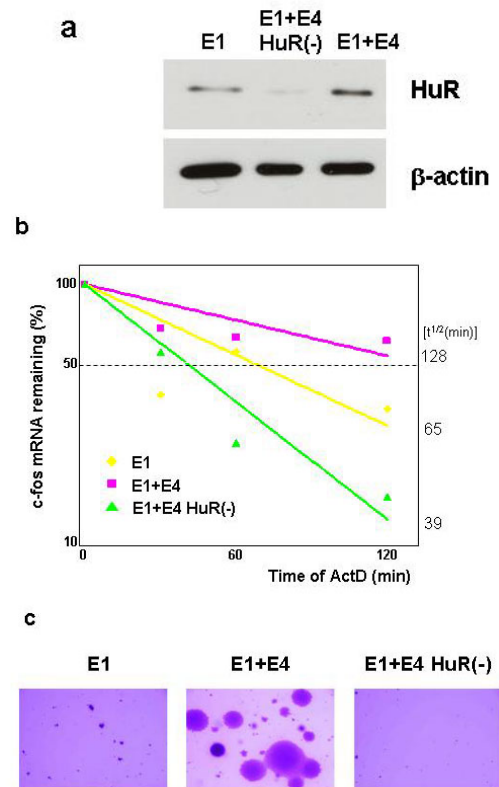


Fig.2

Thus HuR is also necessary for the oncogenic activity of E4orf6. These data suggest that HuR and the stabilization of ARE-mRNA are necessary for the oncogenic activity of E4orf6.

## Discussion & Summary

We present here that E4orf6 stabilized ARE-mRNAs such as *c-fos*, *c-myc* and *COX-2* and that E4orf6 cooperated with *c-myc* to transform 3Y1 cells at least in part by stabilizing *c-myc* mRNA. Moreover, HuR and the stabilization of ARE-mRNA are necessary for the oncogenic activity of E4orf6.

Since the mechanism of action of viral oncoprotein has often led to insights into actual human oncogenesis, we believe that this behavior of mRNA plays an important role in generating human cancer. Recently, a systematic comparison between HuR expression in a variety of cancers and their normal tissue counterparts was carried out and higher HuR expression and cytoplasmic presence in all malignancies examined was shown (2). These data indicate involvement of HuR expression and transportation for human oncogenesis.

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1) Biological Sciences  
1-1) Molecular Biology

## Molecular mechanism of transport of mRNA into dendritic spines

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### Introduction

mRNA localization is a common mechanism operative in cells and has an essential role in localizing cytoplasmic determinants, controlling the direction of protein secretion and allowing the local control of protein synthesis in neurons. In neuronal dendrites the localization and translocation of mRNA is considered as one of the molecular bases of synaptic plasticity. Recent imaging and functional studies revealed that several RNA-binding proteins form a large messenger ribonucleoprotein (mRNP) complex that is involved in transport and translation of mRNA in dendrites. However, the mechanism of mRNA translocation into dendritic spines, a site of local synthesis of proteins, is unknown.

### Results

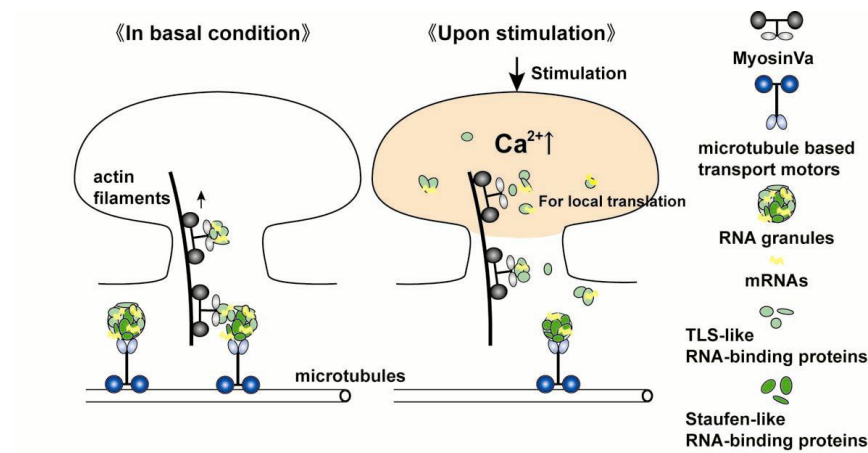
To examine if TLS interacts with myosin-Va, we performed immunoprecipitation (IP) – immunoblotting (IB) of mouse brain lysates with specific antibodies against TLS and myosin-Va. In the presence of EDTA and EGTA, TLS was coimmunoprecipitated with myosin-Va. However, no significant association of TLS with myosin-Va was observed in the presence of 100  $\mu$ M  $\text{CaCl}_2$ . To see whether myosin-Va forms a complex with a specific target of TLS, Nd1-L mRNA, we performed IP/RT-PCR. The amplified PCR products for Nd1-L mRNA migrated to the same position as the control cDNA products obtained from mouse brain RNA. To test whether myosin-Va plays a role in the transport of TLS into dendritic spines, we analyzed the localization of GFP- or red fluorescent protein (RFP)-tagged TLS (TLS-GFP or TLS-RFP) in cultured mouse hippocampal neurons together with that of full-length myosin-Va (BRMV) or 3 kinds of tail domains of its brain-specific isoforms, i.e., brain short tail (BRST), brain stalk (BRSTK) and globular tail domain (GTD). Whereas BRSTK-RFP and GTD-RFP did not affect the localization of TLS-GFP (BRSTK and GTD), overexpression of BRST-RFP, containing both medial tail (stalk) and globular tail domains of myosin-Va, inhibited the translocation of TLS-GFP into spines and resulted in a granular expression pattern within the dendrites, called “Granular” (BRST). To clarify whether the endogenous myosin-Va plays a critical role in the transport of RNA-binding proteins into spines, we adopted

RNA interference (RNAi) technique. We introduced myosin-Va or control- short hairpin (sh)RNA together with TLS-GFP by microinjection into the nucleus of primary hippocampal neurons. In neurons expressing control-shRNA, TLS-GFP accumulated in dendritic spines (~90%). In about 50% of the neurons challenged by 4623MyoVa-shRNA with or without RFP, TLS-GFP formed dense clusters in dendritic shafts ( $p < 0.0001$ ), as observed in neurons expressing the dominant-negative myosin-Va tail domain. To investigate how TLS dynamics would be affected by the loss-of-function of myosin-Va, we stimulated cultured hippocampal neurons expressing TLS-GFP with 100  $\mu$ M DHPG over 60 min and monitored the movement of TLS-GFP by time-lapse confocal microscopy. The relative enrichment at the indicated time span ( $E_k$ ) of TLS-GFP in spines was significantly increased after mGluR stimulation ( $p < 0.0005$  for 40 min,  $p < 0.0001$  for 60 min). On the contrary, in neurons expressing the dominant-negative myosin-Va tail domain (BRST-RFP), TLS-GFP clusters were localized in dendritic shafts, and the intensity of TLS-GFP in the spines remained low even after synaptic stimulation with DHPG. In myosin-Va-knockdown neurons obtained by injection of 4623MyoVa-shRNA, the TLS-GFP clusters formed granules and were located within the dendritic shafts when stimulation with DHPG. The intensity of TLS-GFP in spines exhibited larger fluctuations than in control neurons. Nevertheless, the translocation of TLS-GFP in spines was impaired by knocking down myosin-Va expression. We further verified the results of the above knockdown experiments by using neurons derived from myosin-Va-deficient *dilute-lethal* (*dl/dl*) mice. The time-lapse imaging experiments substantiated this assumption and demonstrated that DHPG stimulation induced TLS-GFP enrichment in spines of  $+/+$  (and/or *dl/+*) neurons, whereas the induction was impaired in *dl/dl* neurons.

## Discussion & Summary

We show that an actin-based motor, myosin-Va, plays a significant role in mRNP transport in neuronal dendrites and spines. Myosin-Va was  $\text{Ca}^{2+}$ -dependently associated with TLS, an RNA-binding protein, and its target RNA Nd1-L, an actin stabilizer. A dominant-negative mutant or RNAi of myosin-Va in neurons suppressed TLS accumulation in spines and further impaired TLS dynamics upon activation of mGluRs. The TLS translocation into spines was impeded also in neurons prepared from myosin-Va-null *dilute-lethal* (*dl*) mice, which exhibit neurological defects as well as dilute fur color. Our results demonstrate that myosin-Va facilitates the transport/accumulation of TLS-containing mRNP complexes in spines and may function in synaptic plasticity through  $\text{Ca}^{2+}$  signaling.

## Figures&Tables



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1) Biological Sciences  
1-1) Molecular Biology

## Functional analysis of ZNRF1 and 2, a novel family of ubiquitin ligases induced by nerve degeneration.

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### Introduction

We aimed to identify proteins targeted by ZNRF (zinc finger/RING finger)1 and ZNRF2, a novel family of ubiquitin ligases, which we previously identified to be expressed in the nervous system. ZNRF1 was originally found to be highly induced after nerve injury in Schwann cells(1). ZNRF1 and 2 are both constitutively expressed in most neuronal cells and implicated in the synaptic vesicle exocytosis(2). Identification of molecules targeted by ZNRF family is critically important for clarifying ZNRF-dependent protein metabolism and cellular functions regulated by ZNRF family of E3 ligases.

### Results

To characterize the biological function of ZNRF family of E3 ubiquitin ligases, it is critically important to identify proteins which are degraded by ZNRF-dependent proteasomal function in neurons as well as Schwann cells. To identify proteins targeted by ZNRF-dependent ubiquitin-proteasome mechanism, we purified proteins which bind to ZNRF1 or ZNRF2 from a brain protein lysate. We found two proteins from the eluate of an affinity column in which ZNRF proteins were cross-linked to the column matrix, and identity of the proteins was determined by mass-spectrometry.

One of the proteins was identified as glutamine synthetase (GS)(Fig1a). GS is a metabolic enzyme which generates glutamine using glutamate as a substrate. We examined whether GS can bind to

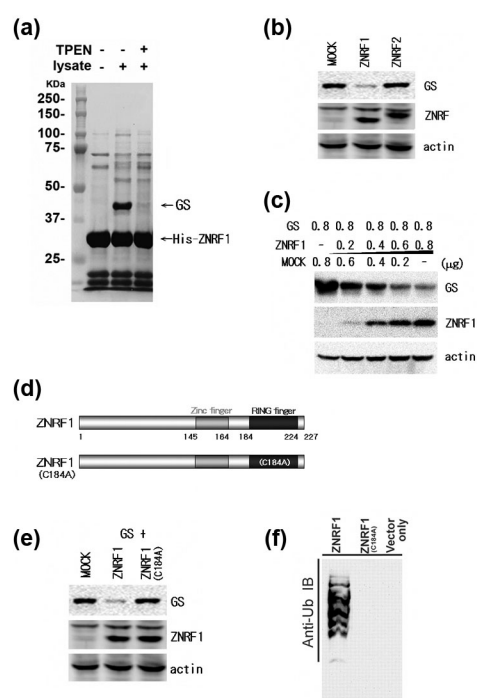


Fig. 1



both ZNRF1 and ZNRF2 by using recombinant GS and ZNRF proteins. We found that GS protein binds to ZNRF1 but not to ZNRF2(Fig 1b). To confirm that GS is degraded by proteasome in a ZNRF1-dependent manner, we examined whether the expression level of GS protein is related to that of ZNRF1 or proteasomal function in cultured cells. We found that in neuro2a cells, forced expression of ZNRF1 reduced the GS protein level in a dose-dependent manner(Fig 1c). The ZNRF1-dependent decrease of GS expression was not observed by introducing C184A mutation in the ZNRF1 protein, which disrupt RING finger of the E3 ligase (Fig 1d-f). These results suggest that GS serves as a substrate of ZNRF1 (but not ZNRF2) E3 ubiquitin ligase.

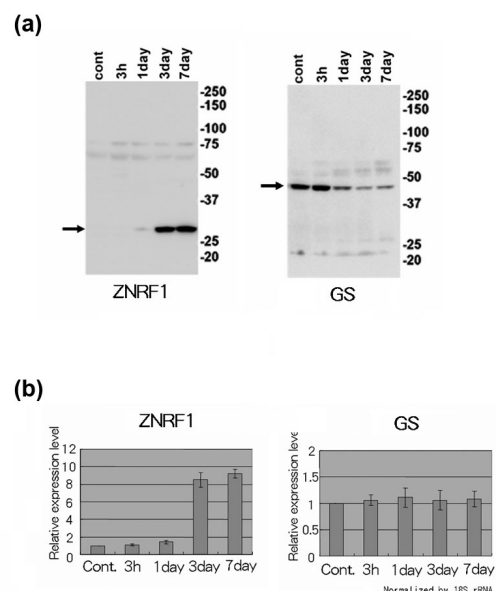
GS is regarded as a “marker” of astrocytes and is not expressed in neurons in the central nervous system. In the peripheral nervous system, GS is known to be expressed in satellite cells of the dorsal root ganglia, which are regarded as non-myelinating Schwann cells, which show a phenotype similar to Schwann cells in injured peripheral nerves. To examine whether GS is a physiological target of ZNRF1-dependent proteasomal degradation, we first examined GS expression time course after nerve injury in Schwann cells. We found that, in a mouse sciatic nerve transection model, ZNRF1 expression is highly induced in a segment distal to the injury site(Fig 2a-b). Side by side with this change, we found that GS protein expression is decreased in injured nerve Schwann cell(Fig 2a). Surprisingly, we also found that, in spite of the significant decrease of the GS protein expression, we found that GS mRNA remains the same level before and after nerve injury in Schwann cells(Fig 2b). This observation suggests that GS is physiological target of ZNRF1 in Schwann cells, and ZNRF1 is responsible for regulation of GS protein expression.

Schwann cells dramatically change their phenotype during development as well as after nerve injury. Protein expression profile designates each phenotype of Schwann cells, and the expression of each protein is usually transcriptionally regulated.

Unlike most other proteins characterizing Schwann cell phenotypes, we found that the GS expression is regulated post-translationally by ZNRF1 E3 ligase-dependent ubiquitin-proteasome degradation mechanism.

We are now determining the function of ZNRF1 and GS during Schwann cell proliferation and peripheral nerve myelination.

We are also examining the nature of the second candidate which binds to ZNRF family of proteins. We will confirm that the second protein also binds to



**Fig. 2**

ZNRF1 or 2, and characterize the function of the candidate protein as well as ZNRF in the nervous system function.

## Discussion & Summary

ZNRF1 was first discovered as a molecule up-regulated in Schwann cells of injured nerve, and ZNRF2 was identified as a molecule highly similar to ZNRF1 in C-terminal zinc-RING finger region. ZNRF1 and ZNRF2 sequences are diverse in the N-terminal area, which suggest each ZNRF ligase has its own target molecule. Our data is compatible with this idea.

GS is now determined as a target of ZNRF1 E3 ligase. ZNRF1 is constitutively expressed in most neurons in both central and peripheral nervous system, but GS is expressed only in glial cells. This suggests that ZNRF1 has substrate(s) other than GS.

To identify more candidate proteins which binds to ZNRF1 or ZNRF2, we may need to change conditions when we screen proteins which bind to ZNRF.

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1) Biological Sciences  
1-1) Molecular Biology

## Study on mechanism of genomic imprinting establishment in YAC transgenic mice

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### Introduction

The mouse *Igf2/H19* locus is subject to genomic imprinting; *H19* gene is expressed only when maternally inherited while the neighboring *Igf2* gene is paternally transcribed. This reciprocally controlled mono-allelic expression requires parental-specific methylation of the imprinting control region (ICR) that is located 5' to the *H19* gene. Although it is reported that methylation imprinting of the *H19* ICR is established during gametogenesis and maintained after fertilization, underlying molecular mechanism in this process is not fully understood. Here, we explored the mechanism of methylation imprinting by using transgenic mouse (TgM) methodology.

### Results

The differentially methylated *H19* ICR functions as a landmark for the parental origin of the chromosomes, and regulates the imprinted expression of the *H19* and *Igf2* genes. The hypo-methylated maternal ICR carries an enhancer blocking insulator activity as a consequence of a CTCF protein binding to it and prevents the distally located *Igf2* gene from being activated by a shared 3' enhancer. In contrast, a hyper-methylated paternal ICR suppresses *H19* gene transcription through promoter DNA methylation, while abolishing CTCF binding, thereby allowing *Igf2* gene expression (Figure 1).

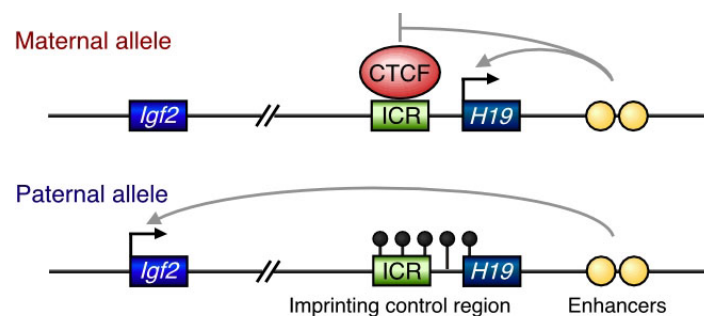


Figure 1. Imprinting model at the *Igf2/H19* locus

To test if the ICR activity is autonomous, we introduced a 2.9-kbp ICR DNA fragment into a heterologous genomic context, *i.e.* 3' end of the locus control region in the human  $\beta$ -globin yeast artificial chromosome (YAC), and established single-copy YAC TgM lines. In these mice, paternally inherited transgenic  $\beta$ -like globin genes were more abundantly expressed than that on the maternal allele. In accord with this result, the paternally transmitted transgenic ICR was more heavily methylated, and CTCF was preferentially recruited to the maternally inherited transgenic ICR in nucleated erythrocytes. These results demonstrated that the ICR could autonomously recapitulate genomic imprinting within a normally non-imprinted locus. An unexpected observation was that hyper-methylation of the transgenic ICR was not observed in male germ cells, suggesting that methylation imprinting in the paternal transgenic ICR was established after fertilization, and “primary mark” other than the DNA methylation may be a prerequisite for a post-fertile, parent-of-origin specific methylation acquisition.

We then tested if the *H19* ICR could establish methylation imprinting by itself. To this end, we injected the 2.9-kbp ICR DNA fragment into pronuclei of fertilized mouse eggs and generated six TgM lines, each of which carried from one to five copies of transgenes inserted randomly at non-imprinted genomic loci. In order to distinguish transgenic from endogenous ICR sequences, the transgenic ICR fragment was flanked by the ~0.5-kbp human  $\beta$ -globin sequences on both sides. Methylation status of the transgenic ICR DNA was then analyzed by Southern blotting and bisulfite sequencing. In nucleated erythroid cells, paternally inherited transgenic ICR was more heavily methylated than the maternal one in five out of six TgM lines. These results suggested that the transgenic ICR fragment could mark its parental origin independent of its copy number and its genomic integration site.

## Discussion & Summary

In this work, we investigated how methylation imprinting of *H19* ICR is established by using TgM methodology, and showed that 2.9-kbp ICR fragment could autonomously establish methylation imprinting at the normally non-imprinted human  $\beta$ -globin locus as well as other random genomic loci. These results suggested that the 2.9-kbp ICR fragment carried sufficient information for marking its parental origin. Interestingly, transgenic ICR at human  $\beta$ -globin locus was hypo-methylated in germ cells, while differential, parent-of-origin specific ICR methylation was observed in somatic cells. Thus, it is likely that establishment of post-fertile methylation imprinting does not always require the differential ICR methylation in germ cells. Distinct molecular mechanisms may independently control establishment of methylation imprinting in somatic or germ cells.

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1) Biological Sciences  
1-2) Struktural Biology

## Intelligent deoxyribozyme and aptamer for the development of side effect-free drugs

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### Introduction

$K^+$  concentration in cells is much higher than that of the outside of cells. Structure of some DNA changes drastically when the  $K^+$  concentration increases. Guanine-rich DNA is a typical example. Its structure is an extended single strand under low  $K^+$  conditions, while the structure becomes compact quadruplex under high  $K^+$  conditions. We call this kind of DNA as a switching unit. Functional nucleic acids such as deoxyribozyme and aptamer can be divided to two sub-units. Suppose these two sub-units are joined to both the ends of guanine-rich DNA (switching unit). Under low  $K^+$  conditions (outside of cells), guanine-rich DNA is extended and two sub-units are separated. Therefore, no deoxyribozyme or aptamer activity is exerted. Under high  $K^+$  conditions (inside of cells), guanine-rich DNA forms a compact structure and thus two sub-units are located closely. Then, deoxyribozyme or aptamer activity is exerted. We already demonstrated that guanine-rich DNA composed of GGA sequences can be used for this scheme. In order to find more functional switching unit, we have studied the structure of human telomeric DNA under physiological high  $K^+$  conditions.

### Results

The structure of human telomeric DNA has been controversial: the solution structure in the presence of  $Na^+$  has been determined to be an antiparallel quadruplex, while the crystal structure in the presence of  $K^+$  has been reported to be a parallel quadruplex. The solution structure in the presence of  $K^+$  has drawn intense interest, as the intracellular  $K^+$  concentration is higher than that of  $Na^+$ , but the structure remains to be elucidated. The difficulty of the structural determination is due to conformational heterogeneity. Here, we have stabilized the major structure in a  $K^+$  solution by the unique method of proper substitutions of guanosines with 8-bromoguanosines and determined the structure at high resolution. Then it was feasible to determine the major structure by NMR. The root-mean-square deviations (rmsd) of the 10 final structures versus the mean structure for all heavy atoms were  $1.3 \pm 0.3$  Å for a whole molecule. They were  $0.8 \pm 0.1$  Å for a core region. The atomic

coordinates have been deposited in the RCSB under Protein Data Bank accession code 2E4I. This demonstrates that the use of 8-bromoguanosine is a powerful tool to overcome the difficulty of the structure determination arising from conformational heterogeneity.

We have examined the possible effect of the substitution with 8-bromoguanosines on a native structure. We substituted the guanines which take on syn in a native structure. It was pointed out that the 8-bromoguanosine substitution at a syn position does not cause a steric hindrance because the Br atom is placed in the groove. Therefore, the effect of the substitution on a native structure is expected to be rather moderate.

The obtained structure is neither the antiparallel quadruplex nor the parallel quadruplex. It is a mixed-parallel/antiparallel quadruplex, in which three strands of the quadruplex are aligned in parallel, while one strand is aligned antiparallel. The positions of both the ends of DNA are close enough. So, when two sub-units of functional nucleic acids are joined to both the ends, re-formation of the functional body is expected. Therefore, human telomeric DNA is supposed to be applicable as a switching unit.

Recently, the structure of telomeric DNA was reported by other group, in which stabilization was brought about by mutation and resultant additional interactions. The structure of the guanine tracts was similar between the two structures. However, difference was seen for loops connecting guanine tracts, which may play a role in higher order arrangement of telomeres. The loop, a TTA segment, does not contain 8-bromoguanosine. Thus, the effect of the substitution on a native loop structure should be moderate. Our lower loop is more compact than that of the other group and three residues of the loop in our structure are located even closer to the guanine-tetrad, which contains the 8-bromoguanosine. Therefore, the steric hindrance involving 8-bromoguanosine can not be the cause of different loop structures. Similarly, the difference in the upper loop structure is not caused by the steric hindrance involving 8-bromoguanosines. Difference was also seen for the sugar conformation of some residues. Our structure which does not contain the mutation can be utilized to design a small molecule which stabilizes the quadruplex. This kind of molecule is supposed to inhibit a telomerase and thus is expected to be a candidate of an anticancer drug.

## Discussion & Summary

Elucidated structure of human telomeric DNA has indicated that this DNA can be used as a switching unit as DNA composed of GGA sequences. The positions of the both ends of this DNA is ideal to join two sub-units of functional nucleic acids.

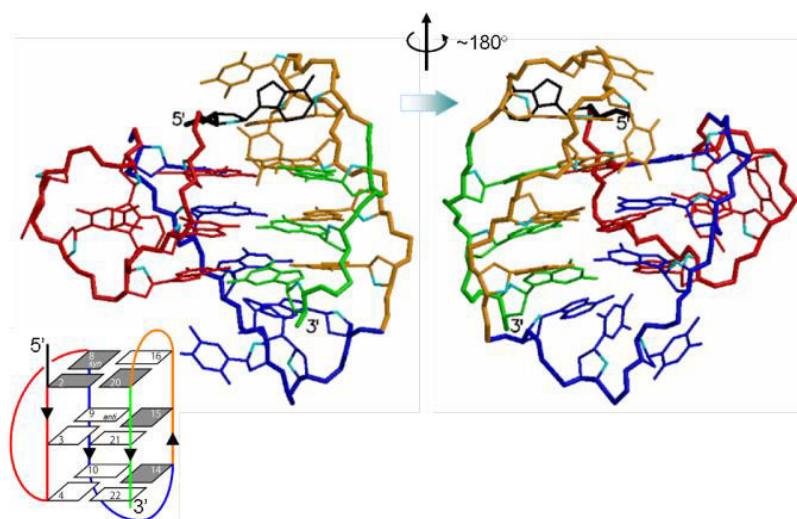
It is found that the activity of telomerase is inhibited by the formation of the quadruplex. Therefore, a small molecule that binds to and stabilizes the quadruplex is expected to be a candidate of an anticancer drug. Our structure determined in a  $K^+$  solution can be utilized to design and develop such a molecule. Precise structures of loops and sugars may be useful for such design and

development. The determination of the unique quadruplex structure must also serve as a basis to understand the interactions of various telomere-binding proteins with telomeres.

There are differences regarding loops and sugars between two structures by our group and the other group. Our structure does not include the mutation and resultant additional base pair which may be responsible for these differences. When the structure is utilized to understand higher order arrangement of telomeres and their interactions with telomere-binding proteins, and also to develop an anticancer drug, these differences may be critical. Therefore, it is important to present the structure stabilized by proper incorporation of 8-bromoguanosines, without introducing the mutation.

## Figures & Tables

**Structure of telomeric DNA in K<sup>+</sup> solution**



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1) Biological Sciences  
1-2) Struktural Biology

## NMR Structural Biology for the elucidation of the mechanisms of Parkinson's disease

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### Introduction

Recent advances in NMR structural biology have made possible the high throughput structural determination of small simple proteins and protein domains. However, it remains as tasks with more challenge to carry out structure biology studies of intrinsically disordered proteins, which are associated with various neurodegenerative disorders. In the present study, we demonstrate that 920 MHz ultra-high field NMR spectroscopy enables us to cope with the difficulty in dealing with those troublesome proteins.

### Results

$\alpha$ -synuclein is a 140-amino acid protein abundantly expressed in presynaptic nerve terminals and is studied as a model of intrinsically disordered proteins. This protein has been identified as the major component of the Lewy bodies (LBs) and Lewy neurites of Parkinson's disease, dementia with LBs and multiple system atrophy. It has been reported that  $\alpha$ -synucleins in synucleinopathy lesions are selectively and extensively phosphorylated at Ser129. Furthermore, phosphorylation of  $\alpha$ -synuclein at Ser 129 promoted fibril formation *in vitro*. However, the conformational effects of phosphorylation of  $\alpha$ -synuclein remain to be understood. In the present study, we perform ultra-high field NMR spectral analyses of  $\alpha$ -synuclein focusing on its phosphorylation at Ser129.

We first attempted to assign  $^1\text{H}$ - $^{15}\text{N}$  HSQC peaks originating from the backbone amide groups of  $\alpha$ -synuclein by use of 920 MHz NMR spectroscopy. While the peaks severely overlap in the 500 MHz spectrum, they were well dispersed in the 920 MHz spectrum. The improvement of the spectral resolution enables us to achieve assignments of the backbone resonances of the  $\alpha$ -synuclein.

We have successfully prepared a large amount of [ $^{15}\text{N}$ ] $\alpha$ -synuclein phosphorylated at Ser129 for detailed NMR analyses of effects of the phosphorylation. Inspection of the  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectral data indicated that the C-terminal phosphorylation site interacts with the middle parts in  $\alpha$ -synuclein molecules. On the basis of these data, we suggest that the phosphorylation destabilizes

an intramolecular interaction of  $\alpha$ -synuclein, rendering this protein more aggregatable.

We also conducted NMR analyses of ataxin-3, a product of the causative gene of Machado-Joseph disease. Ataxin-3 is composed of the N-terminal Josephin domain, two or three ubiquitin (Ub) interacting motifs and a polyglutamine tract. The Josephin domain, which acts as a deubiquitinating enzyme, assumes a papain-like fold with a cysteine in the catalytic site and with a flexible helical hairpin. To provide the structural basis for the substrate recognition by ataxin-3, we performed NMR analyses of the interaction between the Josephin domain and Lys48-linked di-Ub (K48-Ub<sub>2</sub>), which was prepared an enzymatic reaction *in vitro* using Ub mutants as substrates.

NMR chemical shift perturbation data indicated that the hydrophobic surfaces of both Ub units, which surround the isopeptide linkage, were involved in the interaction with the Josephin domain. To determine the orientation of K48-Ub<sub>2</sub> with respect to the Josephin domain, we made a site-specific spin labeling as a source of long-range distance information. For this purpose, either Lys48 of the distal Ub or the C-terminal Gly76 of the proximal Ub was substituted with cysteine, which was subsequently attached to a spin label. On the basis of the paramagnetic relaxation enhancement data, we identified the amino acids residues of the Josephin domain in close spatial proximity of the unpaired electron in the complex. Upon complex formation, the HSQC peak originating from Leu93 exhibited line broadening due to the effect of the spin label attached to the distal Ub, whereas the spin label at the C-terminal of the proximal Ub induced line broadening of the HSQC peaks from the catalytic residues of the Josephin domain.

On the basis of these NMR data, we propose a hypothetical model of the interaction between ataxin-3 and Lys48-linked polyubiquitin chain, which provides insights into the deubiquitinating function of ataxin-3.

We also provided structural basis of molecular recognition by Ub and a variety of Ub-like proteins, including the proteasome-binding domain of parkin, on the basis of NMR spectral data.

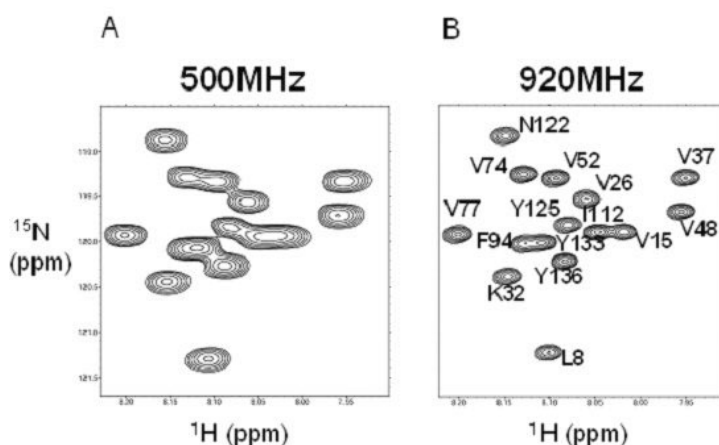


Figure 1 A part of  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectra of [ $^{15}\text{N}$ ] $\alpha$ -synuclein measured at the  $^1\text{H}$  frequencies of (A) 500 MHz and (B) 920 MHz.

## Discussion & Summary

In summary, we successfully applied ultra-high field NMR spectroscopic techniques for structural analyses of proteins as causative gene products of neurodegenerative disorders. This spectroscopic approach allows us to discuss molecular properties of phosphorylated  $\alpha$ -synuclein from the views of structural biology. Based on the results obtained in the present study, we are further carrying out NMR analyses focusing on interactions between small molecule inhibitors and isotopically labeled  $\alpha$ -synuclein. We also succeeded in determination of the mode of interaction between ubiquitin chain and deubiquitinating enzyme using the Josephin domain of ataxin-3 as a model enzyme.

The NMR spectral data will provide structural basis of the pathogenic mechanisms of neurodegenerative caused by protein dysfunction and/or aggregations. This will open up a new avenue for developing design and discovery of drugs for therapy and diagnosis of neurodegenerative disorders.

1) Biological Sciences  
1-3) Cell Biology

## Analysis of molecular machinery for autophagy

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### Introduction

Autophagy is a bulk degradation process that is conserved in eukaryotic cells and functions in the turnover of cytoplasmic materials and organelles. When eukaryotic cells face nutrient starvation, the autophagosome, a double-membraned organelle, is generated from the pre-autophagosomal structure (PAS). In the yeast *Saccharomyces cerevisiae*, 16 *ATG* (autophagy-related) genes are essential for autophagosome formation. Most of the Atg proteins are involved in the PAS, leading to autophagosome production. However, the mechanism of PAS organization remains to be elucidated. Here, we performed a systematic and quantitative analysis by fluorescence microscopy to develop a hierarchy map of Atg proteins involved in PAS organization.

### Results

#### **Comprehensive analysis of Atg protein localization**

Our systematic analysis aims to elucidate the mechanisms of PAS organization. First, we examined whether each Atg protein localizes to the PAS. Second, we investigated whether PAS localization of each Atg protein is perturbed by disruption of another *ATG* gene using quantitative fluorescence microscopy.

We constructed 16 yeast strains expressing GFP-fused Atg proteins from their native loci and examined these Atg-GFP strains using fluorescence microscopy and found that 11 Atg proteins exhibited perivacuolar punctuate structures that colocalized with Ape1p (a PAS marker), showing that these Atg proteins were indeed localized to the PAS (Fig. 1). To determine which specific Atg proteins were required for the PAS localization of the various Atg proteins, all of the *ATG* genes were individually disrupted in each Atg-GFP strain, for a total of 165 (11 x 15) disruptants. These cells were treated with rapamycin for five hours to induce autophagy and analyzed by fluorescence microscopy. The PAS targeting index (PTI) was introduced to quantify the level of Atg protein recruitment to the PAS.

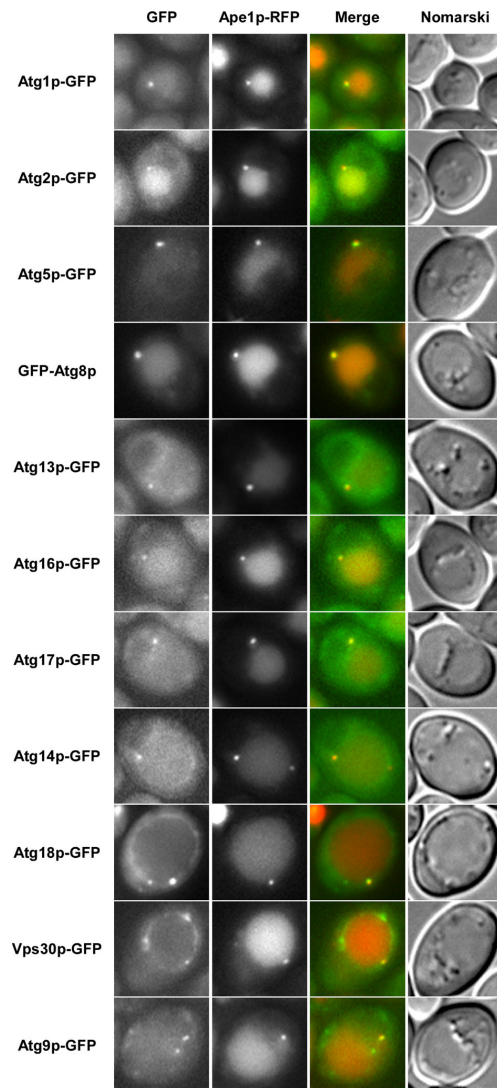
### Atg11p recruits Atg proteins for the Cvt pathway under autophagy-inducing conditions

The PTI analysis showed that localization of most Atg proteins was impaired in the absence of Atg17p. This fact led us to hypothesize that Atg17p is a scaffold protein for PAS organization. If this is true, no Atg proteins should localize to the PAS in the absence of Atg17p. However, the PTI of Atg8p in  $\Delta atg17$  cells was at the similar level as in wild-type cells. Interestingly, GFP-Atg8p dots disappeared in  $\Delta atg11\Delta atg17$  cells. We concluded that Atg11p is a scaffold protein for the Cvt pathway, a kind of selective autophagy that transports Ape1p to the vacuole. In combination with this prior study, our data suggest that Atg17p plays a role in the recruitment of Atg proteins for the autophagic pathway and that Atg11p plays a similar role for the Cvt pathway.

### Atg17p is a scaffold protein that is responsible for PAS organization

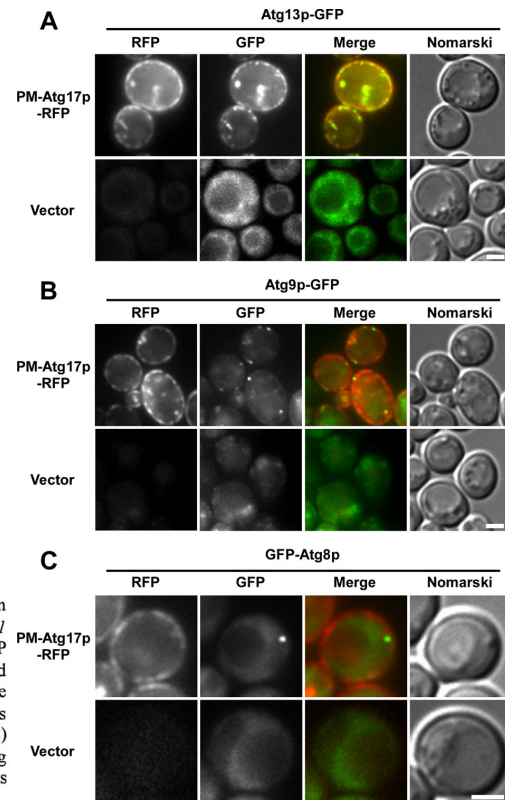
The PAS localization of Atg proteins is completely absent in the  $\Delta atg11\Delta atg17$  mutant. If Atg17p actually functions as a scaffolding protein in PAS organization, Atg proteins must be recruited to the site where Atg17p localizes. We constructed Atg17p fused with a plasma membrane-targeting sequence (PM-Atg17p), then examined whether other Atg proteins were targeted to the plasma membrane in  $\Delta atg11\Delta atg17$  cells expressing PM-Atg17p.

Atg9p, Atg13p, and Atg8p exhibited a cytoplasmic expression pattern in  $\Delta atg11\Delta atg17$  cells carrying an empty vector (Fig. 2, Vector). C-terminally mRFP1-fused PM-Atg17p (PM-Atg17p-RFP) thoroughly localized to the plasma membrane (Fig. 2, left column). Intriguingly, other Atg proteins were also recruited to the plasma membrane and essentially colocalized with Atg17p (Fig. 2), showing that Atg17p indeed acts as a scaffold to recruit Atg proteins. Also, we found slight differences in the localization of Atg proteins: whereas Atg13p was closely colocalized with PM-Atg17p (Fig. 2A), Atg9p was recruited to the plasma membrane, as visualized by the presence of a small number of punctate fluorescence (Fig. 2B), and Atg8p formed a single dot just inside the plasma membrane (Fig. 2C).



**Figure 1** Localization of Atg proteins to the PAS. Each Atg-GFP strain was mated with an Ape1p-RFP strain of the opposite mating type. The resulting diploids were grown in a synthetic medium, incubated with rapamycin for three hours, and then analyzed. Bar represents 2  $\mu$ m.

Notably, PM-Atg17p-RFP did not recover the autophagic activity of the  $\Delta atg11\Delta atg17$  mutant, as determined by the failure of GFP-Atg8p delivery to the vacuole (Fig. 2C), suggesting that these plasma membrane-targeted Atg proteins are unable to generate autophagosomes. Presumably, PAS organization at the proper site (next to the vacuole) is required for normal autophagy. Again, this experiment clearly showed that Atg17p is a determinant of the site of PAS organization during autophagy.



**Figure 2** Atg17p is a scaffold protein that organizes Atg proteins. Cells were grown in SD + CA medium containing 250  $\mu$ M  $\text{CuSO}_4$  to drive the  $\text{Cu}^{2+}$ -inducible *CUP1* promoter, and treated with rapamycin for five hours. (A) Localization of Atg13p-GFP in the  $\Delta atg17\Delta atg11$  mutant. Cells expressing plasma membrane-targeted Atg17p-RFP1 (PM-Atg17p-RFP) and those carrying an empty vector (Vector) were analyzed. (B) Localization of Atg9p-GFP in the  $\Delta atg17\Delta atg11$  mutant. Cells expressing PM-Atg17p-RFP and those carrying an empty vector (Vector). (C) Localization of GFP-Atg8p in the  $\Delta atg17\Delta atg11$  mutant. Cells expressing PM-Atg17p-RFP and those carrying an empty vector (Vector) were analyzed. Bars represent 2  $\mu$ m.

## Discussion & Summary

Sixteen *ATG* genes are essential for autophagosome formation. Extensive analyses have elucidated five functional groups among the Atg proteins—two ubiquitin-like systems, the PtdIns(3)-kinase complex, Atg1 protein kinase and its regulators, and the Atg2p•Atg18p complex—but little has been determined regarding the interrelationships between each unit in autophagosome formation. In this study, we show that every functional group is organized within the PAS, which plays a pivotal role in autophagosome formation. Key to understanding Atg-mediated autophagy is elucidation of how Atg proteins interact to organize and form the PAS. Therefore, we used fluorescence microscopy to systematically analyze the PAS localization of Atg proteins.

From this analysis, we have developed a hierarchy diagram of Atg proteins in PAS organization. Atg1p protein kinase and its regulators, Atg9p, and the PtdIns(3)-kinase complex I act in initial Atg protein recruitment to the PAS. Atg8p-PE, the Atg12p-Atg5p•Atg16p complex, and the Atg2p•Atg18p complex are recruited to the PAS with the help of the Atg proteins belonging to the above group, and work at the PAS to generate autophagosomes. The two ubiquitin-like systems confer autophagic activity to Atg8p and Atg12p by modifying them. This diagram suggests that Atg17p is the most upstream component in PAS organization.

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1) Biological Sciences

1-3) Cell Biology

## Cell cycle regulation by Apollon

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### Introduction

Apollon is a 530K protein containing baculoviral IAP-repeat (BIR) and ubiquitin-conjugating enzyme (UBC) domain, and regulates apoptosis by ubiquitylating caspase9, SMAC and HtrA2 for proteasomal degradation. Gene targeting study showed that apollon deficiency results in growth retardation in some embryos, while abnormality in developmental apoptosis was not obvious, suggesting that apollon plays a role in regulating cell proliferation. We found in this study that apollon regulates mitosis by ubiquitylating cyclin A.

### Results

When primary MEFs were cultured in vitro, the proliferation of apollon-deficient MEFs halted at around 10 days after cultivation, while wild-type MEFs proliferated approximately 30 days (Fig. 1). The apollon-deficient MEFs cultured in vitro for 11 days showed typical features of senescence, flattened cell morphology and acid  $\beta$ -galactosidase activity (Fig. 2). These observations indicate that apollon-deficient MEFs undergo earlier replicative senescence in culture.

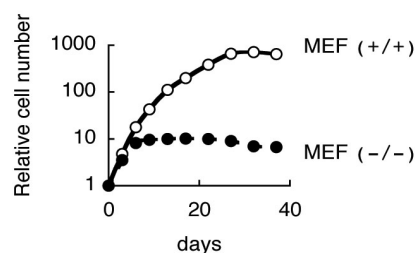


Fig. 1 Apollon-deficient MEFs show earlier replicative senescence.

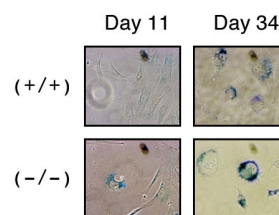


Fig. 2  $\beta$ -Galactosidase staining of Apollon-deficient MEFs

From the senescent MEFs, we developed a pair of immortalized MEFs and observed their proliferation under microscopy equipped with time-lapse video recording system and incubation system. We found that apollon-deficient MEFs require longer period for mitosis than wild type MEFs, while the period for whole cell cycle was almost comparable. These results suggest that



Apollon regulates mitosis.

To study the mechanism how apollon regulates mitosis, we searched for apollon interacting proteins with antibody array, and found that cyclin A but not cyclin B strongly interacted apollon in this array. Co-immunoprecipitation experiment confirms apollon binds to cyclin A but not to cyclin B in cells. Deletion analysis indicated that carboxy-terminal deletions to 1-2690 apollon fragment binds to cyclin A but further deletion seriously affected the binding. Mutation in a conserved cysteine in the BIR domain did not affect the binding to cyclin A while it completely abolished the binding to SMAC. These results indicate that BIR and UBC domains of apollon are not required to bind cyclin A. They also suggest that cyclin A binds to apollon at a different site from SMAC binding.

The domain of cyclin A for binding to apollon was determined to cyclin box 1, to which CDK1 and 2 bind. Accordingly, expression of CDK1 and 2, but not CDK 4 and 6, inhibited the binding of apollon to cyclin A. These results indicate that apollon binds to cyclin A, which is competed by CDK1 and 2.

Since apollon contains a UBC domain, we examined if cyclin A is ubiquitylated by apollon. When co-expressed with FLAG-cyclin A and HA-ubiquitin, apollon, but not survivin or ubc3, enhanced ubiquitylation of cyclin A. Unexpectedly, apollon C4638A mutant, in which a conserved cysteine residue in the UBC domain was substituted to alanine, enhanced the ubiquitylation of cyclin A as well as wild-type apollon, which suggests that the UBC domain of apollon is not essential to ubiquitylate cyclin A in cells. They also suggest the role of apollon as an E3 ubiquitin ligase involving other E2-UBC enzymes for cyclin A ubiquitylation.

To study the role of apollon in cyclin A degradation during mitosis, 293T cells were co-transfected with cyclin A-GFP, cyclin B-DsRed and siRNA against apollon. After synchronization at S-phase by aphidicolin, cells were released and observed for degradation of cyclin A-GFP by fluorescence microscope. During G2-phase, cyclin A-GFP and cyclin B-DsRed localizes to nuclei and cytoplasm, respectively. At prometaphase cyclin B-DsRed translocates to nuclei, giving yellow signal in nuclei, and then cyclin A-GFP was degraded within 20 min in control siRNA treated cells. In apollon siRNA treated cells, on the other hand, cyclin B-DsRed translocate to nuclei at time 0, but the degradation of cyclin A-GFP was delayed to 210 min. These results indicate that apollon regulates mitosis by ubiquitylating cyclin A for proteasomal degradation.

## Discussion & Summary

In mammalian cell cycle, cyclin A and cyclin B are required to enter into mitosis, while their elimination is also essential to complete mitosis. During mitosis, cyclin A and cyclin B are ubiquitylated by anaphase promoting complex/cyclosome (APC/C) and subjected for proteasomal degradation. However, cyclin A begins to be degraded at prometaphase when spindle checkpoint inhibits the activation of APC/C, the mechanism of which was not elucidated. We found in this study

that apollon binds and ubiquitylates cyclin A for proteasomal degradation. In apollon-deficient cells, the degradation of cyclin A and progression through mitosis are delayed. These results indicate that apollon is a ubiquitin ligase for cyclin A involved in its mitotic degradation.

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1) Biological Sciences  
1-3) Cell Biology

## Spatio-temporal analysis of lipid second messengers with probes based on fluorescence resonance energy transfer

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### Introduction

Phosphoinositides (PtdIns) are a family of phospholipids containing *myo*-inositol as their head group. Despite a relatively low abundance in biological membranes, PtdIns have been reported to regulate a myriad of cellular process depending on their metabolites. However, due to lack of proper probes, their subcellular localization remains largely unknown. To visualize and quantitate the distribution of phosphoinositides, we developed probes based on the principle of fluorescence resonance energy transfer.

### Results

FRET probes:

FRET probes for PtdIns(3,4)P<sub>2</sub> and PtdIns(3,4,5)P<sub>3</sub> have been reported previously. These probes consists of a PH domain of TAPP-1 for PtdIns(3,4)P<sub>2</sub> or GRP for PtdIns (3,4,5)P<sub>3</sub>, which is sandwiched with yellow fluorescence protein and cyan fluorescence protein. To modify these probes for PtdIns (4,5)P<sub>2</sub>, PtdIns(4)P, or diacylglycerol (DAG), we used PH domain of PLC- $\delta$  or FAPP1, or C1 domain of PKC $\beta$ . In addition, to monitor the distribution of these enzymes at the plasma membrane, we the probes were localized exclusively at the plasma membrane by using K-Ras C-terminal region.

Distribution of PtdIns in migrating cells:

MDCK cells expressing these FRET probes were time-lapse imaged by fluorescence microscopes. We found that all of PtdIns(4)P, PtdIns(4,5)P<sub>2</sub>, PtdIns(3,4)P<sub>2</sub>, PtdIns(3,4,5)P<sub>3</sub>, and DAG are enriched at the leading edge of migrating cells. Inhibitors that perturb the production of any of these metabolites significantly impaired the cell migration. This observation strongly suggested that either production of PtdIns (4)P or translocation PtdIns (4)P from ER to plasma membrane is accelerated.

Growth factor-induced changes in phosphoinositides:

Growth factor-induced changes in the level of PtdIns(4,5)P<sub>2</sub>, PtdIns(3,4)P<sub>2</sub>, and PtdIns(3,4,5)P<sub>3</sub> were found to be very rapid and transient. This observation suggests that the mechanism to hold the homeostasis of phospholipids are tightly regulated even in growth-factor stimulated cells.

## Discussion & Summary

We could successfully visualized the spatio-temporal regulation of PtdIns(4)P, PtdIns(4,5)P<sub>2</sub>, PtdIns(3,4)P<sub>2</sub>, PtdIns(3,4,5)P<sub>3</sub>, and DAG in migrating cells and growth factor-stimulated cells. By adding proper internal standards, the FRET images could be evaluated in a quantitative manner. Such data will provide versatile information on how the metabolism of phosphoinositides is regulated in the cells. More importantly, such data can be converted into parameters that are essential to operate kinetic simulation models. With this approach, we believe, we could understand the metabolism of and the signaling cascades mediated by phosphoinositides as a integrated system.

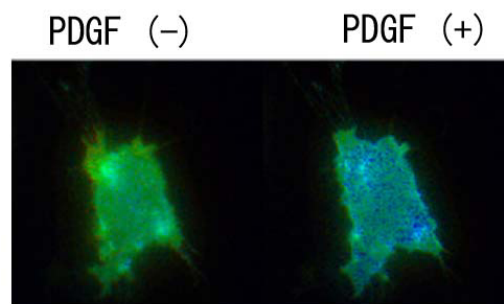


Figure 1. Pippi-PI(4,5)P<sub>2</sub> detects PDGF-induced decrease in PI(4,5)P<sub>2</sub>. NIH3T3 cells were stimulated with PDGF and time-lapse imaged for FRET. Here the cold and warm colors indicate low and high PI(4,5)P<sub>2</sub> levels.

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1) Biological Sciences

1-3) Cell Biology

## Analysis of molecular mechanisms for regeneration of retina and neural tissues by stem cells and clinical application

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### Introduction

Recently, increasing attention is paid to the regenerative medicine exploiting various stem cells including neural or retinal stem cells (1). Neural stem cells are critical as a source of the cell replacement therapy for the neurodegenerative disease or injured brain and spinal cord. Retinal stem cells also have potential to be used for treatment of retinal diseases including retinal degeneration. Therefore, it is important to clarify the molecular mechanisms underling the biology of neural stem cells, such as the control of proliferation and differentiation. We have previously demonstrated that FRS2alpha docking protein plays critical roles for growth and maintenance of several stem cells including neural or retinal stem cells (2). The aim of this study is to clarify the molecular mechanisms for FRS2alpha to maintain these stem cells and to test if FRS2alpha can be used for cell replacement therapy.

### Results

#### **Overexpression of wild type Frs2 $\alpha$ or 8V, but not 6F, increases the size of the neurospheres derived from E12 telencephalon.**

To analyze the function of Frs2 $\alpha$  in NSPCs, neurospheres derived from the telencephalon of E12 mouse embryos were infected with retrovirus expressing wild type or mutant Frs2 $\alpha$ , and cultured in the presence of 2, 5, 20 ng/ml FGF2. Control was infected with empty vector, and viral infection was monitored by the signal from EGFP. At every dose of FGF2, overexpression of wild type Frs2 $\alpha$  or its constitutive active form 8V apparently increased the size of neurospheres compared with control. In contrast, overexpression of 6F, that lacks all tyrosine phosphorylation sites, showed no apparent effect on size of neurospheres. The distribution of diameter shows that neurospheres expressing wild type Frs2 $\alpha$  or 8V contains larger neurospheres than control, but not 6F. In addition, average diameter of neurospheres expressing wild type Frs2 $\alpha$  or 8V, but not 6F, was significantly larger than control at every dose of FGF2.

### **Overexpression of wild type Frs2 $\alpha$ or 8V, but not 6F, increases the number of neurospheres bFGF-specifically**

FGF2 is a commonly used mitogen of NSPCs in vitro. However, it is unclear whether FGF signaling also contributes to the self-renewal of neural stem cell. To examine whether Fgf signaling through Frs2 $\alpha$  plays role in the self-renewal ability of neural stem cells, we examined the number of neurospheres formed when neurospheres were infected with retrovirus expressing wild type or mutant Frs2 $\alpha$ .

In neurospheres derived from the telencephalon of E12 embryos, overexpression of wild type Frs2 $\alpha$  or 8V significantly increased the number of neurospheres, but not 6F. Similar result was obtained when wild type or mutant Frs2 $\alpha$  were overexpressed in neurospheres derived from the telencephalon of E14 embryos, and cultured in the presence of FGF2. In contrast, when neurospheres were cultured in the presence of EGF, overexpression of wild type or mutant Frs2 $\alpha$  had no effect on the number of neurospheres.

To determine whether the effect of overexpression of Frs2 $\alpha$  is FGF-specific, neurospheres cultured in the presence of EGF was passed, and cultured again in the presence of EGF or FGF. When neurospheres were cultured again in medium containing EGF, over expression of wild type or mutant of Frs2 $\alpha$  did not show effect on the number of neurospheres. In contrast, when neurospheres were cultured again in the presence of FGF2, average number of the neurospheres expressing wild type Frs2 $\alpha$  or 8V was significantly larger than control, but not 6F.

### **Activity of Erk and Akt is enhanced in neurospheres expressing wild type Frs2 $\alpha$ or 8V but not Frs2 $\alpha$ -6F.**

To examine the activation of the FGF signaling pathways down stream of Frs2 $\alpha$  in the neurospheres overexpressing wild type of mutant Frs2 $\alpha$ , components of those pathways were analyzed by western blotting. Control neurospheres and neurospheres overexpressing 6F showed similar kinetics with respect to the activation of Erk1/2 and Akt. In contrasts, neurospheres overexpressing wild type Frs2 $\alpha$  or 8V showed strong and long standing activation of both Erk and Akt. In addition, activation of Shp2 and Gab1, two proteins down stream of Frs2 $\alpha$  that are responsible for the activation of Ras-Erk and PI3K-Akt pathway respectively, was enhanced in the neurospheres overexpressing wild type Frs2 $\alpha$  or 8V compared with control or 6F.

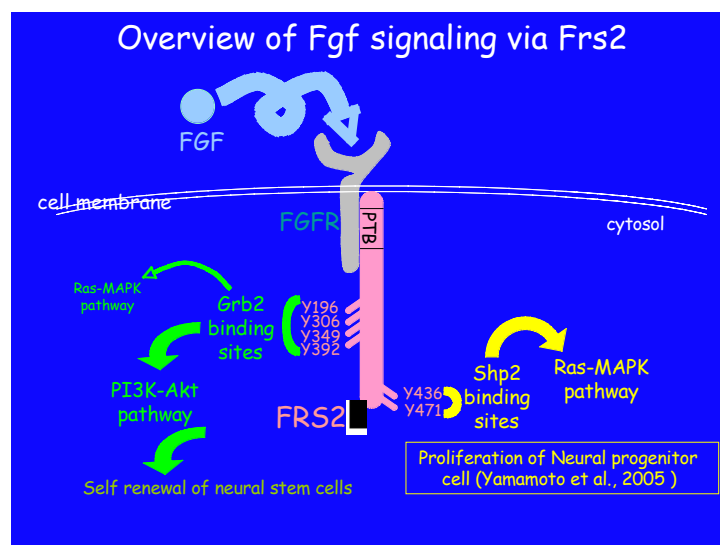
## **Discussion & Summary**

### **Discussion**

In the present study, wild type or mutant forms of Frs2 $\alpha$  were overexpressed in the neurospheres with retrovirus system to analyze the function of Frs2 $\alpha$  in NSPCs. In the presence of FGF2, overexpression of wild type Frs2 $\alpha$  or 8V in E12 or E14 neurospheres increased the size of

neurospheres, but overexpression of 6F did not. These results indicate that FGF2-induced tyrosine phosphorylation of Frs2 $\alpha$  plays an important role for the proliferation of NSPCs regardless of developmental stages examined. In contrast, overexpression of wild type or mutant forms of Frs2 $\alpha$  did not show effect on the proliferation of the neurospheres in the presence of EGF, suggesting that overexpression of Frs2 $\alpha$  or 8V potentiates the response of NSPCs to FGF2 stimulation specifically. Moreover, when neurospheres overexpressing wild type or mutant forms of Frs2 $\alpha$  cultured in the presence of EGF was passed and cultured again in the presence of FGF2 or EGF, neurospheres expressing wild type or 8V showed efficient growth FGF2-specifically, but not 6F. These results further confirm that the effect of overexpression of Frs2 $\alpha$  is FGF2 specific, and indicating that signaling pathways down stream of Frs2 $\alpha$  are activated strongly by the overexpression of Frs2 $\alpha$  or 8V.

Interestingly, overexpression of Frs2 $\alpha$  or 8V increased the number of neurospheres formed, but not 6F. This effect was also FGF2 specific, and independent of developmental stages neurospheres derived from. This indicates that overexpression of Frs2 $\alpha$  or 8V promotes not only the proliferation of NSPCs, but also the self renewal of neural stem cells (Figure).



## Summary

It is important to manipulate neural stem/progenitor cells (NSPCs) in vitro in order to apply them for cell replacement therapy. Fibroblast growth factor (FGF) 2 is a commonly used mitogen for NSPCs in vitro, however, it is still unclear how FGF2 controls NSPCs. Here, we provide evidence that FGF2 controls not only proliferation but also self-renewal of NSPCs through FRS2 $\alpha$  docking protein. We expressed wild type FRS2 $\alpha$  or its mutant forms in neurospheres, spherical cell clusters of NSPCs

using a retrovirus system. Expression of wild type FRS2 $\alpha$  or its constitutive active form of FRS2 $\alpha$ -8V promoted the proliferation of NSPCs in the presence of FGF2 but not epidermal growth factor (EGF). Interestingly, the number of FGF2-induced neurospheres, an indicator of self-renewing ability, was also increased by expression of either protein. In contrast, expression of FRS2 $\alpha$ -6F, the tyrosine (Y) to phenylalanine (F) mutant of all six tyrosine phosphorylation sites, did not show these effects on NSPCs. Moreover, we found that activation of both ERK and AKT was enhanced in neurosphere cells expressing wild type FRS2 $\alpha$  or FRS2 $\alpha$ -8V but not FRS2 $\alpha$ -6F. These results suggest that FGF2-induced tyrosine phosphorylation of FRS2 $\alpha$  plays an important role for proliferation and self-renewal of NSPCs and raise a possibility to make use of FRS2 $\alpha$  for manipulating NSPCs in vitro.

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1) Biological Sciences  
1-3) Cell Biology

## Development of regulatory strategies for neuronal death induced by malfolded proteins.

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### Introduction

The endoplasmic reticulum (ER) stress response is a defense system for dealing with the accumulation of unfolded proteins in the ER lumen. Recent reports have shown that ER stress is involved in the pathology of some neurodegenerative diseases and cerebral ischemia.

### Results

In a screen for compounds that induce the ER-mediated chaperone BiP/GRP 78 (BiP), we identified BiP inducer X (BIX). BIX induced BiP only, in a dose-dependent manner, without induction of other molecules involved in the ER stress response. The induction of BiP mRNA by BIX was mediated by activation of ER stress response element (ERSE) through ATF6 pathway. Pretreatment of neuroblastoma cells with BIX reduced cell death induced by ER stress. Intracerebroventricular pretreatment with BIX reduced the area of infarction due to focal cerebral ischemia in mice. In the penumbra of BIX-treated mice, ER stress-induced apoptosis was suppressed, leading to a reduction in the number of apoptotic cells.

### Figures&Tables

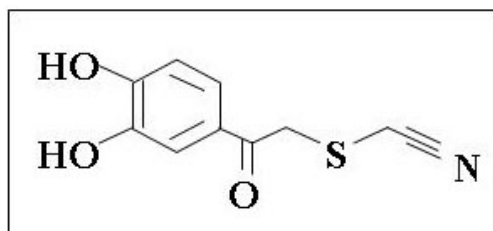
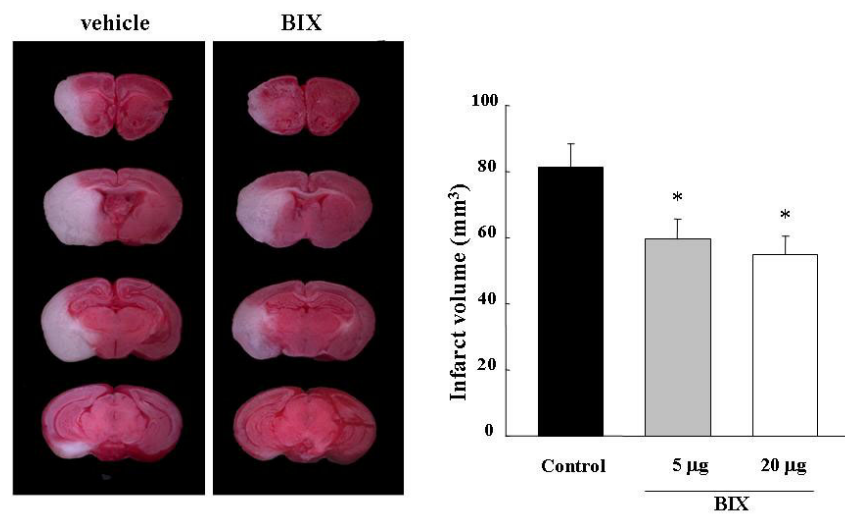


Figure 1. Chemical structure of BIX



**Figure 2. BIX suppresses neuronal death after brain ischemia.**

**A:** BIX or vehicle was administered to brain ventricle of mice. After 30 min following drug administration, mice were operated for the permanent middle cerebral artery occlusion (MCAO). Another 24 h after surgery, the brains of mice were subjected to 2,3,5-triphenyltetrazolium chloride (TTC) staining. **B:** Quantitative analysis of infarct area volume of MCAO mice was shown. Data are the means  $\pm$  S.D.. Significant differences between BIX and vehicle were indicated as asterisks (\*:  $p < 0.05$ , T-test).  $n = 11$  or  $12$ .

## Discussion & Summary

BIX induces BiP to prevent neuronal death by ER stress, suggesting that it may be a potential therapeutic agent for cerebral diseases caused by ER stress.

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1) Biological Sciences  
 1-4) Developmental Biology

## Analysis of molecular mechanism of fusion process intermediating Izumo factor on sperm surface.

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### Introduction

It is said that one tenth of human couples are suffering from infertility. In fact, in our country, at least one out of hundred new born babies are treated with ART (Assisted Reproductive Technology). However, the molecular basis for fertilization is poorly understood, especially in the final sperm-egg fusion step. During the long history of research in fertilization, many factors involved in sperm-egg fusion have been reported. Until now, almost of these factors have been assessed by utilizing gene targeting. Representative of factor, CD9, which belongs to tetraspan-membrane protein family on the egg membrane was found to be essential for fusion, but sperm-related fusion factors remain unknown. Using a fusion-inhibiting monoclonal antibody, we identified a mouse sperm fusion-related antigen, Izumo. Izumo  $-/-$  mice were healthy but were incapable of fusing with eggs. Here we show that we identified novel Izumo-binding factor by using sperm originated from Izumo- 6 x His-transgenic mice line.

### Results

A sperm and an egg meet, recognize each other, and fuse to create a new generation of life. The factors involved in this important membrane fusion event, fertilization, have been sought for a long time. Recently, CD9 on the egg membrane was found to be essential for fusion (1), but sperm-related fusion factors remain unknown. Using a fusion-inhibiting monoclonal antibody, we identified a mouse sperm fusion-related antigen, cloned the gene and discovered that the antigen was a novel immunoglobulin superfamily protein. We termed the gene Izumo and produced a gene-disrupted mouse line. Izumo  $-/-$  mice were healthy but males were sterile. They produced normal looking sperm that bind to and penetrate the zona pellucida, but were incapable of fusing with eggs. Human sperm also contain Izumo and addition of the anti-human Izumo antibody left the sperm unable to fuse with zona-free hamster eggs (2).

To examine whether if the phenotype was directly derived from the lack of Izumo on sperm, we performed the rescue experiment by crossing Izumo  $-/-$  mice with transgenic mouse lines generated

to express Izumo- 6 x His-tag using the testis-specific calmegin promoter. The sterile phenotype was rescued with the transgenically expressed Izumo on mouse sperm.

To identify the factor (s), which interact to Izumo on sperm, we employed His-tag pull down assay. We prepared sperm lysate containing 1% Brij 97 detergent in Izumo knockout background mouse Izumo transgenic mice with 6 x His-tag obtaining from intercrossing beforehand. Then, magnetic beads conjugated with anti-His-tag antibody were added to sperm lysate. The protein (s) coupled with anti-His-tag were passed through the magnetic column to attach the Izumo and Izumo binding protein, the factor was identified by separation of the extracts by SDS-PAGE, sequentially, subject it to liquid chromatography tandem mass spectrometry (LC-MS/MS). Consequentially, these 9 peptides which were 100% identical to a part of the sequence listed in RIKEN full length database were found.

The novel protein was 80 kDa and highly similar to testicular ACE (angiotensin-converting enzyme), which is involved in sperm-zona interaction owing to glycosylphosphatidylinositol (GPI)-releasing activity (3), but, this protein was partially conserved important zinc binding motif regulating center of active site. Besides, this gene showed testis specific expression pattern in mRNA level, therefore we named it testicular ACE 2 (tACE2). We promptly produced anti-tACE2 polyclonal antibody using recombinant tACE2. After that, we could obtain the data that confirm the evidence of directly binding tACE2 to Izumo each other by performing immunoprecipitation experiment.

Taken together, these data suggest that there is possibility that both Izumo and tACE2 are in cooperation with each other to maintain membrane order attributing to trigger the membrane fusion, and are essential factors to accomplish sperm-egg fusion on the sperm side.

## Discussion & Summary

So far, it has been reported that testis ACE functions not only a key regulator of blood pressure together with dipeptidyl carboxypeptidase, but also sperm-zona pellucida binding utilizing a glycosylphosphatidylinositol (GPI)-anchored protein releasing activity (3). Thus, taken together with our data suggests that ACE seems to be multi-functional gene rather than only act as an angiotensin-converting. If tACE2 also involves in fertilization, in particular, sperm-egg fusion process, although we need further gene targeting experiment as proved in Izumo knock out mouse, the study of ACE would be step into new stage of expansion. Hopefully, these finding not only provides insight into the enigmatic fusion mechanism but also promises future benefits for the clinical treatment of infertility and the potential development of novel contraceptive strategies.

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1) Biological Sciences  
1-4) Developmental Biology

## Periodic gene expression generates spatial pattern in mouse development

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### Introduction

In the mouse development, the most prominent repetitive structure is somite, which gives rise to repetitive pattern of mouse body such as vertebrae, ribs and muscles. A bilateral pair of somites buds off from the anteriormost end of the presomitic mesoderm (PSM). A new somite is formed every 2 h in the mouse, and this periodic event is govern by a molecular clock. Previously, we revealed the mechanism of the molecular clock, which includes a negative feedback loop of gene expressions. In this project, we aimed at elucidating how the molecular clock generates the regular interval pattern of somotes.

### Results

In the mouse PSM cells, the expression of several genes, including basic helix-loop-helix gene *Hes7*, oscillates synchronously with somite formation[1,2]. Notch signaling induces the transcription of *Hes7*, and induced *Hes7* inhibits its own promoter to repress the transcription of *Hes7*. Notch signaling also activates *Lunatic fringe* (*Lfng*), which encodes a Notch modulator, and *Hes7* inhibits the expression of *Lfng* periodically [3]. Additionally, gain- or loss-of function of *Hes7* disrupts both oscillatory gene expression and periodic segmentation of somites as well as the regular interval pattern of somites[2](Bessho, unpublished data). Thus, this negative feedback loop of *Hes7* serves as the central mechanism of the gene oscillation, which controls periodic segmentation as a biological clock.

*Fgf8* is expressed in the posterior end of the PSM and displays a gradient to anterior PSM. Because excess FGF signaling pushes the segmentation border to anterior, the gradient of FGF signaling serves as spatial information to determine the position of the segmentation[4,5]. In the tail bud region, the posterior end of the embryo, mesenchymal cells proliferate extensively, and consequently the embryo elongates posteriorly. At the same time the gradient of FGF signaling gradually sifts posteriorly and decides the new segmentation border every 2 h, which the segmentation clock counts, to generate even grained somites. Thus, gene oscillation and FGF

gradient determine the timing and the position of segmentation, respectively.

To elucidate how gene oscillation and FGF gradient are combined and how their information is converted to precise morphological pattern, we assumed putative mediates, which should modulate the FGF signaling periodically in a 2 h cycle depending on the gene oscillation. FGF signaling has several feedback inhibitors. We investigated the expression pattern of those genes in the mouse PSM. Among them, we found that *Sprouty4* is expressed prominently in the PSM and the pattern is similar to that of *Fgf8*. But the expression pattern of *Sprouty4* is various in different embryos. Some embryos have strong signal in the posterior PSM, some show in the middle, the others display strong staining in the anterior PSM (Fig. 1).

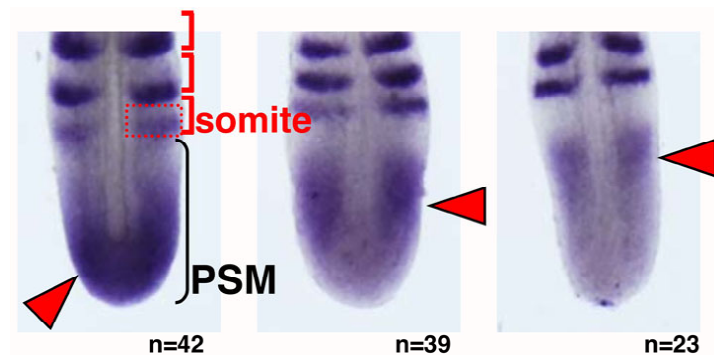


Fig. 1 *Sprouty4* shows different expression pattern in each embryo. E10.5 embryos show variable expression pattern of *Sprouty4*. *Uncx4.1* is used as a marker for formed somites. Among 104 embryos, 42 show strong *Sprouty4* expression in the posterior PSM, 39 show in the middle and 23 show in the anterior PSM respectively.

Next we compared the expression of *Sprouty4* with that of that of *Lfng*, which oscillates in the same phase as *Hes7*[2]. All embryos *Sprouty4* and *Lfng* are co-expressed in the same region of the PSM (Fig. 2), indicating that *Sprouty4* oscillates in the same phase as *Hes7* and *Lfng*. In *Hes7* KO embryos, in which the expression of *Hes7* does not oscillate[2], we saw uniform expression of *Sprouty4* in the PSM (data not shown). Thus, *Hes7* is essential for the oscillation of *Sprouty4*, suggesting *Hes7* may periodically inhibit the transcription of *Sprouty4*. Taken all together, *Sprouty4* could be a candidate of a mediator that combines the temporal information of gene oscillation and spatial information of FGF gradient in the mechanism of periodic somite formation.

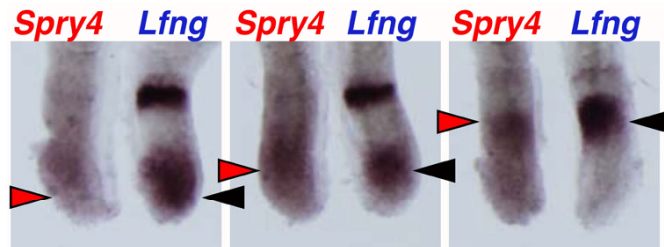


Fig. 2 *Sprouty4* oscillates in phase with *Lfng*. The posterior embryos including PSM were cut into half sagittally, and *Sprouty4* and *Lfng* were detected separately. In each phase, both signals are similar except the anterior most signal of *Lfng*.

## Discussion & Summary

Sprouty4 protein is implicated as an inhibitor that blocks the FGF/MAPK cascade, although the precise molecular mechanism remains elusive[6]. In this study, we showed that the expression of *Sprouty4* mRNA oscillates in the mouse PSM, synchronously with somite segmentation under the control by *Hes7*. It strongly suggests that the expression of Sprouty4 protein also oscillates in the same cycle, and that Sprouty4 protein inhibits the FGF signaling periodically in the PSM. Thus next steps in this project are examining the expression pattern of Sprouty4 protein and measurement of the FGF activity spatiotemporally in the PSM. At the same time, we need to examine whether the oscillation of *Sprouty4* is essential for the somite formation by gain- and loss-of function experiment of *Sprouty4*.

In this project, we identified a candidate molecule that combines temporal and spatial information, and it might be a critical mechanism of formation of repetitive pattern in mouse development.

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1) Biological Sciences

1-6) Pharmacology

## Genomic Drug Target Validation Study for Cerebrovascular Disorders.

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### Introduction

Delayed cerebral vasospasm often develops 3 to 9 days after aneurysmal SAH. In 27% to 38% of patients with SAH, a delayed, neurological ischemic deficit occurs. Despite successful surgical treatment for intracranial aneurysm and postoperative management, 26% to 38% of such patients suffer sequelae or die of symptomatic severe vasospasm. Although extensive research has been carried out into the mechanisms underlying delayed cerebral vasospasm and many advanced therapies have been attempted, we still have no successful treatment for this condition.

### Results

The hydroxyl radical scavenger ( $\pm$ )-N, N'-propylenediniticotinamide is an agent that is especially beneficial in delayed vasospasm. We found that intravenous injection of ( $\pm$ )-N, N'-propylenediniticotinamide exhibit spasmolytic effects with induction of Heme Oxygenase-1 (HO-1) in basilar arteries in rat model of SAH, but not in normal rat. Furthermore, ( $\pm$ )-N, N'-propylenediniticotinamide promotes cGMP accumulation as a downstream of HO-1 in basilar arteries.

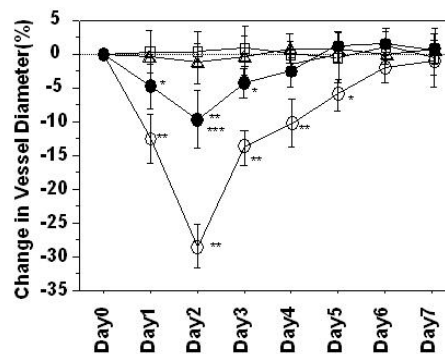
### Discussion & Summary

The supposed pathogenetic role of oxygen free radicals in SAH and other type of neuronal injuries has stimulated interest in antioxidants and free radical scavengers as potential therapeutic agents. [ $\pm$ ]-N, N'-propylenediniticotinamide; nicaraven, a water- and oil-soluble derivative of nicotinamide demonstrates a radical scavenging action putatively specific to the hydroxyl radical. In regard to SAH pathophysiology, AVS pharmacological profile can be summarized as follows: it scavenges hydroxyl radicals; exhibits anticontractile activity of 15-hydroperoxyarachidonic acid; suppresses arterial spasm following SAH in dogs; improves neurological deficits after SAH in rats by decreased

blood-brain barrier permeability change. Furthermore, in a multicenter placebo-controlled double-blind clinical trial, AVS significantly ameliorated the delayed ischemic neurologic deficits following aneurysmal SAH, with a marked improvement in the Glasgow Outcome Scale (GOS) scores at 1 month, and a marked decreased in the overall mortality.

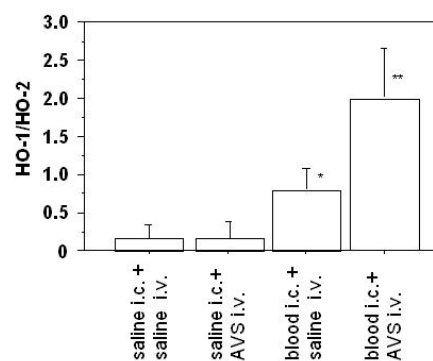
The present study used intracisternal injection of antisense HO-1 ODN to identify the protective effects of HO-1 in cerebral vasospasm in rat model of SAH. In conclusion, the therapeutic mechanism of ( $\pm$ )-N, N'-propylenediniticotinamide is owed its enhancement of intrinsic HO-1 induction in pathophysiological state, subsequently to increased cGMP-induced vascular smooth cell relaxation.

## Figures & Tables



**Figure 1**

The percent change from baseline in the diameter of the basilar artery after the treatment with 0.5 ml of saline Intracisternal injection into cisterna magna (saline i.c.) and administration of 0.2 ml / hr of saline intravenously (saline i.v.) (open triangles, n=3), saline i.c. and 0.2 ml / hr, 1mg / kg / min) of AVS intravenously (AVS i.v.) (open squares, n=3), 0.5ml of autologous blood injection into cisterna magna (blood i.c.) and saline i.v. (open circles, n=3) and blood i.c. and AVS i.v. (closed circles, n=3). Significantly different from the baseline values on day 0 (\* p < 0.05, \*\* p < 0.01). AVS ameliorated the delayed vasospasm on day 2 (\*\* p < 0.01).



**Figure 2**

HO-1 mRNA levels in the basilar arteries were determined using real-time RT-PCR analysis 2days after saline or blood injection into cisterna magna and were expressed as a ratio of HO-1 mRNA to HO-2 mRNA, which did not change appreciably after either blood or saline. Data presented are means of duplicate determinations (n=5). HO-1 mRNA levels after the treatment with 0.5 ml of blood injection into cisterna magna and saline administration intravenously for 2days(blood i.c. + saline i.v.) were induced as compared with saline i.c. + saline i.v. or saline i.c. + AVS i.v. Significantly HO-1 mRNA levels were enhanced by AVS administration after blood injection (blood i.c. + AVS i.v.). (\* p<0.05 vs. saline i.c., \*\* P < 0.01 vs. blood i.c. + saline i.v.)

1) Biological Sciences

1-7) Plant Biology

## Molecular mechanisms of photoreceptors-regulated growth of plant through phytohormone auxin

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### Introduction

The phototropic response of *Arabidopsis thaliana* seedlings is initiated by the blue-light (BL) photoreceptors, phototropin (phot) 1 and phot2, and by their signal transducers, NPH3 and RPT2. Although previous studies have indicated that phot1 contains BL-activated autophosphorylation activity and binds to NPH3 and RPT2, its biochemical function during the induction of the phototropic response remains unclear. In addition, photoreceptors, phytochromes and cryptochromes regulate hypocotyl growth under specific conditions by suppressing negative-gravitropism, modulating phototropism and inhibiting elongation. Although those effects seem to be partially caused via the regulation of the phytohormone auxin, the molecular mechanisms underlying this process are still poorly understood.

### Results

In our current study, we show that phot1 regulates the functional expression of NPH3 and RPT2 in a different manner. Our results indicate that the NPH3 protein is phosphorylated in seedlings under dark or red-light conditions and is dephosphorylated under BL. Phot1 is necessary for this BL-dependent dephosphorylation of NPH3. Our immunoprecipitation analysis further demonstrates that NPH3 dephosphorylation facilitates the effective association of this protein with RPT2 *in vivo*. We determine the phosphorylation domain of NPH3 and reveal the negative effect of NPH3 on the gravitropic response. In addition, phot1 induces the expression of RPT2 in seedlings under BL conditions through post-transcriptional regulation. In contrast, we show that phot2 is not involved in these regulatory pathways for NPH3 and RPT2.

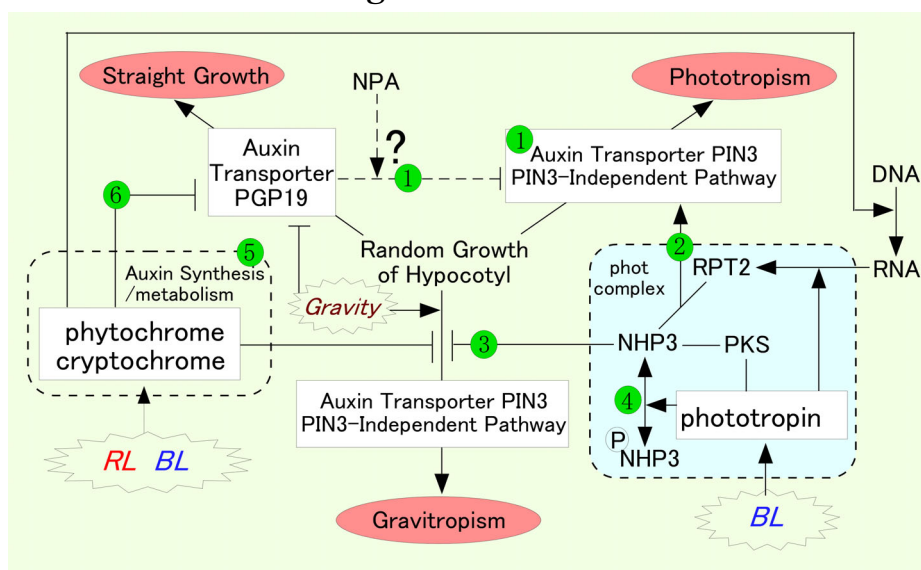
On the functional analyses of phytochrome and cryptochrome on the tropic responses, we demonstrate that the *flabby* mutation enhances the phytochrome-inducible hypocotyl bending in *Arabidopsis*. The *FLABBY* gene encodes the ABC-type auxin transporter, PGP19, and its expression is suppressed by the activation of phytochromes and cryptochromes. These results therefore reveal that phytochromes and cryptochromes have at least two effects upon the tropic responses of the

hypocotyls in *Arabidopsis*; one is the enhancement of hypocotyl bending through the suppression of PGP19, and another is a hypocotyl bending-inducing mechanism, which is independent of PGP19. The auxin polar transport assay and the *DR5::GUS* expression analysis suggest that phytochromes inhibit the basipetal auxin transport and induce the asymmetric distribution of auxin in hypocotyls. Further, activations of phytochromes and cryptochromes decrease auxin content in the aerial portion of the seedlings.

## Discussion & Summary

Our results thus suggest the possibility that the post-transcriptional events of NPH3 and RPT2 are the underlying mechanisms that trigger the phot1-induced phototropic response. In addition, the control of auxin transport and accumulation by phytochromes and cryptochromes may be a critical component of the regulation of hypocotyl growth in response to light.

## Figures & Tables



1) Biological Sciences

1-8) Animal Biology

## Study on processing mechanisms of novel identified neuropeptides.

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### Introduction

Recently, we identified a novel hypothalamic neuropeptide with a C-terminal LPLRFamide sequence in the quail brain (1). This avian neuropeptide was shown to inhibit gonadotropin release from the cultured anterior pituitary. This peptide is the first hypothalamic peptide that inhibited gonadotropin release reported in vertebrates. We, therefore, termed it gonadotropin-inhibitory hormone (GnIH). After this finding, we found that GnIH-related peptides were present in the brains of other vertebrates, such as mammals, birds, amphibians, and fish (2). These GnIH-related peptides possessed a LPXRFamide (X=L or Q) motif at their C-termini in all investigated animals.

### Results

We further characterized the GnIH cDNA from the quail brain and found that the deduced GnIH precursor polypeptide encoded one GnIH and two gene-related peptides (GnIH-RP-1 and GnIH-RP-2). These peptides possessed a LPXRFamide (X=L or Q) motif at their C-termini. Subsequently, GnIH-RP-1 and GnIH-RP-2 were identified as mature endogenous peptides using mass spectrometric analyses. In addition, N-terminally extended forms of GnIH and GnIH-RP-2 were found to be present in the quail hypothalamus and termed as GnIH-18 and GnIH-RP-2-18, respectively.

In the present study, I investigated that GnIH and GnIH-RP-2 were processed from their N-terminally extended forms, GnIH-18 and GnIH-RP-2-18, in the hypothalamus by biochemical and immunohistochemical analyses. Firstly it was found that GnIH-RP-2 was produced by the incubation with GnIH-RP-2-18 and hypothalamic extracts using high performance liquid chromatography (HPLC) and MALDI-TOF mass spectrometric analysis. This result showed that the quail hypothalamus contained a novel processing enzyme which recognize between arginine (R) and serine (S) residues. Secondly I raised specific antiserum against N-terminal region of GnIH-18 and compared the localizations of GnIH and GnIH-18 using the fluorescence double immunohistochemical analysis. This result showed that GnIH-18 was mainly localized in the soma

and GnIH was present in the nerve fibers throughout the brain, suggesting that the mature peptide GnIH was localized in the synapse vesicle of the nerve terminal (Figure 1).

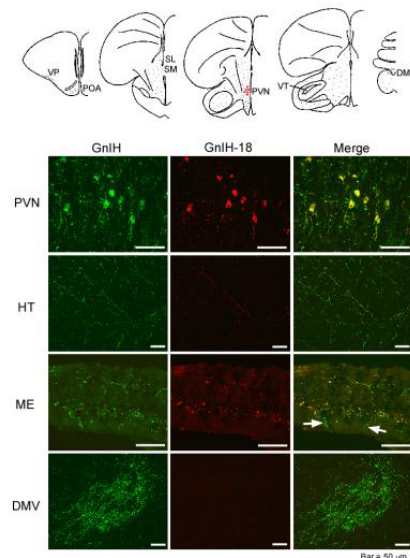


Figure 1

## Discussion & Summary

Before this study, N-terminally extended forms of GnIH and GnIH-RP-2 (GnIH-18 and GnIH-RP-2-18, respectively) have been thought as variants of GnIH and GnIH-RP-2. In this study it was found that GnIH and GnIH-RP-2 were processed from their N-terminally extended forms, GnIH-18 and GnIH-RP-2-18, in the quail hypothalamus by biochemical and immunohistochemical analyses. This result suggests that the unique N-terminal processing mechanism including Kex2-like processing enzyme, carboxypeptidase and peptidylglycine  $\alpha$ -amidating monooxygenase may be present in the precursor polypeptide of GnIH in the avian brain (Figure 2). In addition, this novel processing enzyme may recognize between arginine (R) and serine (S) residues.

## Predicted processing mechanisms of GnIH

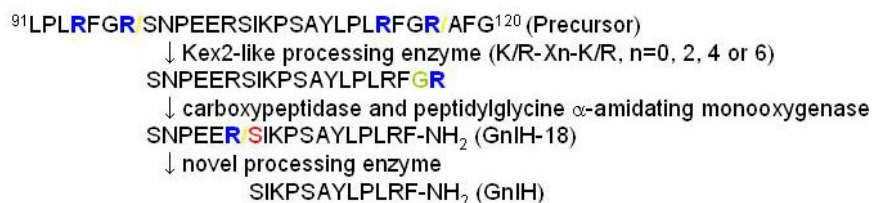


Figure 2

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2) Medical Sciences  
2-1) Immune System

## The role of cortex-to-medulla migration of developing thymocytes in establishing central tolerance

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### Introduction

Immature CD4<sup>+</sup>CD8<sup>+</sup> thymocytes, which are generated in the thymic cortex, are induced upon positive selection to differentiate into mature T lymphocytes and relocate to the thymic medulla. It was recently shown that a chemokine signal via CCR7 is essential for the cortex-to-medulla migration of positively selected thymocytes in the thymus. However, the role of the cortex-to-medulla migration in T-cell development and selection has remained unclear. Using mice that are deficient in CCR7 or its ligands (CCR7L), we addressed whether T cells may be exported from the thymic cortex without accumulation in the medulla and whether central tolerance to organ-specific self-antigens may be affected by the absence of medulla migration.

### Results

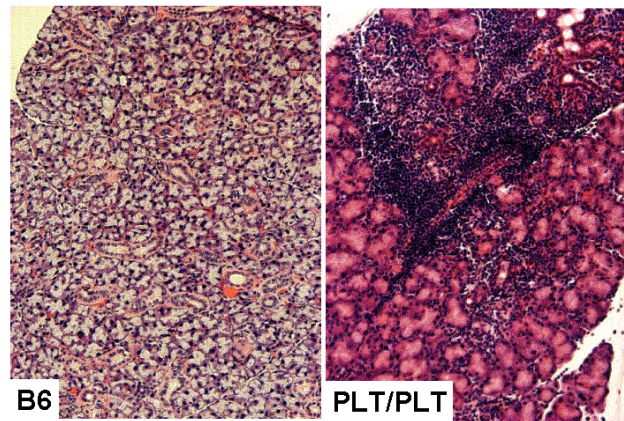
Our results showed that the pharmacological inhibition of S1P-mediated thymocyte egress in CCR7- or CCR7L-deficient mice results in the accumulation of mature thymocytes in the cortex, suggesting that mature thymocytes may be exported via the S1P-dependent mechanism from the cortex in the absence of CCR7 signals. We also showed that in the absence of CCR7-dependent medulla migration, mature thymocytes are incapable of acquiring tolerance to lacrimal and salivary glands and are potent in inducing autoimmune exocrinopathy similar to Sjögren's syndrome.

### Discussion & Summary

The present study showed that the developmental kinetics and the thymic export of mature thymocytes were undisturbed in mice lacking CCR7 or CCR7L. However, the thymocytes that were generated in the absence of CCR7 or CCR7L were potent in causing autoimmune dacryoadenitis and sialadenitis in mice, and were thus incapable of establishing central tolerance to organ-specific antigens. These results indicate that CCR7-mediated cortex-to-medulla migration of thymocytes is essential for establishing central tolerance rather than for supporting the maturation or

export of thymocytes.

## Figures & Tables



Autoimmune dacryoadenitis in mice deficient for CCR7L (PLT/PLT)

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2) Medical Sciences

2-2) Central Nervous System

## Uterine sensitization-associated gene-1 (USAG-1), a novel BMP antagonist expressed in the kidney, accelerates tubular injuries

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### Introduction

Dialysis dependency is one of the leading causes of morbidity and mortality in the world, and once end-stage renal disease develops, it cannot be reversed by currently available therapy. Although the administration of large dose of bone morphogenetic protein-7 (BMP-7) has been shown to repair established renal injuries and improves renal function, regulatory mechanism of endogenous BMP-7 remains elusive.

### Results

Here we show that uterine sensitization-associated gene-1 (USAG-1), novel BMP antagonist abundantly expressed in the kidney, is the central negative regulator of BMP function in the kidney, and that USAG-1 deficient mice (*USAG-1*<sup>-/-</sup> mice) are resistant to kidney injuries. *USAG-1*<sup>-/-</sup> mice exhibited prolonged survival and preserved renal function in acute and chronic renal injuries. Renal BMP signaling, assessed by phosphorylation of Smad proteins, is significantly enhanced in *USAG-1*<sup>-/-</sup> mice during renal injury, and the administration of neutralizing antibody against BMP-7 abolished reno-protection in *USAG-1*<sup>-/-</sup> mice, indicating that renoprotection in *USAG-1*<sup>-/-</sup> mice was at least in part attributed to the enhancement of BMP-7 function. Furthermore, expression analysis of BMP antagonists in the kidney revealed that USAG-1 was the most abundant BMP antagonist in the kidney, and its expression overlapped with that of BMP-7. From these findings, we conclude that USAG-1 plays a critical role in the modulation of reno-protective action of BMP.

### Discussion & Summary

The results in the study justify the therapy targeted towards USAG-1: drugs or neutralizing antibodies that inhibits binding between USAG-1 and BMP, or gene-silencing therapy for USAG-1 would enhance the activities of endogenous BMP, and might be a promising way to develop novel therapeutic methods for severe renal diseases. Because the expression of USAG-1 is confined to the kidney in adult mice and humans, it would be a better target for therapeutic trials than the

administration of BMP-7 itself, whose target cells are widely distributed throughout the body.

## Figures & Tables

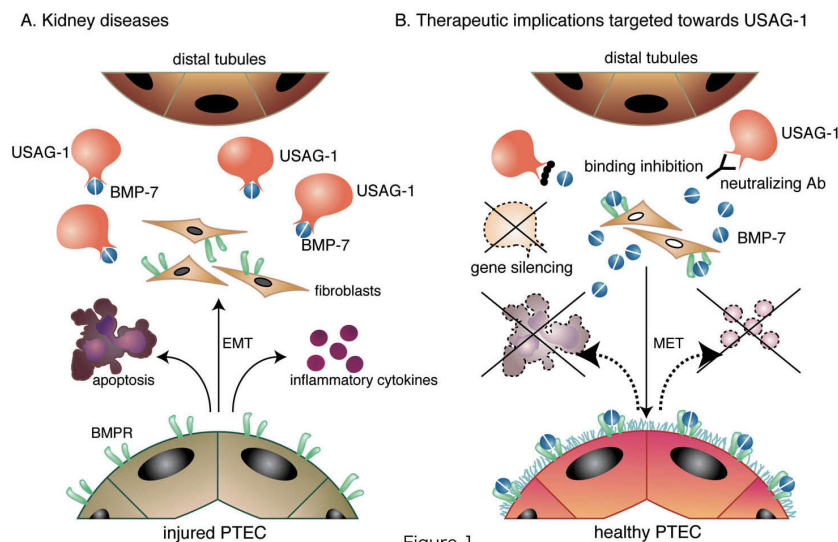


Figure 1

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USAG-1, a novel BMP antagonist abundantly expressed in the kidney, accelerates kidney injuries.  
J Clin Invest. 116: 70-9, 2006.
- 2 Motoko Yanagita  
Modulator of bone morphogenetic protein activity in the progression of kidney diseases.  
Kidney Int. Sep;70(6):989-93, 2006
- 3 Motoko Yanagita  
Uterine Sensitization Associated Gene-1 (USAG-1): A Bone Morphogenetic Protein Antagonist in the Kidney. (book chapter)  
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2) Medical Sciences  
 2-2) Central Nervous System

## Studies on the calcium-dependent mechanisms regulating the activity-dependent release of brain-derived neurotrophic factor (BDNF) at the presynaptic terminal.

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### Introduction

The brain-derived neurotrophic factor (BDNF), one of neurotrophins in the central nervous system, has been known to be involved in the hippocampus-dependent mechanisms of learning and memory. Here we investigated the mechanisms regulating BDNF release at the large mossy fiber presynaptic terminal of hippocampus. Our optical methods have to be improved in signal/noise ratio for further investigation.

### Results

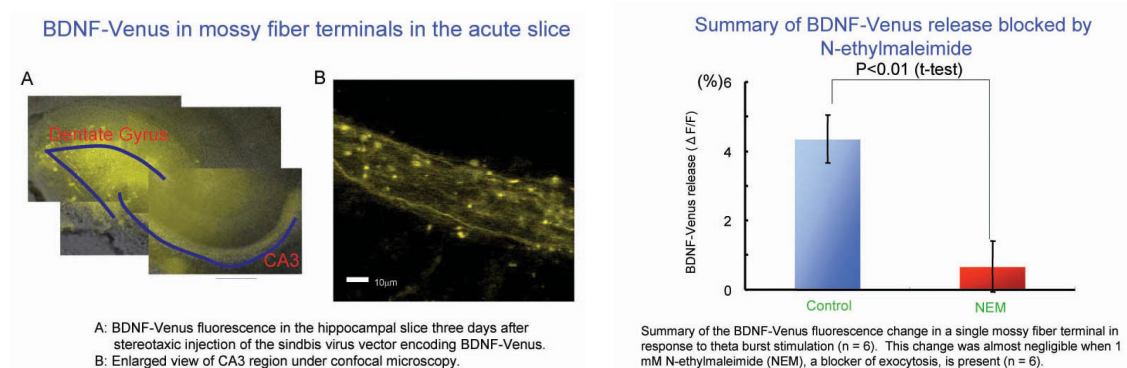
Two Sindbis virus vectors were conventionally made; one with a construct of a fusion protein of preproBDNF and Venus, one of green fluorescent protein derivatives,; the other with a Ds-Red derivative, mCherry (a generous gift from Dr. R. W. Tsien). A mixture of BDNF-Venus vector and mCherry vector were inoculated stereotactically in the dentate gyrus of mouse hippocampus (P18-25) under ketamine-xylazine anesthesia. After 2-3 days, BDNF-Venus was distributed at mossy fiber terminals which was morphologically detected by mCherry in the acute slice under confocal microscopy. Fluorescent intensity of BDNF-Venus (515 nm) and mCherry (630 nm) was measured simultaneously from a single mossy fiber terminal. By an electrical stimulation of mossy fiber, the fluorescence at 515 nm was reduced. But that at 630 nm was not. So the reduction of 515 nm fluorescence reflects BDNF release from the terminal. To remove synchronous signals derived from slice movement, 515/630 ratio was used as the BDNF release-specific signal. This response depended on extracellular calcium, and disappeared by either removing extracellular calcium or treating with N-methylmaleimide (NEM). We made a transgene plasmid which consists of CAG promoter sequence, floxed polyA-Neo and preproBDNF-Venus and obtained six mouse lines which have this transgene in their genomic DNA. The genetically recombinant mice were made with gamma1-Cre knockin mice which express cre recombinase specifically in the hippocampal CA3

pyramidal cells. However, none of six lines were not expressing BDNF-Venus phenotypically. It is suggested that the insertion of transgene may be inadequate for recombination.

## Discussion & Summary

These observations would provide first and direct evidences that BDNF is actually released from the giant mossy fiber boutons through activity-dependent exocytosis. It is suggested that BDNF is released from the MF terminals during induction of long-term potentiation (LTP). This study developed a optical methods to investigate directly the release of BDNF from individual mossy fiber presynaptic terminals of hippocampus. However, the methods have to be improved to reveal the mechanisms regulating BDNF release. Since the transgenic system appear to be ideal for improving the signal/noise ratio, several trials of foundation would be necessary to establish the line expressing BDNF-Venus stably. We are planning to generate the transgenic system which would conditionally express BDNF-Venus and mCherry.

## Figures & Tables



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2) Medical Sciences  
2-2) Central Nervous System

## A study of mechanisms which underlie activity-dependent neural circuit formation

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### Introduction

During development neural connections are formed by not only developmentally regulated guidance molecules but also neural activity such as firing and synaptic activity. Thalamocortical (TC) axon branching in the mammalian neocortex is a well-characterized system in which to investigate the activity-dependent mechanisms. The aim of this study is to reveal how TC axon branching is modified by neural activity by using organotypic cocultures of the thalamus and cortex.

### Results

TC axon branching in cocultures of the thalamus and cortex was examined by introducing enhanced yellow fluorescent protein (EYFP)-encoding plasmid into thalamic cells. Most of the individually distinguishable EYFP-labeled axons formed elaborate branches primarily in the target layer, layer 4 of cortical explants after 2 weeks *in vitro*. This indicates that TC axon branches are formed *in vitro* with laminar specificity resembling that found *in vivo*, in accordance with previous findings. To investigate whether electrical activity was generated in thalamic and cortical cells *in vitro*, firing activity was recorded by the multi-electrodes dishes that were implanted into culture dishes. Indeed, spontaneous firing was observed as single units and field potentials in both thalamic and cortical explants (Fig. 1), and was prominent at the later stages, when axonal branching were formed abundantly. This finding raises the possibility that spontaneous activity may affect axonal branching.

To test this possibility, cocultures were subjected to pharmacological treatment with blockers of sodium channels or glutamatergic synaptic transmission during the second week *in vitro*. As a result, TC axon branching was decreased in these treated cultures. Quantitative

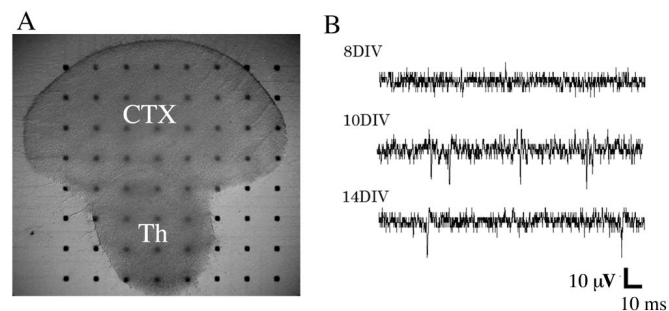


Fig. 1. Development of spontaneous activity of thalamic and cortical neurons.

analysis showed that in the presence of TTX, APV or DNQX, the number of branch points was decreased dramatically. Laminar specificity of axonal branching was also reduced under these treatments. To further examine what aspects of axonal branching were affected by the pharmacological treatments, the dynamics of TC axon branching was studied by time-lapse imaging in the presence or absence of these blockers. Over the observation period, TC axon arbors became larger and more complex. The average number of branch points and the total axon length increased gradually as development progressed, but the analysis of individual axonal branches revealed that TC axonal branches did not increase monotonically but remodeled actively. More than 80% of branches showed changes in length. Most added and elongating branches were enriched in the upper layers including layer 4. In contrast, the distribution of lost and retracting branches was more uniform over all cortical depths. In drug treated slices, the total number of branches was substantially decreased compared with the untreated group, but approximately 90% of the branches were dynamic exhibiting both growth and loss. The laminar distribution of growing and eliminating branches in the treated slices was nearly constant across all cortical layers. As development of axon branching can be attributed to the accumulated difference between the number of added or elongating branches and the number of retracting or lost branches, the differences per day were calculated for the target and non-target layers. In untreated cultures the change in the number of branch points was much higher in layer 4 than the deep layers. The difference in layer 4 was dramatically reduced as a result of drug treatment. On the other hand, we did not find that stable branches were more prevalent in the target layer under any conditions. Furthermore, growth motility was not different between the treated and untreated slices. Thus, our results demonstrate that neural activity is required for lamina-specific TC axon branching.

## Discussion & Summary

Our present results demonstrated that lamina-specific TC axon branching was formed when spontaneous firing activity increased in both thalamic and cortical neurons. The results further showed that TC axon branching was generated dynamically by branch addition and elimination with a preference toward branch accumulation in the upper layers. This is consistent with the previous finding that a branch-promoting molecule is expressed in the target layer, whereas a branch-inhibiting molecule is expressed in all cortical layers. Moreover, the pharmacological study clearly showed that lamina-specific branching with the remodeling process was reduced by blockade of firing or synaptic activity. These findings indicate that neural activity including synaptic transmission could play a role in regulating the proper expression of these branch-regulating molecules. Alternatively, activity might modify the expression and responsiveness of the receptors on growing TC axons or affect downstream signaling mechanisms in TC axons.

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- 2) Medical Sciences
- 2-3) Reproductive System

## Study of epigenetic pathophysiology and effective treatment of aged infertile oocytes

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### Introduction

Oocytes are one of the most sensitive reproductive systems for aging. To clarify molecular mechanism(s) of aging of oocytes, we are trying to estimate epigenetic modifications in detail. Our aims of this study is, 1. Study of molecular mechanisms for epigenetic modification in gametogenesis, and 2. Epigenetic estimation of aged oocytes.

### Results

1. Study of molecular mechanisms for epigenetic modification in gametogenesis.

Dnmt3L (DNA methyltransferase 3 like, a putative regulator of DNA methylation) is an essential molecule for region specific DNA methylation in gametogenesis. We screened candidate molecules which can interact with Dnmt3L by yeast two-hybrid method. Finally we got two genes and now biochemical assay and genetic assay with these candidate genes are proceeding.

2. Epigenetic estimation of aged oocytes.

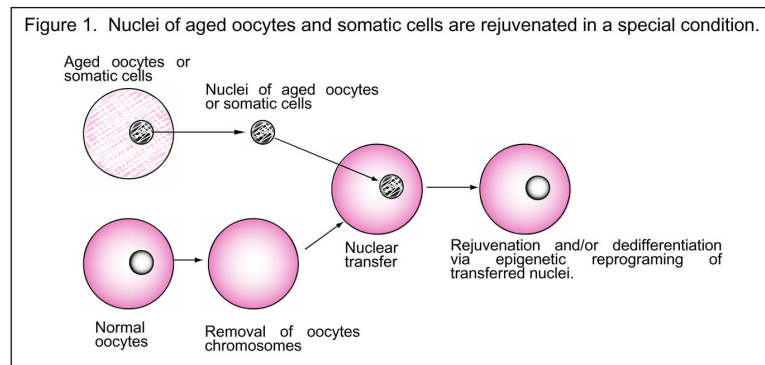
To perform DNA methylation assay with aged oocytes, we have been collecting aged oocytes from old female mice. Since only a few dozens of oocytes are collected from aged mice we still need time to prepare enough number of oocytes. Additionally, we could not get high quality DNA and RNA from small number of oocytes. This could be a constitutional problem of methods and we are trying to modify common methods..

### Discussion & Summary

Aged oocytes can be rescued by transfusion of cytoplasm of donated young oocytes (Figure 1.). Clone organisms have been produced by nuclear transfer of somatic cells. These evidences clearly show that nuclei of aged oocytes or somatic cells can be rejuvenated by unknown factors in cytoplasm of young oocytes. Epigenetic factors such as Dnmt3L, are decreased in aged oocytes and known as essential factors for early mouse development. These evidences strongly suggest that



Dnmt3L regulate region specific DNA methylation in oocytes. On the other hand, aged oocytes lose such epigenetic modification system(s) and aberrant DNA methylation may cause loss of early embryos. We continue to study epigenetic pathophysiology of aged oocytes.



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## 2) Medical Sciences

## 2-5) Cardiovascular Metabolic Endocrine

## Towards development of a novel therapy for atherosclerosis via the apoptosis inhibitory factor, AIM.

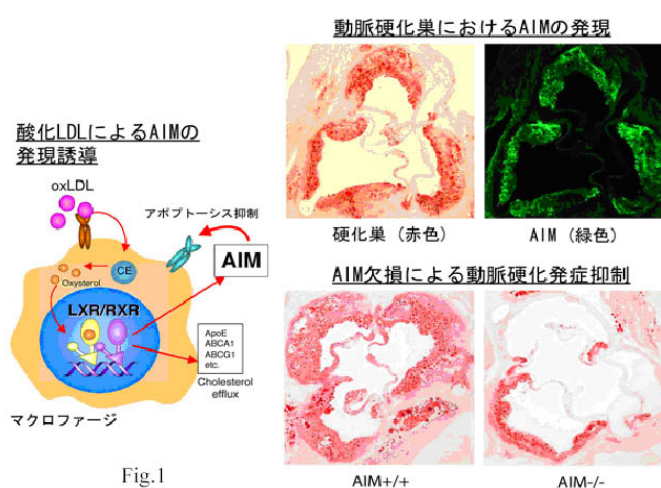
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### Introduction

Atherosclerosis, one of the most representative diseases for the Metabolic Syndrome, reveals the most frequent cause of humans' death in advanced nations (refs. 1, 2). So far, this disease has mainly and almost solely been regulated by controlling cholesterol levels in the body. Due to the recent drastic change of our life style, however, prevention of the increase of cholesterol in the body is becoming more and more difficult. Therefore, it is apparently needed to develop a novel therapy for atherosclerosis independent of control of cholesterol levels. Recently, we have shown that AIM, an apoptosis inhibitory protein, is restrictedly produced in lesional macrophages upon uptake of oxidized LDL (oxLDL), via activation of nuclear receptors LXR/RXR. This response supports macrophage survival and accumulation within the lesion, leading to overt atherosclerosis (Fig. 1). In AIM-deficient mice, lesional macrophages are far more susceptible to apoptosis induced by oxLDL, and in consequence, the lesion size is dramatically reduced compared to wild-type mice, after a challenge with high-fat diet (ref. 3).

Based on these results, our aim is to establish a therapeutic application of AIM-suppression for atherosclerosis in human. For this rationale, our specific aims are: (1) Development of functional anti-AIM antibodies; (2) Establishment of inducible AIM-deficient animals; and (3) Identification of AIM-receptor.



## Results

### (1) Development of functional anti-AIM antibodies:

We have generated recombinant mouse AIM protein (HA-tagged) of up to 2mg, through an antibody (anti-HA) column purification. By immunizing mice with the protein, we have established 25 independent anti-AIM monoclonal antibodies that capture native AIM protein. 15 out of 25 recognize both human and mouse AIM. By using these antibody clones, we first created an Elisa system for serum AIM detection (Fig. 2). Our system sensors AIM in human serum in a very sensitive fashion. We are now analyzing a large numbers of sera from patients who harbor various cardiovascular diseases. Secondly, we are now preparing mouse experiments to test possible therapeutic effect of these antibodies for atherosclerosis in mouse models. We will assess if multiple injections of antibodies could prevent the disease in LDLR-deficient animals with a high-fat diet.

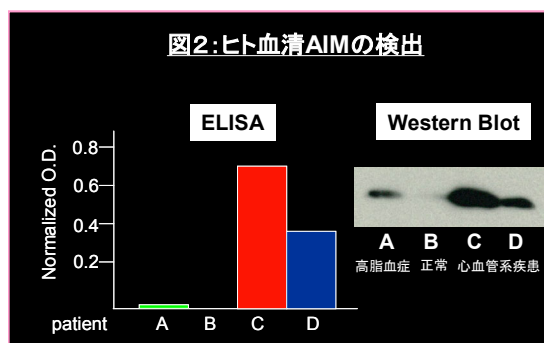


Fig.2

### (2) Establishment of inducible AIM-deficient animals:

A targeting vector was designed to generate a conditional AIM-deficient mouse line. By using the vector, we have obtained several clones of ES cells in which the AIM gene was successfully targeted via homologous recombination. The ES clones were transmitted into germ line of mice, and now the resulting offspring is cross bred with inducible Cre-transgenic mice and LDLR-deficient mice. After the breeding is completed, we will assess whether the shut-off of AIM gene is therapeutically efficient in mice that harbor different levels of atherosclerosis.

### (3) Identification of AIM-receptor:

We have established two types of cDNA expression libraries from mouse macrophage cell lines. By using the recombinant AIM protein described above, we will soon start screening these libraries for AIM-binding (AIM-receptor) protein(s).

## Discussion & Summary

During the grant year, we have achieved a reasonable progress in the proposed researches. Further efforts to complete the whole project will contribute in developing a new therapy for atherosclerosis via functional modulation of AIM, which will definitively be required in a near future. In addition, our Elisa system for serum AIM levels will be a useful diagnostic method for progression of atherosclerosis including early stages.

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2) Medical Sciences

2-5) Cardiovascular Metabolic Endocrine

## The role of RNA polymerase kinases on the pathogenesis of cardiac hypertrophy and heart failure

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### Introduction

The Cdk7/cyclin H/menage-a-trois 1 (MAT1) heterotrimer has proposed functions in transcription as the kinase component of basal transcription factor TFIID and is activated in adult hearts by Gq-, calcineurin-, and biomechanical stress-dependent pathways for hypertrophic growth. This study is designed to investigate the role of Cdk7/cyclin H/MAT-1 in cardiac growth and metabolism.

### Results

Using cardiac-specific Cre, we have ablated *MAT1* in myocardium. Despite reduced Cdk7 activity, MAT1-deficient hearts grew normally, but fatal heart failure ensued at 6–8 weeks.

By microarray profiling, quantitative RT-PCR, and western blotting at 4 weeks, genes for energy metabolism were found to be suppressed selectively, including targets of peroxisome proliferator-activated receptor  $\gamma$  coactivator 1 (PGC-1).

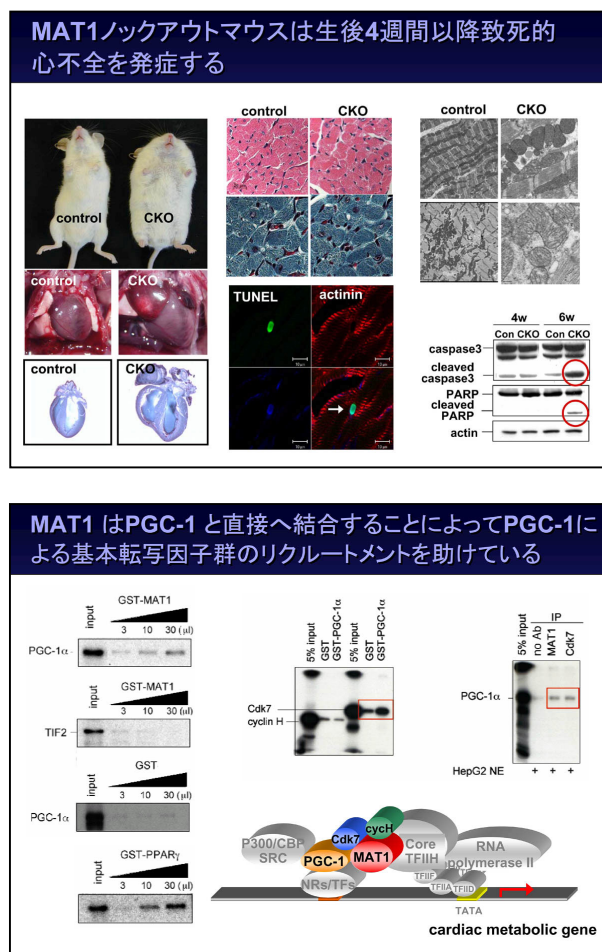
Cardiac metabolic defects were substantiated in isolated perfused hearts and isolated mitochondria. Consistent with the impairments in gene expression, we confirmed reduced enzyme activities of tricarboxylic acid cycle and respiratory chain complexes. In Langendorff-perfused hearts, oleate and glucose oxidation were reduced by at least 60% and 50%, respectively. Likewise, state III respiration and the respiratory control ratio were reduced in mitochondria from MAT1-deficient hearts, with little change in ADP/O.

In culture, deleting *MAT1* with Cre disrupted PGC-1 function: PGC-1 $\alpha$  failed to activate PGC-1-responsive promoters and nuclear receptors, GAL4-PGC-1 $\alpha$  was functionally defective, and PGC-1 $\beta$  was likewise deficient. PGC-1 bound to both MAT1 and Cdk7 in coprecipitation assays.

## Discussion & Summary

By cardiac-specific deletion of *MAT1*, we found that loss of MAT1 and the ensuing loss of TFIIH-associated Cdk7 activity result in a late-onset metabolic cardiomyopathy. We identified a block to the functional activity of PGC-1 $\alpha$  and PGC-1 $\beta$  in MAT1-deficient cells and showed that PGC-1 interacts physically with MAT1 and Cdk7. This block to metabolic gene expression via defective operation of the PGC-1 family implicates MAT1 in the control of cardiac metabolism, with deficient coactivation by PGC-1 the responsible molecular event.

## Figures & Tables



## References

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2) Medical Sciences

2-5) Cardiovascular Metabolic Endocrine

## Elucidation of transcriptional networks in metabolic syndrome and vascular disease

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### Introduction

Metabolic syndrome is an important risk factor for cardiovascular disease. However, exact mechanisms by which metabolic syndrome leads to atherogenesis are not clear. In the present study we analyzed the role played by the transcription factor KLF5, which we have previously shown to play an important role in tissue remodeling in the cardiovascular system, in metabolic stress response. We also analyzed the structural changes in adipose tissue obesity.

### Results

Previously we have shown that KLF5 heterozygous knockout (*KLF5*<sup>+/-</sup>) mice showed much reduced neointima formation in vascular disease models. The mice also exhibited reduced cardiac hypertrophy and fibrosis in the angiotensin II-induced hypertrophy model. Interestingly, KLF5 is also expressed in metabolic tissues, such as adipose tissue, skeletal muscle and pancreatic islets. In adipose tissue, we have shown that KLF5 is an essential component of the transcription factor network that controls adipocyte differentiation. KLF5 is induced by the transcription factors C/EBP $\beta$  and C/EBP $\delta$ , and once expressed it PPAR $\gamma$ <sub>2</sub> transactivates PPAR $\gamma$ <sub>2</sub> in concert with C/EBP $\beta$  and C/EBP $\delta$ , thus mediating the early and late differentiation programs. In the present study, to analyze KLF5's role in metabolic tissues in adult animals, we fed *KLF5*<sup>+/-</sup> mice with high-fat diet. *KLF5*<sup>+/-</sup> mice showed resistance to diet induced obesity and insulin resistance although they ate more food than wild-type mice, suggesting that *KLF5*<sup>+/-</sup> mice is protected from metabolic syndrome. The O<sub>2</sub> consumption was elevated in skeletal muscle of *KLF5*<sup>+/-</sup> mice, suggesting that enhanced energy expenditure partly explain their phenotypes. We also found that the structural integrity of pancreatic islets was maintained in *KLF5*<sup>+/-</sup> fed with high-fat diet, and the mice showed better insulin secretion, suggesting that KLF5 plays a role in stress response in pancreatic islets. These results demonstrate the KLF5 is important for response to metabolic stress. Given that KLF5 plays an essential role in pathogenesis of cardiovascular diseases, the results suggest that there are common molecular mechanisms that control response to various stresses in the cardiovascular and

metabolic systems. To address this we developed a novel living tissue imaging method and analyzed obesity of adipose tissue. We found that obesity of adipose tissue involves adipogenesis, angiogenesis, adipocyte death, and macrophage infiltration. We further addressed the role of angiogenesis in obesity by administering anti-VEGF antibody to db/db mice. The anti-VEGF antibody treatment inhibited not only angiogenesis but also adipogenesis, indicating that adipogenesis is closely coupled with angiogenesis in obesity of adipose tissue. Because adipogenesis takes place in the cell clusters and is coupled with angiogenesis, we designated these adipogenic cell clusters as adipo-/angiogenic cell clusters. The adipo-/angiogenic cell clusters consisted of differentiating adipocytes, blood vessels and lectin<sup>+</sup> CD68<sup>+</sup> small stromal cells. Adipo-/angiogenic cell clusters were most often found in 8-week-old db/db mice, and in elder mice, another type of cell clusters called crown-like structure were found. Crown-like structures contained macrophages engulfing dead adipocytes. As such, our live imaging demonstrated that obesity of adipose tissue involves dynamic interplays between adipocytes, endothelial cells, and stromal cells including macrophages and lectin<sup>+</sup> CD68<sup>+</sup> cells. The changes taking place in adipose tissue obesity can also be found in atherogenesis. Results of the present study strongly suggest that there are the common molecular mechanisms operating both in the cardiovascular and metabolic systems in obesity, metabolic syndrome, and atherosclerosis.

## Discussion & Summary

Our living tissue imaging demonstrated the dynamic structural changes in obesity of adipose tissue. The changes are similar to those found in atherosclerosis and can be considered as chronic inflammation (Fig. 2). These findings and the findings that KLF5 plays roles in stress responses in both cardiovascular and metabolic systems suggest common molecular mechanisms that operate in obesity and adipogenesis. KLF5 appears to be such a transcription factor that is involved in development of metabolic syndrome as well as atherosclerosis. Future studies on the common molecular mechanisms would elucidate how metabolic dysfunction in metabolic syndrome lead to atherosclerosis.



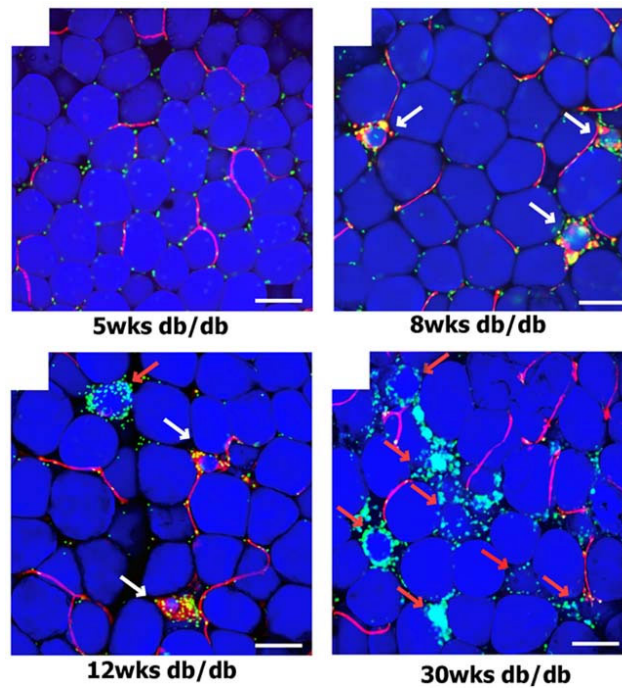


Fig.1 Adipo-/angiogenic cell clusters and crown-like structures  
White arrow, adipo-/angiogenic cell clusters.  
Red arrow, crown-like structures.

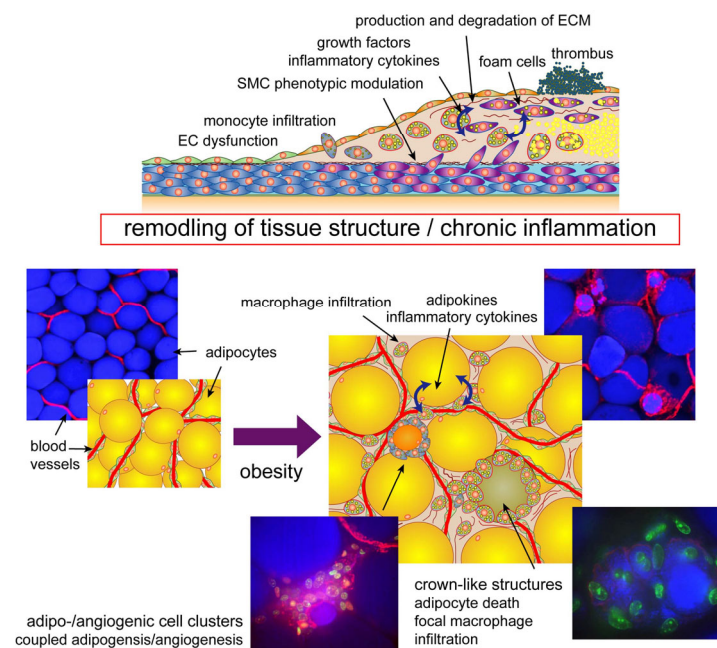


Fig.2 Obesity of adipose tissue and atherosclerosis

2) Medical Sciences  
2-6) Gastro-intestinal

## Role of IKK/NF-kappaB activation in inflammatory bowel diseases

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### Introduction

Inflammatory bowel diseases (IBD) are associated with the infiltration into the intestinal lamina propria of mononuclear cells, such as macrophages or lymphocytes, which overexpress the products of nuclear factor-kappa B (NF- $\kappa$ B) target genes, including the proinflammatory cytokines tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) and IL-6.

The present study was conducted to investigate the effect of specific inhibition of IKK $\beta$ -mediated NF- $\kappa$ B activation on murine colitis using the well-characterized NEMO-binding domain (NBD) peptide (1) and IKK $\beta$  knockout mice.

### Results

#### *Inhibition of IKK blocks LPS-induced NF- $\kappa$ B activity and proinflammatory cytokine expression*

Initially, we examined the effect of NBD peptide on NF- $\kappa$ B activation by LPS in J774.1 murine macrophages, which is the major cell type that accumulates in murine colitis. I $\kappa$ B $\alpha$  phosphorylation and degradation were observed after 15-30 min of LPS treatment, indicating that the NF- $\kappa$ B signaling pathway had been activated. The NBD peptide (10  $\mu$ M) inhibited the phosphorylation and degradation of I $\kappa$ B $\alpha$  by LPS treatment. NF- $\kappa$ B binding activity was also inhibited by NBD in the EMSA analysis (Figure 1). To explore the effect of NBD on LPS-mediated inflammatory cytokine expression in macrophages, we treated J774.1 cells with LPS with or without NBD peptide, collected the supernatant, and determined the IL-6, IL-1 $\beta$ , and TNF $\alpha$  concentrations by ELISA. The IL-6, IL-1 $\beta$ , and TNF $\alpha$  levels were markedly increased, and the wtNBD peptide reduced the concentrations of IL-6, IL-1 $\beta$ , and TNF $\alpha$  in dose-dependent manners. Almost same results were obtained in IKK $\beta$  knockout macrophages isolated from the *Ikk $\beta$ <sup>F/F</sup>* mice crossed with *Mx1-Cre* transgenic mice.

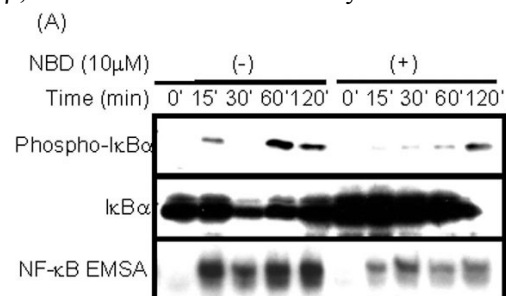


Fig.1

### *Effect of IKK blockade on DSS-induced colitis*

To determine the function of IKK $\beta$ /NF- $\kappa$ B in colitis, mice were given 3% DSS in their drinking water for 5 days with NBD peptide, and were monitored for weight loss, which is a characteristic of severe intestinal inflammation. After 5 days, the body weights of mice without NBD peptide started to decrease, until day 10 when they were sacrificed and analyzed; a 14% total decrease in body weight was recorded on Day 10. In contrast, the body weights of mice with the NBD peptide showed only a 5% total decrease on Day 8 and thereafter, they started to recover. Histologic analyses revealed that the severity and extent of inflammatory lesions in the colons of NBD peptide-treated mice were significantly ( $p < 0.05$ ) lower than in untreated mice, and that they had smaller areas of ulceration. Numerous macrophages had infiltrated in the DSS-mediated colitis mice, as determined by F4/80 immunostaining (Figure 2). After DSS exposure, IKK $\beta$ /NF- $\kappa$ B activity determined by phospho-I $\kappa$ B $\alpha$  and nuclear p65 immunostaining was found to have decreased in the colons of NBD peptide-treated mice compared to untreated mice. The amounts of cyclooxygenase-2 (Cox-2) and IL-6 protein, which are expressed in colitis, were significantly lower in the colons of NBD peptide-treated mice relative to their non-treated counterparts. Phospho-I $\kappa$ B $\alpha$ , nuclear p65, Cox-2, and IL-6 were predominantly expressed in F4/80-positive macrophages within inflammatory lesions. Mice treated with wtNBD peptide expressed lower amounts of mRNAs for the pro-inflammatory cytokines IL-6, IL-1 $\beta$ , and TNF $\alpha$  in their colons relative to untreated mice. Almost same results were obtained in IKK $\beta$  knockout mice generated by crossing *Ikk $\beta$ <sup>F/F</sup>* mice with *Mx1-Cre* transgenic mice.

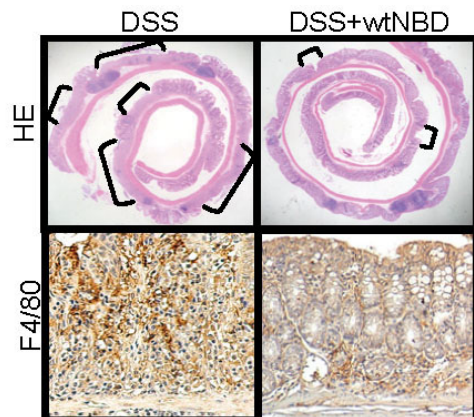


Fig.2

### *Treatment of TNBS-induced colitis by administration of NBD peptide*

We next examined the effects of the NBD peptides in another murine colitis model that involves the administration of TNBS in ethanol into the mouse rectum. Initially, we monitored the survival of mice and found that mice treated with the NBD peptide had a significantly increased survival rate (80%) relative to untreated mice (40%). Treatment with ethanol, which used as the TNBS vehicle, did not result in mortality. Whereas mice that received TNBS exhibited progressive weight loss, which is a characteristic of severe intestinal inflammation, those treated with the NBD peptide showed significantly less weight loss and started to recover on Day 4 after TNBS administration. Histologic analyses revealed that the severity and extent of inflammatory lesions in the colons of NBD peptide-treated mice were significantly ( $p < 0.05$ ) lower than in untreated mice, and that ulcerated areas were smaller than those seen in the DSS model. Mice treated with NBD peptide expressed lower levels of mRNAs for pro-inflammatory cytokines in their colons relative to Et-OH

treated mice. These results indicate that IKK $\beta$ /NF- $\kappa$ B activation is one of the critical regulators in TNBS colitis, and that blockade by NBD peptide may offer a new therapeutic approach for human IBD.

## Discussion & Summary

In the present study, we demonstrate that NBD peptide, which is a well-characterized IKK inhibitor, ameliorates colitis-related disease in two mouse models of IBD. Among the common cellular pathways activated in IBD, NF- $\kappa$ B is thought to be the most common pathway central to cell activation and the production of various inflammatory mediators, including a variety of cytokines. Thus, inhibition of NF- $\kappa$ B activity is a promising strategy for blocking inflammation. To date, several investigators have reported that the use of an NF- $\kappa$ B decoy with or without viral delivery is very effective for ameliorating murine IBD. Compared with other NF- $\kappa$ B inhibitors, the NBD peptide has the important advantage that it works upstream of the master kinase IKK in the cytoplasm. Since NF- $\kappa$ B is a dimer that consists of several NF- $\kappa$ B family members, inhibitors must be designed for several activation domains. In contrast, the NBD peptide blocks IKK activity, which results in the blockade of most NF- $\kappa$ B activity.

Many anti-inflammatory drugs for the treatment of IBD inhibit NF- $\kappa$ B activity, at least partially. Of these, glucocorticoids and 5-aminosalicylic acid are reported to be strong inhibitors of NF- $\kappa$ B activity, which is thought to be one of the reasons behind their efficacy. We have demonstrated that the NBD peptide, which can potently inhibit NF- $\kappa$ B activity, ameliorates murine colitis by preventing the expression of proinflammatory cytokines and may represent a novel therapeutic approach for IBD.

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2) Medical Sciences

2-7) Infection

## Carcinogenesis and progression of gastric cancer by infection of *Helicobacter pylori* in ASC-deficient mice

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### Introduction

ASC is an adaptor molecule originally found in apoptotic cells, and activates Caspase-1 responsible for the maturation of the pro-inflammatory cytokines IL-1 $\beta$  and IL-18. Thus, ASC plays an important role in innate immunity. *Helicobacter pylori* is now known to cause persistent gastritis and is linked to ulcer disease and gastric cancer. Cytokines are important in the host defense against microbial pathogens, including *H. pylori*. And *H. pylori* are known to induce IL-1 $\beta$ . Further more, IL-1 $\beta$  gene cluster polymorphisms is suspected of enhancing production of IL-1 $\beta$ , and is associated with increased risk of gastric cancer.

In this study we'll determine the role of ASC in the host defense against gastric colonization and carcinogenesis by *Helicobacter pylori* using ASC deficient mice.

### Results

Specific-pathogen-free 5 to 8 week-old male mice ASC.KO were inoculated intragastrically with mouse-adapted *H. pylori* isolate (Sydney Strain 1), and assessed for the degree of gastritis and bacterial load at 2 and 10 weeks after the last inoculation (n=6~10). Results were compared with those obtained from *H. pylori*-infected C57BL/6 mice.

Mice were orally inoculated with 0.5ml of *H. pylori* SS1 10<sup>8</sup> CFU/ml concentration 3 times at the intervals of 24 hours. Infection status in inoculated mice was assessed using the gastric biopsy urease test, microbial culture, and by immunohistology. Mice were classified as *H. pylori*-positive if they were positive by histology, culture or biopsy urease test.

#### 1) Colonization of *H. pylori* in wild type and ASC<sup>-/-</sup> mice

The number of colonized bacteria in the stomach was determined at 2 and 10 weeks after the last challenge. The number of *H. pylori* colonized in the stomach of ASC<sup>-/-</sup> mice tended to be higher than that of Wild-type mice, at 2 and 10 weeks after infection. Repeat of this experiment is under way to confirm statistical difference in colonization levels between ASC<sup>-/-</sup> and Wild type mice. This result suggests that ASC may play a protective role against *H. pylori* infection.

## 2) Histopathological examination of Wild type and ASC<sup>-/-</sup> Mice after infection.

To investigate the role of ASC in gastric inflammation induced by *H. pylori* infection, the histology of stomachs samples of Wild type and ASC.KO mice was examined after infection with *H. pylori* SS1. Two weeks after infection, more number of mononuclear and polynuclear neutrophils cells in the submucosa and the lamina propria was observed in ASC.KO mice than in wild-type mice, probably reflecting the nature resistant to programmed cell death in ASC<sup>-/-</sup> mononuclear and polynuclear neutrophils as compared with wild type cells. Ten weeks after infection(more chronically infected-state), strong infiltration of neutrophils and lymphoid follicles appeared in the submucosa and the lamina propria of wild-type mice,; in contrast in ASC.KO mice; only mild infiltration of inflammatory cells recognized as lymphocytes was observed. The existence of *H.pylori* was observed more frequently in ASC<sup>-/-</sup> mice than in wild type mice.

## 3 ) Cell viability under stressful conditions

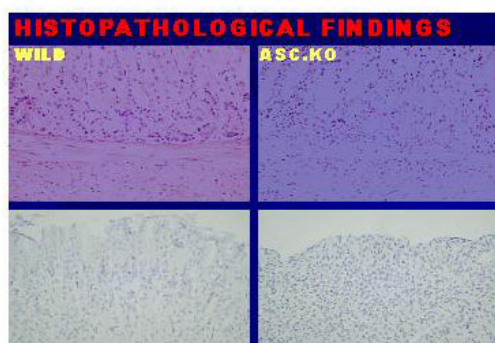
When cells were placed under various stressful conditions, such as starvation, wound, LPS treatment in vitro as well as in vivo, we observed that ASC<sup>-/-</sup> cells(smooth muscle cell, neural cell, cornea cells etc.) were resistant to cell death in association with enhanced autophagy(data not shown).

## Discussion & Summary

Our results of the current studies focused on *H. pylori*-ASC interactions showed that ASC deficiency significantly attenuates chronic inflammation, and increased colonization density, suggesting that ASC may contribute to increase severity of *H. pylori*-induced gastric inflammation, while also contributing to reduce bacterial colonization.

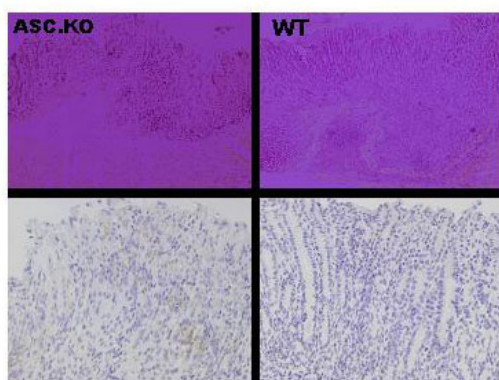
The results are consistent with the findings of higher gastric colonization by *H. pylori* but minimal gastritis in IFN- $\gamma$ <sup>-/-</sup> mice than in wild-type mice. The lowered IFN- $\gamma$  expression is observed in ASC.KO mice, because of the lack IL-18, a strong inducer of IFN- $\gamma$ . The hallmark of the gastric inflammatory response to *H. pylori* is its capacity to persist for decades, with increased risk of corpus atrophy, which is considered as a pre-malignant gastric lesion. It still remains to be clarified, however, whether loss of ASC protects *H. Pylori*-infected ASC.KO mice against carcinogenesis, due to the lower inflammation, or the loss of ASC promotes carcinogenesis, due to the lack of programmed cell death. To answer the above questions, further investigations, such as experiments using ras-transgenic ASC<sup>-/-</sup> mice, are under way.

## Figures & Tables

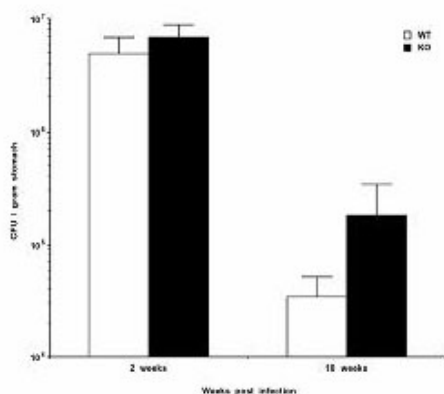


Pathology and immunohistological analysis of gastric mucosa of ASC-/- and Wild-type mice infected with SS1 strain of *H. pylori*.

**FIG-1:** Photomicrographs of H&E-stained and immuno-stained, by 2 weeks after infection. A massive infiltration of inflammatory cells (mostly neutrophils) in the submucosa and lamina propria of ASC.KO mice was observed, whereas only a mild inflammation was seen in Wild-type mice. Uniform moderate bacterial colonization was observed in all experimental groups by immunostaining; a small decline in bacterial colonization was observed in Wild-type mice as compared with ASC.KO mice (FIG-3).



**FIG-2:** By 10 weeks after infection, the bacterial load was significantly reduced in Wild-type mice. The reduced *H. pylori* colonization in Wild-type mice was histomorphologically associated with a strong infiltration of neutrophils and lymphocytes as compared with *H. pylori*-infected ASC.KO mice.



**FIG-3:** Higher bacterial load in the gastric mucosa of ASC.KO mice compared to wild-type C57/BL6 mice. Colony counts were determined from gastric biopsy material encompassing the entire length of the gastric mucosa. At 2 weeks, n=6 ASC.KO mice and n=10 C57BL/6 mice. At 10 weeks, n=6 ASC.KO mice and n=10 C57BL/6 mice. Data are expressed as mean  $\pm$  SD for each experimental group. Statistical studies between groups was determined by student's t-test.

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Guest editor: Michael McDermott (in press)

2) Medical Sciences

2-8) Oncology

## PGE<sub>2</sub>-mediated regulation of gastric epithelial differentiation and proliferation

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### Introduction

In gastric and intestinal cancer tissues, expression of COX-2 and mPGES-1 is induced, which results in production of PGE<sub>2</sub>. Epidemiological and experimental analyses have indicated that inhibition of COX-2 and PGE<sub>2</sub> pathways by treatment with inhibitors suppressed gastrointestinal carcinogenesis, indicating that PGE<sub>2</sub> plays a key role in tumor development. To investigate the role of PGE<sub>2</sub> in gastric epithelial differentiation and proliferation, we have constructed transgenic mice (*K19-C2mE* mice) expressing both COX-2 and mPGES-1 in gastric mucosa simultaneously. Here, we demonstrate that increased PGE<sub>2</sub> signaling impaired normal differentiation in the gastric mucosa of *K19-C2mE* mice.

### Results

We have generated *K19-C2mE* mice using keratin 19 (K19) gene promoter that is transcriptionally active in gastric epithelial cells including undifferentiated cells. In this mouse model, hyperplastic tumorous lesions were developed in the proximal glandular stomach, which was suppressed by treatment of the mice with a COX-2 selective inhibitor, NS-398. Histologically, tumorous lesions were consisting of well-differentiated epithelial cells with increased level of BrdU incorporations, indicating that these lesions were hyperplasia. Major components of such lesions were Alcian-blue positive mucous cells that were not found in the normal gastric mucosa. Moreover, inflammatory cell infiltration was found in submucosa of the hyperplasia and metaplasia lesions. These results indicate that increased PGE<sub>2</sub> signaling causes inflammation-associated hyperplasia and mucous metaplasia in the stomach.

In the glandular stomach of *K19-C2mE* mice, expression of proinflammatory cytokines, TNF- $\alpha$ , IL-1 $\beta$  and IL-6, is elevated significantly. Among these cytokines, it has been reported that TNF- $\alpha$  and IL-1 $\beta$  play important roles in carcinogen-induced tumorigenesis in the skin and hepatic melanoma metastasis, respectively. To investigate the roles of TNF- $\alpha$  and IL-1 $\beta$  in PGE<sub>2</sub>-induced inflammation and metaplastic hyperplasia, we crossed knockout mice of TNF- $\alpha$  (*Tnf*) and IL-1



receptor  $\alpha$  chain (*Il1r1*) genes with *K19-C2mE* mice to construct respective compound mutant mice, and examined gastric phenotypes at 20 weeks of age. In the control *K19-C2mE* mice, metaplastic hyperplasia was found in the proximal glandular stomach with inflammatory infiltration. However, hyperplastic growth was suppressed significantly in the *Tnf* ( $-/-$ ) *K19-C2mE* mice with reduced mucosal thickness to the wild-type level (Fig. 1). Histologically, inflammatory cell infiltration was not found in the submucosa of the *Tnf* ( $-/-$ ) *K19-C2mE* stomach. Consistently, mucous cell metaplasia was not detected in the compound mutant mice. On the other hand, IL-1 $\beta$  inhibition did not affect gastric phenotypes of *K19-C2mE* mice (Fig. 1). Accordingly, it is possible that TNF- $\alpha$ -associated inflammatory response is required for PGE<sub>2</sub>-induced hyperplasia and mucous cell metaplasia in the stomach.

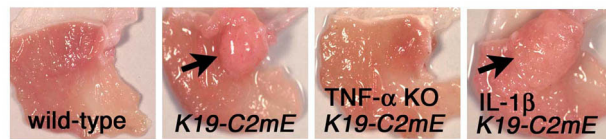


Figure 1

It has been demonstrated that TFF2/spasmodic polypeptide-expressing mucous metaplasia (SPEM) is associated with intestinal-type gastric cancer in human and *Helicobacter*-infected mice, suggesting that SPEM is a preneoplastic metaplastic lesion of gastric cancer. Because mucous cell metaplasia was also found in the *K19-C2mE* mouse stomach, we examined expression of TFF2 by RT-PCR and *in situ* hybridization. The mRNA level of TFF2 in the *K19-C2mE* gastric lesion was increased significantly compared with that of wild-type mice. Consistently, the number of TFF2-expressing cells was increased in the tumor tissues, while TFF2 positive cells were found only in neck region of wild-type (Fig. 2). Importantly, expression of TFF2 was found only in the gland neck of the *Tnf* ( $-/-$ ) *K19-C2mE* mice with reduced level of TFF2 mRNA, suggesting that TNF- $\alpha$ -associated inflammation causes SPEM formation with TFF2 positive epithelial cells (Fig. 2). Recently, it has been reported that MUC6 is also expressed in the TFF2 positive mucous metaplastic cells. In the *K19-C2mE* gastric lesions, MUC6 expression was also found in the TFF2 positive cells, which was suppressed in the *Tnf* ( $-/-$ ) *K19-C2mE* mice. Only mucous neck cells in the stomach are positive for both TFF2 and MUC6. Accordingly, our results suggest that TFF2+ MUC6+ double positive SPEM cells are derived from mucous neck cells.

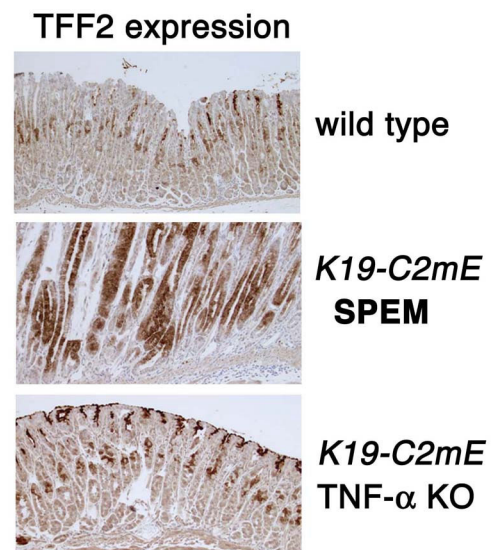


Figure 2

These results, taken together, indicate that increased PGE<sub>2</sub> signaling causes TNF- $\alpha$ -associated

inflammation, which impairs differentiation of gastric epithelial cells, resulting in development of preneoplastic SPEM lesions. Because SPEM cells are frequently found in the *H. pylori*-infected mice, and *H. pylori* infection induces PGE<sub>2</sub> production through induction of COX-2 and mPGES-1, the present results suggest that *H. pylori* infection plays a causal role for SPEM formation by induction of PGE<sub>2</sub> pathway.

## Discussion & Summary

We showed here that transgenic mice expressing both COX-2 and mPGES-1 (*K19-C2mE* mice) develop hyperplasia and mucous metaplasia associated with inflammatory responses. To further investigate possible roles of inflammatory cytokines in gastric tumorigenesis, we introduced knockout mutations for tumor necrosis factor (TNF)- $\alpha$  (*Tnf*) and interleukin (IL)-1 receptor  $\alpha$  chain (*Il1r1*) into *K19-C2mE* mice. *Tnf* ( $-/-$ ) *K19-C2mE* mice showed significant suppression of hyperplasia and metaplasia, while gastric lesions remained unaffected in compound mutants of *K19-C2mE* with *Il1r1*. Importantly, spasmolytic polypeptide/trefoil factor 2-expressing metaplasia (SPEM) in the *K19-C2mE* stomach was also suppressed in the *Tnf* ( $-/-$ ) *K19-C2mE* mice. These results indicate that TNF- $\alpha$ -dependent inflammation is responsible for PGE<sub>2</sub>-induced development of preneoplastic SPEM. Therefore, it is possible that inhibition of TNF- $\alpha$ -dependent inflammation together with eradication of *Helicobacter* is an effective prevention strategy for gastric cancer.

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2) Medical Sciences

2-8) Oncology

## Role of prostaglandin E in the growth and metastasis of cancer cells.

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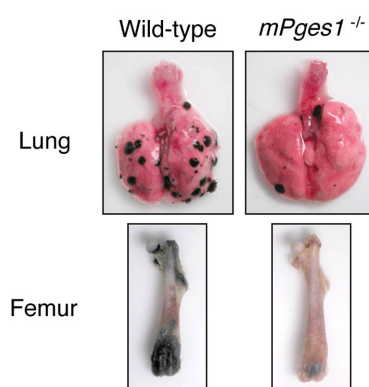
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### Introduction

Prostaglandin E2 (PGE2) has been implicated in tumorigenesis and tumor progression in several human cancers. Membrane-bound PGE synthase-1 (mPGES-1) is an inducible enzyme that regulates the PGE biosynthesis. However, the role of mPGES-1 in cancer growth and metastasis is not clear. Recently, we have reported that lipopolysaccharide-induced inflammatory bone loss is significantly attenuated in mPGES-1-deficient mice (*mPges1*<sup>-/-</sup>) [1]. In this study, we examined the role of PGE2 in cancer growth and metastasis, using *mPges1*<sup>-/-</sup>.

### Results

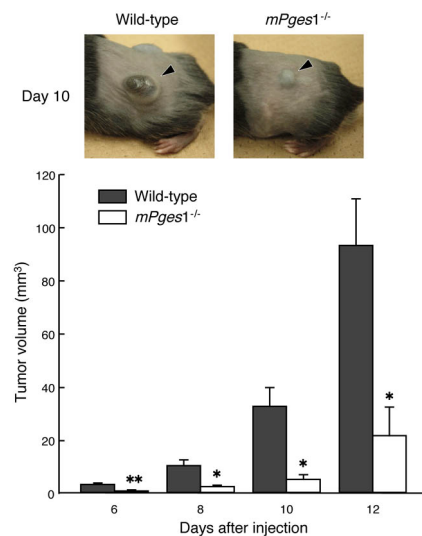
We first examined the role of PGE2 in cancer metastasis to lung and bone, using a cancer metastasis model. By the intravenous injection of mouse malignant melanoma B16 cells (B16) into wild-type mice (WT), a number of metastatic foci were detected as black in lung on day 18 after the injection. In *mPges1*<sup>-/-</sup>, lung metastasis was significantly attenuated compared with WT (Fig. 1). By the intravenous injection of B16 into WT, metastatic regions were detected as black in the distal femurs on day 18 after the injection. In *mPges1*<sup>-/-</sup>, the metastasis of B16 could be detected, but the metastasis area, shown as black, was markedly reduced compared with WT (Fig. 1). To examine the role of the PGE2 in osteolysis due to cancer metastasis, we measured the femoral bone mineral density (BMD) by dual energy X-ray absorptiometry. The BMD was significantly decreased by B16 metastasis in the femurs of the WT, but the femoral BMD did not decrease in *mPges1*<sup>-/-</sup> after the B16 injection. The levels of PGE2 in bone marrow supernatants collected from WT were elevated by the metastasis of B16. However, the



**Fig.1. Lung and bone metastasis of B16 melanoma cells is attenuated in *mPges1*<sup>-/-</sup> mice.**

PGE2 level in bone marrow supernatant was low and not elevated by the metastasis of B16 in *mPges1<sup>-/-</sup>*. These results suggested that the mPGES-1-dependent PGE2 production by host cells is critical for lung and bone metastasis and the osteolysis of cancer cells.

We next examined the role of PGE2 in tumorigenesis, using a subcutaneous tumor formation model. By the dorsal subcutaneous injection of B16 into WT, B16 tumor was formed and grown rapidly from day 6 to 12 (Fig. 2). In *mPges1<sup>-/-</sup>*, B16 tumor could form, but the growth rate of B16 tumor was significantly attenuated compared with WT from day 6 to 12 (Fig. 2). PGE2 did not influence the growth of B16 cells in vitro. Furthermore, we detected mPGES-1 and cyclooxygenase-2 (COX-2) mRNA in B16 tumor in vivo, but not cultured B16 cells in vitro. These results suggested that autocrine action of PGE2 in host cells is essential for the growth of B16 tumor in vivo.



**Fig.2. Subcutaneous tumor growth of B16 melanoma cells is attenuated in *mPges1<sup>-/-</sup>* mice.**

\*P<0.01, \*\*P<0.001 vs Wild-type

## Discussion & Summary

In the present study, we clearly showed the attenuation of cancer growth and metastasis in *mPges1<sup>-/-</sup>*. Therefore, mPGES-1-mediated PGE2 production has the essential roles in cancer growth, metastasis and cancer-induced osteolysis in bone. Recently we have reported that prostaglandin E receptor EP4 antagonist suppresses osteolysis due to cancer metastasis [2]. Therefore, mPGES-1-mediated PGE2 production by host osteoblasts and autocrine action of PGE2 via EP4 on host osteoblasts play an essential role in osteolysis due to cancer metastasis. Our study suggested that the inhibition of PGE synthesis and its signal pathways might provide a possible therapeutic approach to cancer metastasis of various tissues including bone.

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2) Medical Sciences

2-8) Oncology

## Functional analysis of various tumor suppressor genes *in vivo*.

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### Introduction

Suppression of cancer suppressor genes expression is frequently involved not only in various cancers but also in other non-cancerous diseases. We will try to analyze the functions of cancer suppressor genes such as PTEN, ING1, and cancer suppressor candidate PICT1, which stabilizes PTEN, by reverse genetics methods.

### Results

First of all, we had analyzed the functions of PTEN in bronchioalveolar epithelial cells. Because the PTEN<sup>-/-</sup> mice show early embryonic lethality (~E9.0), we turned to generated a bronchioalveolar epithelium-specific null mutation of *Pten* in mice [*SOPten*<sup>fllox/fllox</sup> mice] that was under the control of doxycycline. Ninety percent of *SOPten*<sup>fllox/fllox</sup> mice that received doxycycline *in utero* [*SOPten*<sup>fllox/fllox</sup> (E10-16)] died of hypoxia soon after birth. Most of the surviving *SOPten*<sup>fllox/fllox</sup> (E10-16) mice, as well as mice that received doxycycline postnatally [*SOPten*<sup>fllox/fllox</sup> (P21-27)], developed spontaneous lung adenocarcinomas. Urethane treatment accelerated the number and size of lung tumors that developed in *SOPten*<sup>fllox/fllox</sup> mice of both ages. Histological and biochemical examination of the lungs of *SOPten*<sup>fllox/fllox</sup> (E10-16) mice revealed hyperplasia of bronchioalveolar epithelial cells and mesenchymal cells such as myofibroblasts, enlarged alveolar epithelial cells, and impaired production of surfactant proteins. Thus, abnormal thickness of the blood-air barrier and reduction in the production of surfactant proteins caused by impaired alveolar epithelial cell differentiation may contribute to the respiratory failure of *SOPten*<sup>fllox/fllox</sup> (E10-16) embryos. In addition, numbers of bronchioalveolar stem cells (BASCs), putative initiators of lung adenocarcinomas, were increased. Lungs of both *SOPten*<sup>fllox/fllox</sup> (E10-16) and (P21-27) mice showed increased expression of Spry2, which inhibits the maturation of alveolar epithelial cells. Levels of Akt, c-Myc, Bcl-2 and Shh, which are shown to positively regulate stem cell self-renewal, were also elevated. Furthermore, *K-ras* was frequently mutated in adenocarcinomas observed in *SOPten*<sup>fllox/fllox</sup> (P21-27) lungs. The combined effects of *Pten* deficiency, *K-ras* mutation and perhaps other tumorigenic events may further increase the proliferation of BASCs such that they eventually initiate

lung adenocarcinoma formation. These results indicate that *Pten* is essential for both normal lung morphogenesis and the prevention of lung carcinogenesis, possibly because this tumor suppressor is required for BASC homeostasis (J.Clin. Invest. in revision).

PICT1 stabilizes PTEN protein and its expression is markedly reduced in many brain tumors. Suppression of PICT1 expression causes the instabilization of PTEN protein, and eventually causes the activation of Akt. Therefore PICT1 is thought to be a cancer suppressor candidate. We therefore generated null mutation of PICT1 in mice. PICT1-deficient ES cells show decreased expression of PTEN. However, they showed enhanced apoptosis and reduced cell growth. PICT1<sup>+/-</sup> mutant mice show resistant to carcinogen-induced tumors, and PICT1<sup>-/-</sup> mice show early embryonic lethality. These results suggest that PICT has another target besides PTEN and PICT1 is mainly involved in enhanced cell growth.

Finally, we had generated null mutation of ING1, a cancer suppressor genes which controls the expression of p53 and the chromatin remodeling, in mice. About one third of the ING<sup>-/-</sup> neonates are embryonic lethal but surviving mice began to develop malignant lymphomas at about one-year old age.

## Discussion & Summary

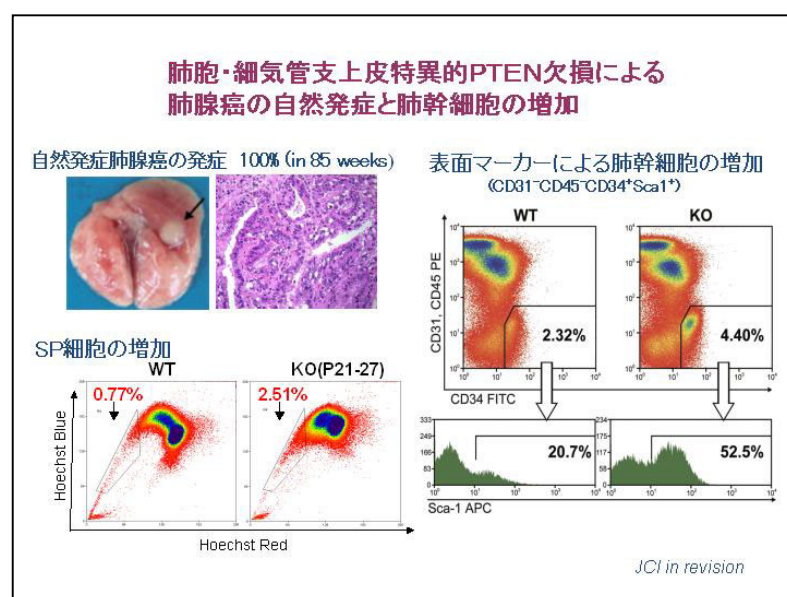
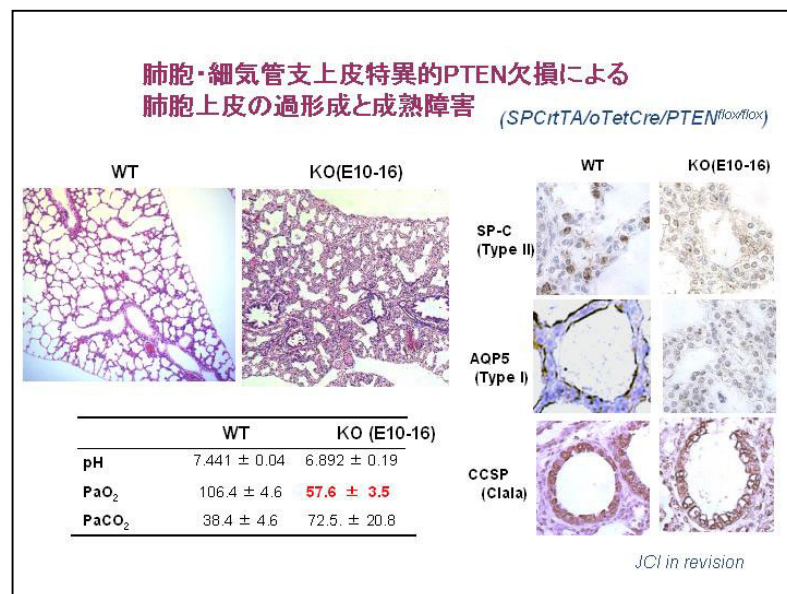
We had analyzed the function of cancer suppressor genes (PTEN and ING1) and cancer suppressor candidate gene (PICT1) in vivo by generating the null mutation of these genes in mice.

Especially using the bronchioalveolar epithelium-specific *Pten* mutant mice, we have demonstrated that an intact PI3K/*Pten*/Akt pathway in bronchioalveolar cells is essential for normal lung morphogenesis and BASC homeostasis as well as for the prevention of lung tumorigenesis. Our results suggest that the PI3K/PTEN/Akt pathway, particularly in BASCs and/or cancer stem cells, may be an attractive therapeutic target for the treatment of lung adenocarcinomas in humans.

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## Figures & Tables





## 2) Medical Sciences

## 2-8) Oncology

## Phenotypic analysis of RAD18-KO and RAD18, XPA double KO mice.

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### Introduction

Cellular DNA is continually damaged by intrinsic sources; reactive oxygen species and extrinsic sources; UV light irradiation. If these DNA damages are not repaired, accumulation of the DNA damages cause cellular death or carcinogenesis. Most DNA lesions are removed prior to DNA replication by the nucleotide excision, in which XPA gene is involved. On the other hand, unrepaired lesions encountered by the DNA replication machinery during S-phase cause replication fork stalling. RAD18 gene is involved in the resumption process, operationally defined as post-replication repair (PRR), and is characterized by re-initiation of DNA replication without removal of the lesion on a template strand

### Results

#### I . UV sensitivity and skin cancer formation of mice

We have prepared RAD18-Knockout mice. At first, we examined MED (minimal erythema dose) of wild-type mice, XPA-KO mice and RAD18KO mice. MED of wild-type mice, XPA-KO mice and RAD18KO mice are 2500, 400 and 2000 J/m<sup>2</sup>, respectively.

XPA-KO mice is known to form skin cancer by irradiation of 400-2000 J/m<sup>2</sup> of UV/day for 20 weeks. We examined whether RAD18-KO mice form skin cancer by the UV-irradiation. After 20 week UV-irradiation to the mice, no skin cancer was observed (Fig. 1). We next prepared XPA-RAD18 double knockout mice by mating. These mice appeared to grow normally. We expected that the formation of skin cancer due to XPA deficiency will be rescued by RAD18 deficiency. However, XPA-RAD18 double knockout mice showed almost same MED as that of XPA-KO mice and form sever ulcer even at low dose of UV (400J/m<sup>2</sup>). These results suggest that formation of skin cancer due to XPA deficiency was not rescued by RAD18 deficiency.

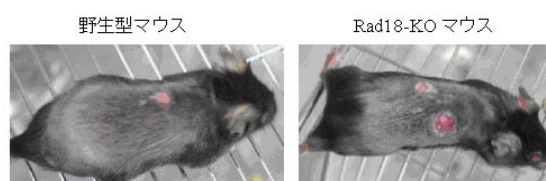


図1 マウスに対する UV照射試験



## II. Progressive degeneration of testicular stem cell of RAD18-KO mice by aging.

We have shown that testicular germ cells in RAD18-KO mice degenerate by aging (Fig. 2). By aging, the weight of testis became lesser and sperm number dramatically decreased at 1 year age. To explore role of RAD18 in testicular stem cell, we examined localization of RAD18 in testis. RAD18 specifically localized at spermatogonial cells.

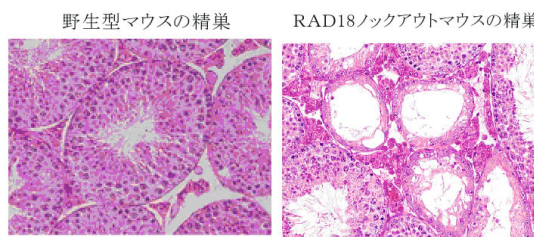


図2. RAD18欠損マウスは老化により、精巣が枯渇する。

## Discussion & Summary

RAD18 have pivotal role in post-replication repair. In yeast, RAD18 regulated various translesion polymerase, such as polymerase  $\eta$ , I and  $\zeta$ . Polymerase  $\eta$ , and  $\zeta$ . are thought to be involved in erro-free and error-prone DNA synthesis, respectively. RAD18 knockout ES cells showed lower induced mutation frequency as compared to that of wild-type (ref.) Thus, we expected that skin cancer formation in XPA-KO mice were suppressed by deficiency of RAD18. However, RAD18-XPA double knockout mice showed low MED and severe ulcer at low dose of UV irradiation. The mechanism of formation of skin cancer was dominant as compared to role of RAD18 in post-replication repair. Actually, aged testicular stem cells appeared to be removed from testis in RAD18KO mice, thus we speculated that RAD18 has a role to maintain genome integrity especially in stem cells.

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2) Medical Sciences

2-8) Oncology

## The role of *Helicobacter pylori* CagA-activated SHP-2 oncoprotein in gastric carcinogenesis

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### Introduction

*Helicobacter pylori* virulence factor CagA is directly injected from *H. pylori* into *H. pylori*-attached gastric epithelial cells via the bacterial type IV secretion system (TFSS) (1). *H. pylori*-injected CagA then undergoes tyrosine phosphorylation by Src family kinases and specifically binds and deregulates SHP-2 tyrosine phosphatase (2), gain-of-function mutations of which have been reported in a variety of human malignancies (3). The purpose of this study was to identify *in vivo* substrates of CagA-activated SHP-2, which contribute to gastric carcinogenesis.

### Results

CagA-deregulated SHP-2 induces cell-morphological transformation showing an extremely elongated cell-shape termed the hummingbird phenotype in gastric epithelial cells. Notably, hummingbird cells easily detach from the culture plate, indicating that CagA impairs the cell adhesion system. We thus decided to investigate the effect of CagA on focal adhesion kinase (FAK), which regulates cell adhesion by controlling the formation of focal adhesion spots. Expression of CagA in AGS human gastric epithelial cells resulted in reduced levels of FAK tyrosine phosphorylation. Decreased FAK phosphorylation was dependent on CagA tyrosine phosphorylation and required a CagA region, which mediates CagA-SHP-2 interaction, indicating a role of CagA-activated SHP-2 in the reduced levels of FAK tyrosine phosphorylation. Consistently, ectopic expression of a constitutively active SHP-2 in gastric epithelial cells resulted in the decrease in FAK phosphorylation levels. A substrate-trapping mutant of SHP-2 was capable of forming a stable complex with CagA. Furthermore, SHP-2 specifically dephosphorylated FAK in an *in vitro* phosphatase assay. From these results, we concluded that FAK is an *in vivo* substrate of CagA-activated SHP-2 phosphatase.

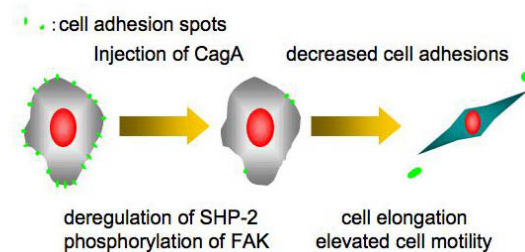
There are six potential tyrosine phosphorylation sites in FAK. To identify tyrosine residues that are dephosphorylated by SHP-2, we generated a series of the FAK mutants that lack various combinations of the potential tyrosine phosphorylation sites. Ectopic expression of these FAK

mutants revealed that FAK is phosphorylated on tyrosine-397, -576, -577 in gastric epithelial cells and CagA-activated SHP-2 dephosphorylated all of these tyrosine residues. Notably, phosphorylation of tyrosine-576 and -577, both of which are located on the activation loop of the kinase catalytic domain, is required for full activation of FAK kinase activity. This in turn indicated that FAK is inhibited when CagA-activated SHP-2 dephosphorylates it. Consistent with this idea, expression of CagA potentially downregulated FAK kinase activity in gastric epithelial cells.

The above observation raised the possibility that inhibition of FAK is responsible at least in part for the induction of the hummingbird phenotype. To address this possibility, we inhibited endogenous FAK kinase activity by expressing two distinct dominant-negative FAK mutants. As expected, expression of these mutants in AGS cells induced elongated cell-shape, which was highly reminiscent of the hummingbird cells induced by CagA. Conversely, expression of a constitutively active FAK mutant abolished the activity of CagA to induce the hummingbird phenotype. These observations collectively indicated that inhibition of the FAK kinase activity by CagA-activated SHP-2 plays an important role in the morphogenetic activity of CagA.

To consolidate inhibition of focal adhesions by CagA, CagA-expressing cells were stained with an anti-phospho-FAK antibody, which specifically recognizes the active form of FAK and found that focal adhesion spots containing active FAK were strongly reduced in cells expressing CagA. Intriguingly, there still remained a small amount of active FAK molecules, which are specifically enriched at the tips of the membrane protrusions, in CagA-expressing cells with the hummingbird phenotype. This observation indicated that a specific compartmentalization of active FAK, which has escaped from CagA-stimulated SHP-2, may promote assembly of new focal adhesion complexes that generate precursor sites for membrane protrusions. Such a polarized localization of active FAK may also be important for a single cell to move from one place to another with a small number of focal adhesions (Figure 1).

Figure 1 A model for the induction of the hummingbird cells by CagA



## Discussion & Summary

In this work, we identified FAK as an *in vivo* substrate of CagA-activated SHP-2 phosphatase. FAK is a legitimate downstream target of CagA because it plays pivotal roles in cell adhesion and

cell morphology as well as cell motility. Two lines of evidence support the idea that reduced FAK activity plays a role in the morphogenetic activity of CagA. First, a constitutively active mutant of FAK, which exhibits phosphorylation-independent enhanced kinase activity, abolished induction of the hummingbird phenotype by CagA. Second, ectopic expression of dominant-negative FAK was capable of inducing hummingbird-like phenotype. Although many studies have implicated FAK as a positive regulator of cell motility in response to integrin signaling, recent studies have also demonstrated that downregulation of FAK activity plays an important role in growth factor-induced changes in cell morphology and cell movement. Impaired cell adhesion and increased motility by CagA may be involved in promotion of gastric carcinogenesis caused by *cagA*-positive *H. pylori* infection.

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2) Medical Sciences

2-8) Oncology

## Isolation of sympathetic neural crest stem cells and neuroblastoma cancer stem cells and the application for neuroblastoma research.

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### Introduction

Neuroblastoma, one of the common solid cancers in child, arises from sympathetic nerve derived from trunk neural crest. Recently, the neural crest stem cells have been identified in sciatic nerve and enteric nerve in rat embryo whose cell surface immunophenotype were p75<sup>+</sup>/p0<sup>-</sup> and p75<sup>+</sup>/alpha 4 integrin<sup>+</sup>, respectively. But, it has been still unknown the relationship between neuroblastoma and neural crest stem cells. We first try to identify the neural crest stem cells in the sympathetic nerve tissue to investigate whether or not, the neural crest stem cells can be the source of neuroblastoma.

### Results

Adrenal gland that contains the largest sympathetic ganglion of rat postnatal day 0 was digested in collagenase 4 and trypsin-EDTA solution in order to disaggregate into single cell. The single cell suspension was incubated with the monoclonal antibody against p75 (low affinity nerve growth factor receptor) and followed by incubation with FITC conjugated anti-mouse antibody. One hundred P75 positive cells per each well were isolated by FACS (fluorescence activated cell sorting) and grown in the self-renewal media which contained rich chicken embryo extract, insulin-growth factor 1 and basic fibroblast growth factor on the fibronectin coated dish under the hypoxic condition (6% CO<sub>2</sub> and 1% O<sub>2</sub>) for seven days. They were incubated in the differentiation media containing lower concentration of growth factor and no chicken embryo extract for another five days to get them differentiated. Plating efficiency was very low and around 3%. The colonies were fixed by 4% paraformaldehyde and immunocytochemically stained using the antibodies against peripherin which is a specific marker for peripheral neurons and GFAP (glial fibrillary acidic protein) which is a specific marker for glia cells. We found that around one-third of colonies contained both peripherin positive neuron and GFAP positive glial cells. Two-third colonies contained only glial cells. Around 1% of p75 positive cells was sympathetic neural crest stem cells. In addition to adherent culture, we performed floating neurosphere culture to make sure the existence of neural crest stem cells in sympathetic nerve tissue. Ten thousand P75 positive cells per each well were grown in the

self-renewal media for floating culture for ten days. The number of neurosphere was around five (The efficiency was around 0.05%). The floating neurospheres were put on the fibronectin coated for adherent culture in the differentiation media for five days and followed by immunocytochemical analysis using both anti-peripherin and anti-GFAP antibodies. We found the differentiated primary neurospheres contained both neuron and glia. A couple of floating primary neurospheres were digested by trypsin-EDTA to disaggregate and incubated in the self-renewal media for ten days and in the differentiation media for another five days and followed by immunocytochemical analysis using both anti-peripherin and anti-GFAP antibodies. We revealed secondary neurospheres gave rise to both neuron and glia as same as primary neurospheres.

## Discussion & Summary

We concluded that some p75 positive cells in the sympathetic nerve were neural crest stem cells. Since the plating efficiency of the adherent culture for sympathetic neural crest stem cells was very low compared to those of neural crest stem cells derived from sciatic nerve and enteric nerve, we have been trying to increase the purity of sympathetic neural crest stem cells using various antibodies, such as CD9, CD81, c-ret and HNK-1.

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2) Medical Sciences

2-14) Others

## Role of protein kinase CK2 in the treatment of glomerulonephritis.

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### Introduction

Glomerulonephritis (GN) is a progressive inflammation that may be caused by a variety of underlying disorders. It is the primary cause of chronic renal failure and end-stage renal disease, which require dialysis and transplantation worldwide. Immunosuppressive therapy has been used to treat GN clinically, but this treatment has had insufficient therapeutic effects. We show that protein kinase CK2 is a key molecule in the progression of GN. cDNA microarray analysis identified CK2 $\alpha$ , the catalytic subunit of CK2, as a GN-related, differentially expressed gene. Our results show that CK2 plays a critical role in the progression of immunogenic renal injury, and therefore, CK2 is a potential target for GN therapy.

### Results

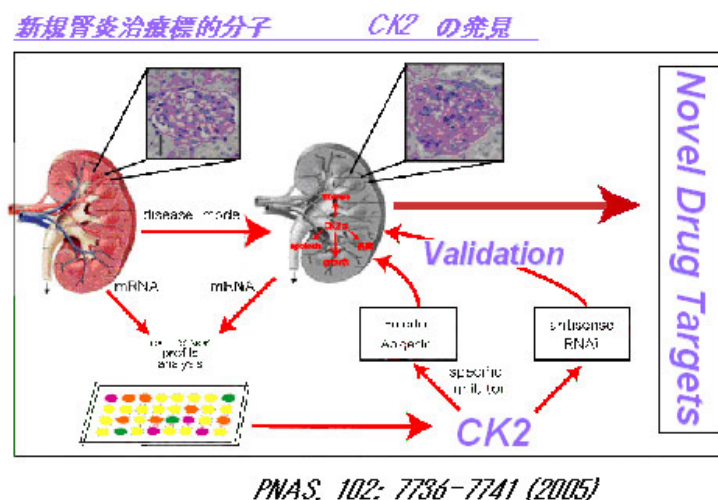
Expression profiling was carried out by using mRNA from the renal cortex of anti-GBM GN or control rats on day 28 and cDNA microarrays enriched for clones representing rat kidney genes. We selected 43 of 3,000 cDNAs that were examined, in which the expression levels differed by >2-fold intensity from controls. The expression of 29 genes, including CK2  $\alpha$ , TGF  $\beta$  1, osteopontin, and collagen IV  $\alpha$  1 were up-regulated, whereas the expression of 14 genes, including pendrin and organic anion transporter 1, were down-regulated. Expression profiling performed in the renal cortex of prednisolone-treated anti-GBM GN rats showed that 18 up-regulated and 7 down-regulated GN-related genes, respectively, were repressed by prednisolone treatment. TGF  $\beta$  1, osteopontin, collagen IV  $\alpha$  1, pendrin, and organic anion transporter 1 were previously reported as genes for which expression levels change during the development of renal disease. Real-time RT-PCR analysis on these genes further verified that the microarray data accurately represented gene expression in anti-GBM GN rats. Among the differentially expressed genes, we focused on one gene, CK2  $\alpha$ , that was overexpressed in the anti-GBM GN rats. Furthermore, the histologic evaluation was conducted on human renal biopsy specimens obtained from untreated lupus nephritis and IgA nephropathy patients. In all specimens examined, CK2  $\alpha$  was overexpressed in the glomeruli, and in some

cases, in the peritubular interstitium. Hence, overexpression of CK2  $\alpha$  appeared to be closely associated with glomerular injury not only in the GN animal models but also in GN patients. We further found that an antisenseDNA against CK2  $\alpha$  was therapeutically effective in anti-GBM GN rats. To provide mechanistic insight into the role of CK2 in GN, we examined *in vivo* the effect of CK2 inhibition on apoptosis, proliferation, inflammation, and fibrosis, all processes that are relevant to resolution and/or progression of GN. The results showed that the protective effects of CK2 inhibition in GN may result from its suppression of ERK-mediated cell proliferation, and its suppression of inflammatory, as well as fibrotic processes that are enhanced in GN; however, CK2 inhibition apparently does not result in increased apoptotic activity. Administration of either antisense oligodeoxynucleotide against CK2 $\alpha$  or low molecular weight CK2-specific inhibitors effectively prevented the progression of renal pathology in the rat GN models. The resolution of GN by CK2 inhibition may result from its suppression of extracellular signal-regulated kinase-mediated cell proliferation, and its suppression of inflammatory and fibrotic processes that are enhanced in GN.

## Discussion & Summary

We have isolated a GN-related gene, CK2, by microarray analysis performed on kidney cDNA from experimental GN model rats, and demonstrated that *in vivo* inhibition of the kinase ameliorates the renal dysfunction and histological progression. Because diverse insults can induce similar clinicopathologic presentations in GN, a marked overlap among downstream molecular and cellular responses has been suggested. Hence, pharmacologic agents that inhibit common underlying cellular mechanisms are expected to prove effective in treating glomerular diseases of diverse etiologies. Our present study indicates that CK2 could be an ideal therapeutic target for treating immunogenic GN.

## Figures & Tables





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2) Medical Sciences

2-14) Others

## Discovery of a protein involving in drug exclusion in the kidney; remedy for renal failure

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### Introduction

It was found that the newly discovered OATP-R is a gene that expresses only in human kidney, and that the protein produced by OATP-R is a membrane protein transporting various drugs from blood into the kidney.

The transporting protein has a function to take in drugs and substances of renal failure into the cell membrane or discharge them to the outside of the cell in the kidney. OATP-R was also confirmed as a transporting protein for digoxin, which is a drug used for the therapy of heart failure and arrhythmia. Digoxin is discharged from the kidney, and its discharge route was previously considered to be controlled mainly by multi-drug resistant MDR genes. OATP-R plays an important role in the uptake of digoxin from the blood to the kidney and controls its concentration in the blood, which shows the possibility of its application to therapy for kidney diseases. The aim of this study is to clarify the mechanism of OATP-R and to develop a new therapy for renal failure.

### Results

Tubular secretion of digoxin appears to be a major route of eliminating into urine. In the past, glomerular filtration was considered as a main elimination pathway, because digoxin excretion is correlated with glomerular filtration rate (GFR). However, co-administration of several drugs with digoxin (i.e. verapamil, quinidine, amiodarone etc.) decreases the renal excretion of digoxin without affecting GFR. Because digoxin secretion system is reported to be different from OAT and OCT systems, these data further suggest that other type(s) of molecule(s) should be involved in the basolateral transport of digoxin. We have recently isolated kidney specific organic anion transporter OATP-R and established MDCK cell line to characterize the pharmacological function. The establishment of OATP-R expressing cells leads to the characterization and development of a drug for renal failure.

In addition, we focused on the manipulation of genes in the proximal tubules. The proximal tubule works for maintaining body fluid homeostasis and drug metabolism. Although there are many

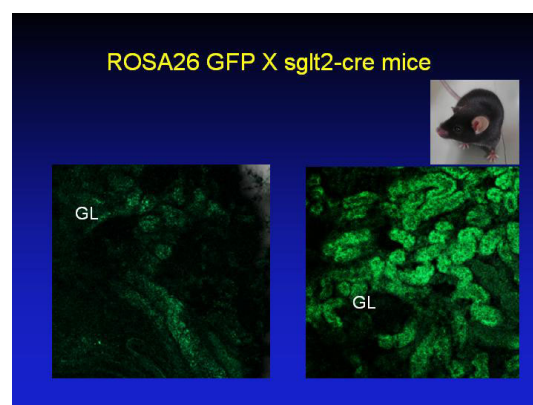
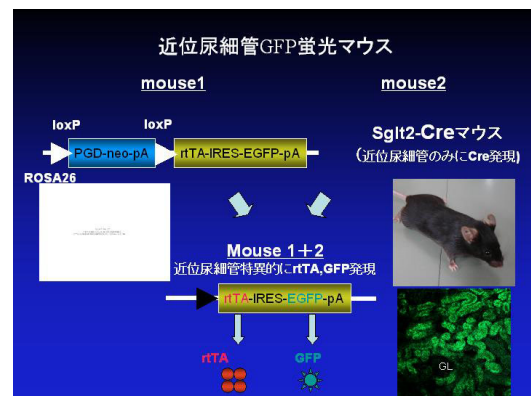
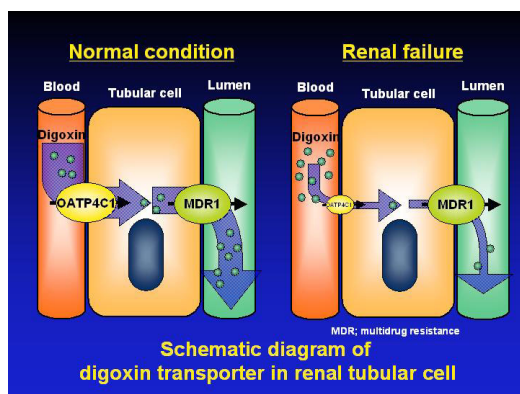
different channels or transporters in proximal tubule, which work for this aim, it remains unclear how they work *in vivo* on account of difficulty of separation and gene manipulation of intact proximal tubule. To resolve this problem, we made a new genetically modified mouse which express GFP and rtTA in proximal tubule specifically by mating ROSA-rtTA-GFP-loxP mouse and Sglt2-Cre mouse which has specific expression of Cre protein in proximal tubule. Thus, we will be able to use tet-on system to express any target gene in proximal tubule specifically under the presence of doxycycline by mating this new mouse and the other mouse which is inserted the target gene into the downstream area of tet-o gene. We would clarify the mechanism of OATP-R at proximal tubule *in vivo*.

## Discussion & Summary

The expression level of rat oatp-R is decreased in the renal failure model. This reduction might give one of the reasons for reduction of digoxin and renal failure substances excretion in the renal failure state.

The manipulation of OATP-R might play a key role to eliminate and be a clue to develop a new remedy for renal failure patients.

## Figures & Tables



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3) Chemical Sciences  
3-1) Organic Chemistry

## Studies on the Biomimetic Cascade Synthesis of Polycyclic Terpenoids

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### Introduction

Biomimetic polyene cyclization is an important key step in the concise total synthesis of polycyclic natural products. In particular, the Lewis acid-promoted diastereoselective cyclization of polyenic aldehyde acetals to 4 $\beta$ (axial)-alkoxypolycycles has been established by Johnson et al. However, excess SnCl<sub>4</sub> is often required as Lewis acid and there are no methods available for the synthesis of 4 $\alpha$ (equatorial)-alkoxypolycycles. We report here the SnCl<sub>4</sub> (10 mol%)-catalyzed polycyclization of homo(polyprenyl)arene analogues bearing terminal siloxyvinyl groups, which were much more reactive than other initiators such as acetals, aldehydes, and ketones

### Results

The  $\alpha$ (equatorial)/ $\beta$ (axial) selectivity of 4-siloxy group at polycycles could be controlled by the nucleophilicity of *pro*-C(9) and the steric effect of a silyl group.

The proposed mechanism is shown in **Figure 1**. 4 $\alpha$ -Selective cyclization of polyenic silyl enol ethers would concertedly proceed through antiperiplanar (chair–chair-like) transition state (TS) **1**. On the other hand, 4 $\beta$ -selective cyclization of polyenic silyl enol ethers would proceed stepwise through synclinal TS-**2** or **3** stabilized by Coulomb attractive interaction (minimalization of charge separation) between O and *pro*-C(10). A strong nucleophilicity of *pro*-C(9), a (6*E*)-geometry and a bulky silyl group would effectively favor TS-**1**, while a weak nucleophilicity of *pro*-C(9), a (6*Z*)-geometry and less steric hindrance of a silyl group would favor TS-**2** or **3**.

Based on the experimental results, two natural diterpenoids, 18-norabieta-8,11,13-trien-4-ol (**6**), which has antibacterial activity, and its epimer **11** were synthesized from **4** and **7** with >99% 4 $\alpha$  and >99% 4 $\beta$ , respectively (**Scheme 1**). The anti-herpes active diterpenoid **9**, a synthetic intermediate of **11**, was also synthesized with >99% ds.

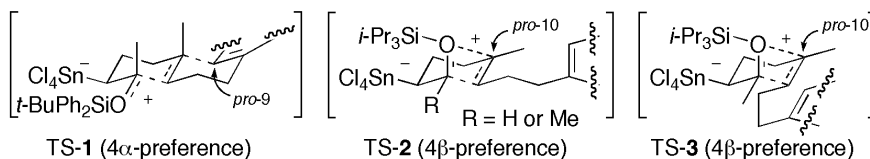
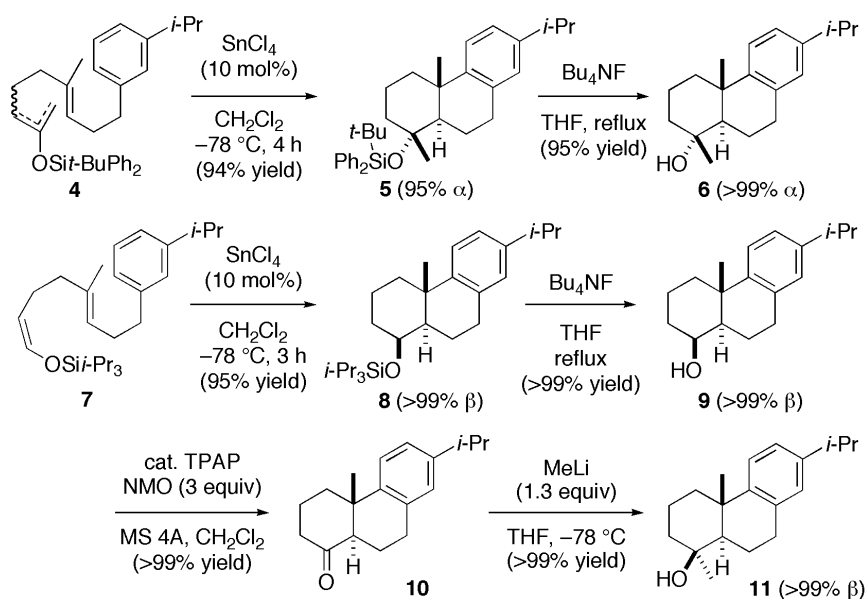


Figure 1. Proposed transition-state assemblies 19-21



Scheme 1. Total Syntheses of Epimers 6 and 11

## Discussion & Summary

Although it was difficult to directly generate silyloxocarbenium ion intermediates from aldehydes and ketones with silyl Lewis acids, we succeeded in their catalytic generation with  $\text{SnCl}_4$  from silyl enol ethers instead of carbonyl compounds. The present results demonstrate the synthetic advantages of using polyprenoid analogues bearing a terminal siloxyvinyl group as substrates of polyene cyclization with respect to both the reactivity and 4 $\alpha$ /4 $\beta$ -diastereocontrol.

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3) Chemical Sciences  
3-1) Organic Chemistry

## Development of Novel Asymmetric Reactions Based on Unprecedented Asymmetric Amplification

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### Introduction

The development of practical and efficient methods for construction of chiral molecules is an essential part of programs to explore new medicinal and agrochemical agents. We have used a strategy to design novel chiral system derived from metal salts of tartaric acid ester, in which two substrates bound to the metal centers might be ideally oriented and/or activated for subsequent reaction. Based on this proposal, we studied a nucleophilic addition of alkynylzinc reagents to acyclic nitrones, and unprecedented asymmetric amplification was realized.

### Results

The asymmetric addition of alkynylzinc reagents, prepared *in situ* from dimethylzinc and 1-alkynes, to acyclic nitrones was investigated by utilizing di(*t*-butyl) (*R,R*)-tartrate as a chiral auxiliary to afford the corresponding optically active (*R*)-*N*-(propargylic)hydroxylamines. In order to confirm how the reaction proceeded, the time course of the reaction was observed and the enhancement of enantioselectivity was found along the progress of the reaction. Based on this observation, unprecedented enhancement of enantioselectivity was realized by the addition of methylzinc salt of product-like hydroxylamine to afford the (*R*)-*N*-(propargylic)hydroxylamines up to 95% ee.

The catalytic version of the present reaction of alkynylzinc reagents was also achieved. The influence of the stereochemistry of the product-like additive was investigated. When the methylzinc salt of (*R*)- or (*S*)-product-like hydroxylamine was used as the additive instead of that of racemic product-like hydroxylamine, the enantioselectivity was surprisingly decreased. By the addition of methylzinc salt of the product-like racemic hydroxylamine, enantioselectivity of the *N*-(propargylic)hydroxylamines was again enhanced up to 96% ee.

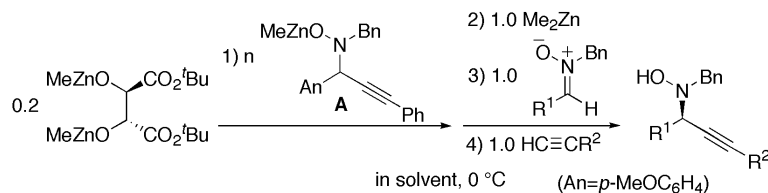
To the contrary, the asymmetric addition reaction of diphenylzinc to *C*-alkynyl nitrone was also investigated by utilizing di(*t*-butyl) (*R,R*)-tartrate as a chiral auxiliary. In this case, the corresponding (*S*)-*N*-(propargylic)hydroxylamines was obtained. By the addition of the same methylzinc salt of product-like hydroxylamine again realized enantiomeric enhancement.

Furthermore, in the catalytic version of this phenylation of *C*-alkynyl nitron was examined. Although chiral induction was very poor without the product-like additive, the presence of the product-like additive dramatically improved the enantioselectivity.

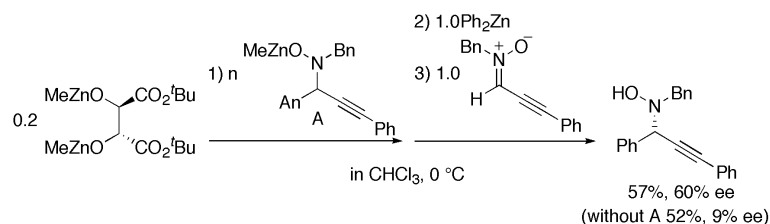
## Discussion & Summary

In conclusion, by the addition of a product-like racemic substrate, the asymmetric amplification was observed, and the corresponding *N*-(propargylic)hydroxylamines were obtained with excellent enantioselectivities. So far, the precise mechanism and the reason why such unprecedented enantiomeric enhancement was observed are not yet clear. The products *N*-(propargylic)hydroxylamines were found to be readily crystallized in racemic form rather than in enantiomerically pure form. In  $^1\text{H}$  NMR, chemical shifts of racemic *N*-(propargylic)hydroxylamines and enantiomerically pure ones are not same each other. These observation suggested that even the methylzinc salts of *N*-(propargylic)hydroxylamines readily dimerize in solution, which induced the formation of active chiral environment containing methylzinc salt of di(*t*-butyl) (*R,R*)-tartrate.

## Figures & Tables



Entry	R <sup>1</sup>	R <sup>2</sup>	A	<i>n</i>	solvent	Yield/%	ee/%
1	Ph	Ph	<i>racemic</i>	0.0	CH <sub>2</sub> Cl <sub>2</sub>	87	68
2			<i>racemic</i>	0.3	CH <sub>2</sub> Cl <sub>2</sub>	90	83
3			<i>R</i>	0.3	CH <sub>2</sub> Cl <sub>2</sub>	74	51
4			<i>S</i>	0.3	CH <sub>2</sub> Cl <sub>2</sub>	86	73
5			<i>racemic</i>	0.3	Et <sub>2</sub> O	89	83
6			<i>racemic</i>	0.3	toluene	92	91
7			<i>racemic</i>	0.3	EtPh	84	95
8	<i>p</i> -BrC <sub>6</sub> H <sub>4</sub>	Ph	<i>racemic</i>	0.3	EtPh	73	90
9	<i>o</i> -BrC <sub>6</sub> H <sub>4</sub>	Ph	<i>racemic</i>	0.3	EtPh	77	96
10	Ph	SiMe <sub>3</sub>	<i>racemic</i>	0.3	toluene	57	88





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3) Chemical Sciences  
3-1) Organic Chemistry

## Efficient Synthesis of Biologically Active Nitrogen-Containing Heterocyclic Compounds by the Carbocyanation Reactions

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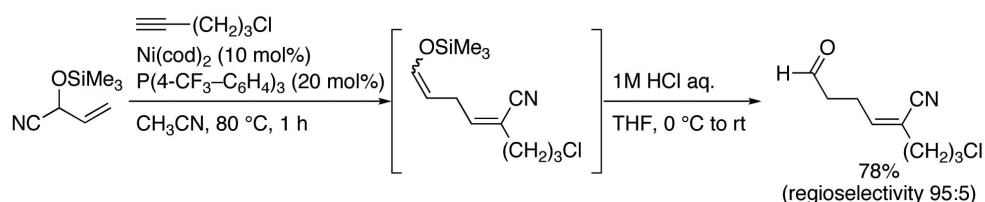
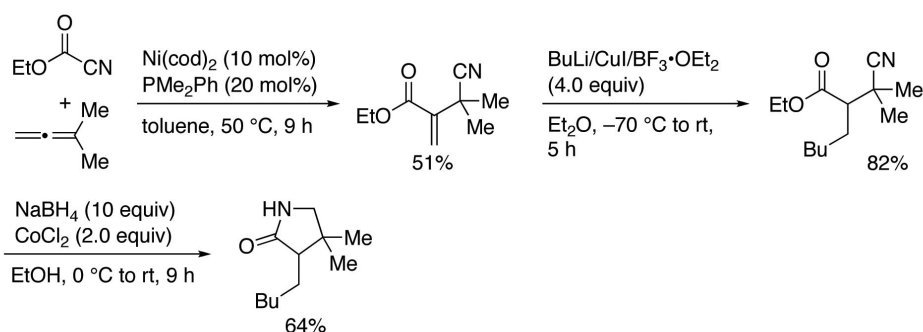
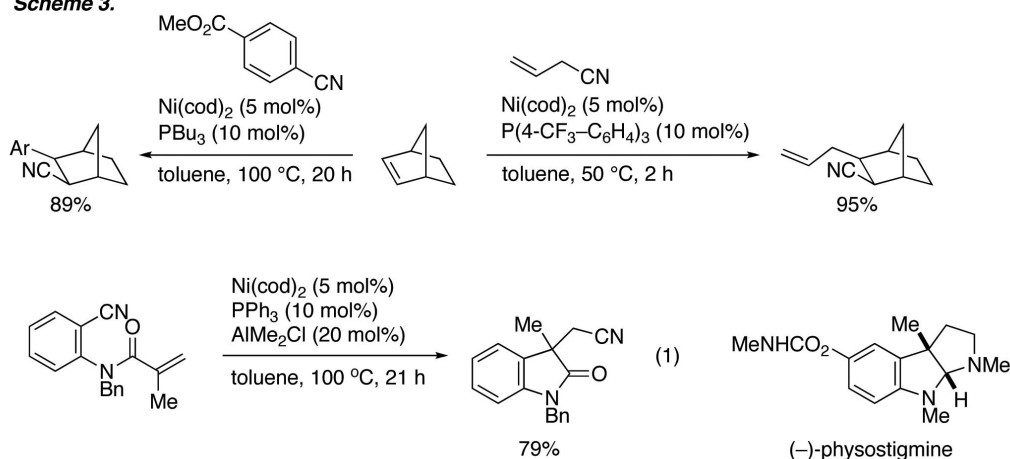
### Introduction

Direct cleavage of a R–CN bond followed by reconnection of both R and CN with two carbons in unsaturated carbon–carbon bonds, namely carbocyanation reaction,<sup>1</sup> provides ready access to highly functionalized nitriles from simple ones with perfect atom economy and, thus, should be of great synthetic value. The purpose of this research is to develop and apply the carbocyanation reaction to concise synthesis of nitrogen-containing heterocyclic compounds.

### Results

We have extensively studied the carbocyanation reaction in the last three years, and found for the first time that aryl cyanides add across alkynes in the presence of Ni/PMe<sub>3</sub> catalyst.<sup>1</sup> To expand the scope of nitriles applicable to the carbocyanation reaction, we first examined the addition of allyl cyanides across alkynes. After screening of several combinations of a metal catalyst and a ligand, we have found that the combination of Ni(cod)<sub>2</sub> and P(4-CF<sub>3</sub>–C<sub>6</sub>H<sub>4</sub>)<sub>3</sub> is optimum to achieve the allylcyanation reaction in highly stereo-, regio-, and chemoselective manners.<sup>2</sup> The addition of  $\alpha$ -siloxyallyl cyanides across alkynes proceeded at the  $\gamma$ -carbon exclusively to give aldehyde products after acidic hydrolysis (Scheme 1). It is worth noting that functional groups such as a C–Cl bond, which readily undergoes the oxidative addition to nickel(0), are tolerated under the present reaction conditions. We then turned our attention to the carbocyanation reaction across other unsaturated compounds, and found that 1,2-dienes undergo the addition of cyanoformate esters (Scheme 2).<sup>3</sup> For example, the addition of ethyl cyanoformate across 3-methyl-1,2-butadiene took place exclusively at the internal double bond in the presence of Ni/PMe<sub>2</sub>Ph catalyst to afford ethyl 2-(2-cyanoprop-2-yl)acrylate in 51% yield. The adduct underwent 1,4-addition reaction with butylcopper/BF<sub>3</sub>•OEt<sub>2</sub> to give the corresponding  $\beta$ -cyanoester, which further afforded a substituted  $\gamma$ -lactam, a versatile synthetic intermediate for five-membered nitrogen-containing heterocyclic compounds, upon treatment with NaBH<sub>4</sub> in the presence of CoCl<sub>2</sub>. Whereas the carbocyanation

reaction of simple 1-alkenes has been elusive due to competitive  $\beta$ -hydrogen elimination to give Heck-type adducts, norbornene and norbornadiene undergo the aryl- and allylcyanation reaction in a *cis*-fashion from the *exo*-face (Scheme 3).<sup>4</sup> On the other hand, intramolecular arylcyanation of alkenes works well, giving oxyindole product having a quaternary carbon (Eq. 1). The use of a catalytic amount of  $\text{AlMe}_2\text{Cl}$ , which would act as a Lewis acid cocatalyst to coordinate to the cyano group and promote the oxidative addition step,<sup>5</sup> is crucial for this particular reaction. This intramolecular reaction would be useful for the synthesis of alkaloids having a hexahydropyrrolo[2,3-*b*]indole structure such as (–)-physostigmine.

**Scheme 1.****Scheme 2.****Scheme 3.**

## Discussion & Summary

In summary, we have demonstrated several types of carbocyanation reactions of unsaturated compounds. The transformations described above have no piece of precedents and, thus, have introduced an important innovation in retro-synthetic analysis of target molecules. The reaction should find many applications to production of not only nitrogen-containing heterocycles but also fine chemicals, synthetic intermediates for pharmaceuticals, and monomers for functional polymers. Further efforts to expand the scope of the chemistry to other nitriles and unsaturated compounds including enantioselective version are ongoing subjects in our laboratories.

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3) Chemical Sciences  
3-1) Organic Chemistry

## Studies on the Synthesis and Biological Activity of Neurotoxins Produced by Marine Algae

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### Introduction

Polycyclic ethers, produced by marine algae, show potent neurotoxicity by binding to the ion channels and cause massive fish kills and human food poisoning. Since further studies are hampered by the limited availability from nature, chemical synthesis has been the sole realistic way to obtain sufficient amounts of the natural products. Moreover, the unusual structures of these compounds are particularly attractive targets for synthetic chemists.

### Results

Yessotoxin is a disulfated polycyclic ether isolated from the digestive glands of scallops, *Patinopecten yessoensis*.<sup>1</sup> Due to its novel structural features and biological activities, yessotoxin has attracted the attention of synthetic chemists. As a part of total synthetic study of yessotoxin, we investigated the stereocontrolled synthesis of the IJK ring segment. A cyclization precursor, having an aldehyde and acrylate moieties, was synthesized from 2-deoxyribose. SmI<sub>2</sub> mediated cyclization of the substrate gave the IJ ring system with high stereoselectivity in good yield. Further modification on the side chain was carried out to give an epoxy alcohol, which was subjected to the acid catalyzed cyclization to afford the IJK ring segment in a highly region- and stereocontrolled manner.<sup>2</sup>

Next we investigated the formal total synthesis of hemibrevetoxin B. This compound was isolated from the cultured cells of the red tide organism *Gymnodinium breve* by Shimizu in 1989.<sup>3</sup> The unique structural features have attracted the attention of synthetic chemists, and a number of strategies have been investigated. The synthesis was started from triacetyl glucal as achioral source, and the AB ring segment was prepared by known procedures including an acid catalyzed epoxy-alcohol cyclization. The C ring was constructed by the intramolecular allylation of  $\alpha$ -acetoxy ether prepared from the corresponding ester derivative. The D ring moiety was successfully prepared by the ring-closing olefin metathesis mediated by the second generation Grubbs catalyst. Finally, hydrogenation of the D ring olefin and removal of the protective group on

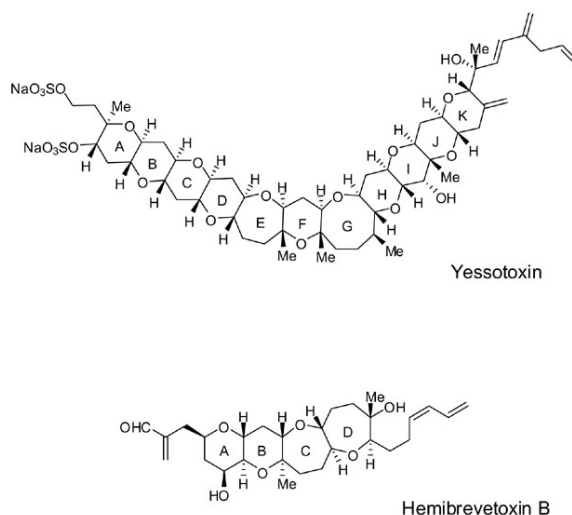
the side chain was performed with H<sub>2</sub>/Pd-C to give the known synthetic intermediate.<sup>4</sup>

## Discussion & Summary

In conclusion, we have achieved the synthesis of the IJK ring segment of yessotoxin via SmI<sub>2</sub> mediated cyclization and acid catalyzed cyclization as key ring-closure processes in highly stereocontrolled manner. The present work is the first example of the synthesis of the IJK ring segment of yessotoxin.

Furthermore, a formal total synthesis of hemibrevetoxin B was performed by the intramolecular allylation of an  $\alpha$ -chloroacetoxy ether and ring-closing metathesis as key reactions. The convergent strategy employed makes the synthesis highly efficient. Further application of these strategy to the synthesis of other marine natural products is in progress.

## Figures & Tables



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3) Chemical Sciences  
3-1) Organic Chemistry

## Novel Synthesis of N-Containing Heterocycles via Transition Metal-Catalyzed [2+2+2] Cocyclization

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### Introduction

A [2+2+2] cocyclization is one of the promising methodology for the synthesis of various aromatic compounds. Recently, we have reported a Pd-catalyzed [2+2+2] cocyclization of  $\alpha,\omega$ -diynes and arynes and its application to the synthesis of natural aryl-naphthalene lignans, such as taiwanins C, E, and dehydrosesoxypodophyllotoxin.<sup>1</sup> This project mainly focused on the development of a transition metal-catalyzed [2+2+2] cocyclization of  $\alpha,\omega$ -diynes and heteroarynes.

### Results

It is known that a heteroaryne such as 2,3-pyridine **2** or 3,4-pyridyne **4** can be produced *in situ* by treatment of the corresponding precursors **1** or **3** with CsF, respectively (Scheme 1).<sup>2</sup> The pyridines are homologues of the arynes such as a benzyne, and it is expected to have the unique property. However, there is only a few examples of Diels-Alder type reactions of 1,3-dienes and pyridines in the literature, and a [2+2+2] cocyclization of the pyridines has not been reported so far. First, we investigated a [2+2+2] cocyclization of  $\alpha,\omega$ -diynes **5** and 3,4-pyridynes **4** generated the precursors **3** under the various conditions. As the results, it was found that a nickel complex could promote the [2+2+2] cocyclization of diynes and 3,4-pyridynes affording isoquinoline derivatives in good yields (Table 1). In this reaction, an electron-donating substituent on the pyridine ring of the 3,4-pyridyne precursor was tolerated, and the reaction of diyne **5c** and **3b** or **3c** gave the corresponding substituted isoquinoline **6cb** or **6cc** in good yields, respectively. On the other hand, it was found that an electron-withdrawing substituent on the pyridine ring of the precursor retarded the [2+2+2] cocyclization. Thus, the reaction of **5c** and **3d** gave isoquinoline **6cd** in a moderate yield.

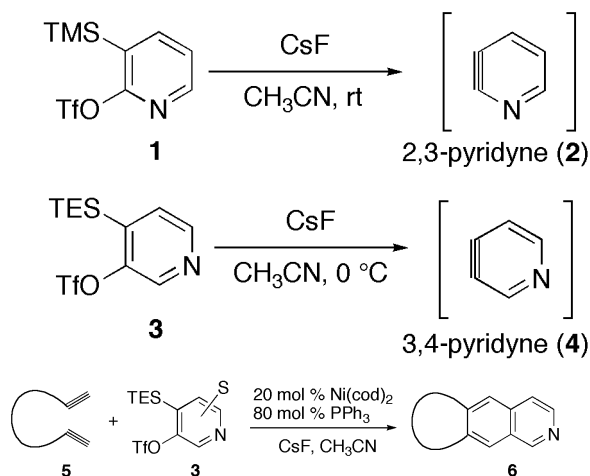
### Discussion & Summary

In summary, we succeeded in developing the novel nickel-catalyzed [2+2+2] cocyclization of  $\alpha,\omega$ -diynes and 3,4-pyridynes for the first time, affording isoquinoline derivatives in good yields.<sup>3</sup>

The present convergent strategy should pave the way for the novel synthesis of various isoquinoline derivatives by virtue of a variety of combinations of diynes and 3,4-pyridyne precursors.

## Figures & Tables

**Scheme 1.** Formation of Pyridynes



**Table 1.** [2+2+2] Cocyclization of various diynes and pyridyne precursors<sup>a</sup>

run	diyne	pyridyne precursor	product	yield (%)
1				49
2				63
3				61
4				57
5				20

<sup>a</sup> An CH<sub>3</sub>CN solution of diyne was added over a period of 3 h (syringe pump) to a mixture of pyridyne precursor (4 eq), Ni(cod)<sub>2</sub> (20 mol%), Ph<sub>3</sub>P (80 mol%), and CsF in CH<sub>3</sub>CN at room temperature.

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3) Chemical Sciences  
3-1) Organic Chemistry

## Development of External Stimulus-triggered Auto-processing Peptides

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### Introduction

Control of the function of peptides by external stimuli has been receiving increasing attention due to its potential utility in chemical biology. An external stimulus-induced peptide bond cleavage seems to allow peptides to be converted to their corresponding active (or inactive) forms. Therefore, we attempted to synthesize an auto-processing peptide which can be changed to other form by an external stimulus.

### Results

Inspired by the trimethyl lock system,<sup>1,2</sup> we designed stimulus-responsive peptide **1**, which can release a functional peptide after a stimulus-induced removal of a phenolic protective group (PG) and subsequent processing reaction (Scheme 1). Designed peptide **1** features nucleophilic involvement of a regenerated phenolic hydroxyl group to an adjacent peptide bond to release functional peptides. In this paper, we chose a photo-removable *o*-nitrobenzyl (*o*-NB) and phosphatase-removable phosphate group for phenolic protection to afford stimulus-responsive model peptides.

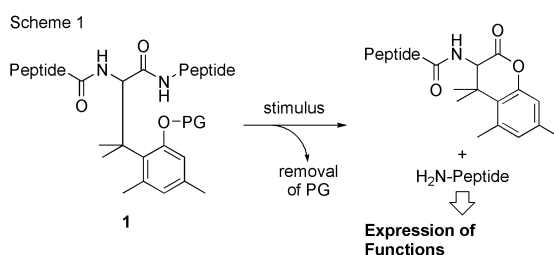
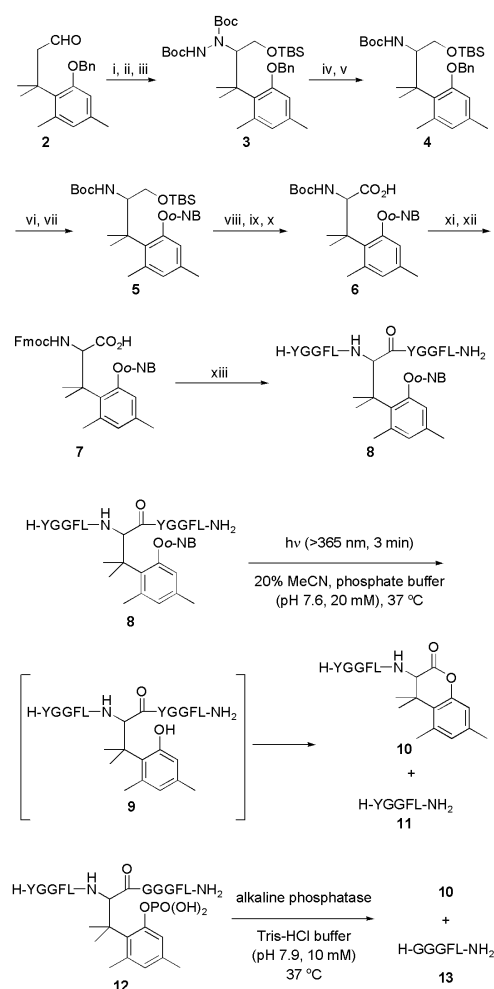


Photo-responsive model peptide **8** was synthesized as shown in Scheme 2. Aldehyde **2**<sup>[2]</sup> was  $\alpha$ -aminated with di-*tert*-butyl azodicarboxylate in the presence of pyrrolidine. After reduction of the aldehyde group with sodium borohydride, the resulting alcohol was protected with a TBS group to give silyl ether **3**. Trifluoroacetylation of the terminal nitrogen in **3** with trifluoroacetic anhydride (TFAA) and subsequent N-N bond cleavage by SmI<sub>2</sub> in the presence of HMPA and *tert*-BuOH gave

amino alcohol **4**. The benzyl group on **4** was removed by hydrogenolysis and the generated phenolic hydroxyl group was protected with an *o*-nitrobenzyl group to afford *o*-NB ether **5**. After removal of the TBS group of **5** under acidic condition, a two-step oxidation was performed to give carboxylic acid **6**. The Boc group on **6** was removed with hydrogen chloride in ethyl acetate to yield an amine, which was reprotected with an Fmoc group to give Fmoc protected photo-responsive processing device **7**. The total yield of Fmoc derivative **7** amounted to 11% over 12 steps starting from aldehyde **2**. Finally, incorporating amino acid **7** into the peptide by standard Fmoc solid phase peptide synthesis (SPPS) afforded photo-responsive model peptide **8** as a diastereomeric mixture.

To examine the photo-reactivity of model peptide **8**, we performed the photo-processing reaction outlined in Scheme 2. Peptide **8** in 20% MeCN/phosphate buffer (pH 7.6) was irradiated by UV light (>365 nm) for 3 min and then incubated at 37 °C. The reaction progress was monitored by HPLC and the peptides were characterized by ESI-MS. The *o*-nitrobenzyl group of peptide **8** was completely removed with 3 min of UV irradiation to generate deprotected intermediate **9**. Afterward, intermediate **9** was converted to corresponding processing products **10** and **11** via incubation for 2 h. Monitoring the processing reaction clarified that half-life of intermediate **9** was approximately 34 min.

To demonstrate the applicability of this processing system to other stimuli, we designed phosphatase-responsive model peptide **12** in which the phenolic hydroxyl group was protected with phosphate (Scheme 2). Peptide **12** was synthesized by Boc SPPS. A phosphatase-responsive processing reaction of peptide **12** was examined in the presence of alkaline phosphatase derived from calf intestine. Model peptide **12** was incubated with alkaline phosphatase in Tris-HCl buffer (pH 7.9) at 37 °C. After 24 h, HPLC and ESI-MS analyses clarified that substrate **12** completely disappeared and processing products **10** and **13** were obtained in good purity. In the absence of an alkaline phosphatase, hydrolysis of the phosphate moiety was not detected under these conditions.



Scheme 2. Synthesis of amino acids and processing reactions. (i) *di-tert*-butyl azodicarboxylate, pyrrolidine, CH<sub>2</sub>Cl<sub>2</sub>, 85%; (ii) NaBH<sub>4</sub>, MeOH, 100%; (iii) TBSOTf, Et<sub>3</sub>N, CH<sub>2</sub>Cl<sub>2</sub>, 89%; (iv) TFAA, pyridine, CH<sub>2</sub>Cl<sub>2</sub>; (v) Sml<sub>2</sub>, HMPA, *tert*-BuOH, THF, 74% (2 steps); (vi) H<sub>2</sub>, Pd/C, MeOH, 88%; (vii) *o*-nitrobenzyl bromide, K<sub>2</sub>CO<sub>3</sub>, DMF, 98%; (viii) AcOH, THF, H<sub>2</sub>O, 94%; (ix) PCC, CH<sub>2</sub>Cl<sub>2</sub>, 60%; (x) NaClO<sub>2</sub>, NaH<sub>2</sub>PO<sub>4</sub>, *tert*-BuOH, H<sub>2</sub>O, acetone, 2-methyl-2-butene; (xi) HCl, AcOEt; (xii) FmocOSu, Na<sub>2</sub>CO<sub>3</sub>, 1,4-dioxane, H<sub>2</sub>O, 40% (3 steps); (xiii) Fmoc SPPS.

## Discussion & Summary

In conclusion, we developed photo- and phosphatase-responsive self-processing peptide. The processing reaction was triggered by the deprotection of the protective group on the phenolic hydroxyl moiety and processing products were obtained in good purity. Reaction monitoring revealed that the reaction rate depends on the amino acid next to the stimulus-responsive residue. Application of the stimulus-responsive processing device-containing functional peptides in chemical biology field is in progress.

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3) Chemical Sciences  
3-1) Organic Chemistry

## Synthesis of Native Glycoprotein by *in vitro* Protein Synthesis

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### Introduction

For investigation of oligosaccharide modification of protein, homogenous structure glycoprotein is strongly required. By pure organic synthetic methodology, the limitation of preparation of large molecule glycoprotein exists. In this project, the synthetic approach will be investigated by combining the synthetic organic chemistry and *in vitro* protein synthesis to overcome the problem.

### Results

We chose the *C*-mannosylated tryptophan (*C*-Man-Trp) and *O*-linked *N*-acetyl glucosamine (*O*-GlcNAc) as target molecules. The target sugar modifications are related to diseases and thought to be important molecules from biological point of view.

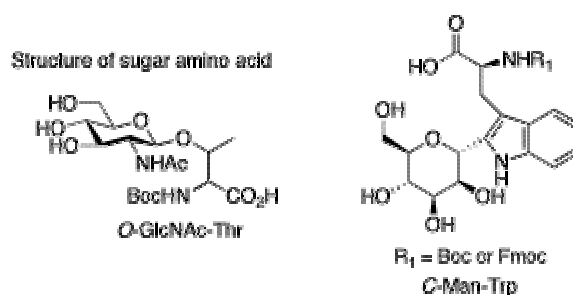
Firstly, the amino group protection group of *C*-Man-Trp was investigated. After synthesis of *C*-Man-Trp by our own strategy, the amino group of amino acid moiety was protected either Boc or Fmoc group. The aminoacylation reaction of the protected *C*-Man-Trp to the suppressor t-RNA was achieved by use of ribozyme developed by Suga in high yields. After aminoacylation reaction, removal of protecting group was found to be difficult. In the case of Boc group, the instability of *C*-Man-Trp under acidic conditions is the problem, while in the case of Fmoc group, the ester group between RNA and amino acid was not stable during deprotection of Fmoc under basic conditions. Then, we turned out the target to the *O*-GlcNAc. After preparation of *O*-GlcNAc in an efficient manner, the amino group was protected by Boc group. After aminoacylation to suppressor RNA, the Boc group was removed. The synthesis of RNA linked sugar amino acid was achieved. By using above RNA-linked sugar amino acid, the preparation of protein was attempted. However, *in vitro* protein synthesis is difficult at this stage, probably because of the steric bulkiness and hydrophilicity of the sugar moiety.

### Discussion & Summary

We have achieved the efficient synthesis of glyco-amino acid, Fmoc *C*-Man-Trp, Boc-*C*-Man-Trp,

Boc-*O*-GlcNAc-Thr. By using the ribozyme developed by Suga, the amino acylation reaction of the sugar-amino acids to the suppressor RNA was achieved in a efficient manner and in high yields. The limitation of *in vitro* protein synthesis was also found. Probably because of the steric bulkiness or the hydrophilic nature of the sugar part, the protein formation. The protein synthesis is currently under investigation.

## Figures & Tables



3) Chemical Sciences  
3-4) Analytical Chemistry

## Design, Synthesis and Biological Application of Molecular Probes Which Convert Cellular Biological Responses to Chemical Output

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### Introduction

Phosphorylation and dephosphorylation are general biochemical modifications seen on a wide variety of biomolecules. Phosphorylation is catalyzed by kinases, and dephosphorylation by various phosphatases, for instance, alkaline phosphatase, acid phosphatase, serine/threonine phosphatases, and tyrosine phosphatases, etc. For studying about dephosphorylation of biomolecules, fluorometric assays that can be applicable to bioimaging experiments are paid more attention by many researchers.

### Results

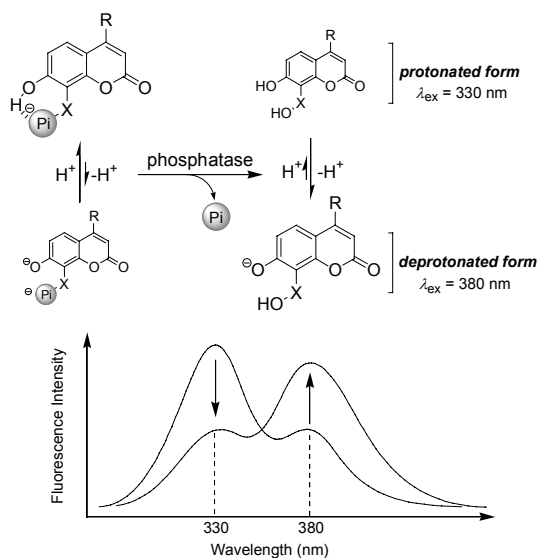
Development of fluorescent probes detecting phosphatase activity is important, because phosphorylation and dephosphorylation regulate a lot of metabolic pathways in living organism. For clarifying the biological role of each phosphatase by fluorescence measurement, it is desirable that the fluorescent probe has the specificity toward the target enzyme. However, widely-used fluorescent probes such as DiFMUP have almost no enzyme specificity among phosphatases. This is in part because all phosphatase probes are aryl phosphate monoesters, and the aryl monoester-based structure does not possess flexibility to modify the basic structures for having an enzyme specificity. Here, we report a novel design strategy for fluorescent phosphatase probes which have an alkyl phosphate monoester. There were no fluorescent probes based on alkyl monoester because dephosphorylation does not change its electronic characters much as aryl monoester. The design strategy is based on a phenomenon that an anionic group adjacent to 7-hydroxyl group of 7-hydroxycoumarin increases the  $pK_a$  of the hydroxyl group. Using this strategy, we developed novel fluorescent probes for phosphatases and investigated their photophysical and enzymological properties. They showed ratiometric changes of the excitation spectra by addition of acid phosphatase (ACP). They showed no spectral changes by any protein tyrosine phosphatase or serine/threonine phosphatase and very small changes by alkaline phosphatase (ALP). In conclusion, we developed fluorescent probes detecting phosphatase activity

using a novel design strategy. This strategy would lead to development of specific probes for various phosphatases. The  $pK_a$  modulation approach is widely applicable strategy for designing fluorescent probes targeted for other enzymes.

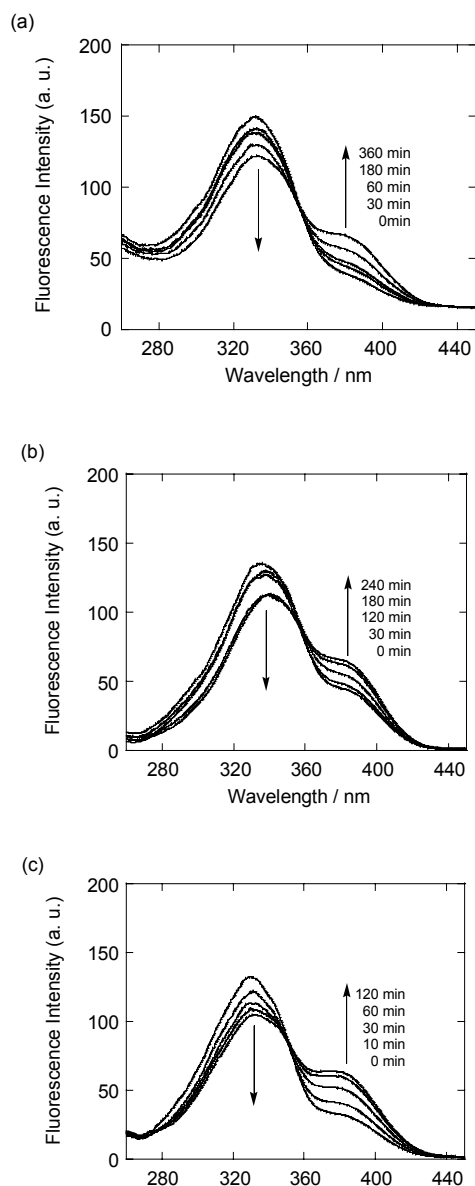
## Discussion & Summary

A novel design strategy for development of fluorescent probes detecting phosphatase activities was introduced. This design principle is based on that the  $pK_a$  change of 7-hydroxyl group of 7-hydroxycoumarin derivatives are induced by neighboring anionic groups such as a phosphate monoester group, and that the  $pK_a$  change induces the excitation spectral change. We applied this principle to the probe design and developed fluorescent phosphatase probes that have an alkyl phosphate monoester as the enzyme-recognition group. Our synthesized probes were dephosphorylated by acid phosphatase (ACP) efficiently and showed their excitation spectral shift. The result indicates that the modification of the linker can change the affinity to enzyme dramatically.

## Figures & Tables



**Figure 1.** Hypothesized scheme of the excitation spectral change of 7-hydroxycoumarin derivatives by phosphatases. R: alkyl group. X: linker.



**Figure 2.** Excitation spectra of (a) **3a**, (b) **4a**, and (c) **5a** before and after addition of acid phosphatase (ACP) in 100 mM HEPES buffer solution (pH 7.4). Emission wavelength was 470 nm.

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3) Chemical Sciences

3-5) Others

## Elucidation of Functional Properties of Thermostable Electron Transfer Proteins and Their Application to Biomaterials

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### Introduction

MonoHEME Class I cytochromes *c* (cyts *c*), in which heme Fe is coordinated to His N and Met S atoms as axial ligands at the redox center, are some of the best characterized redox active proteins. Homologous Class I cyts *c*, thermophilic *Hydrogenobacter thermophilus* cytochrome *c*<sub>552</sub> (HT) and mesophilic *Pseudomonas aeruginosa* cytochrome *c*<sub>551</sub> (PA), exhibit a unique thermodynamic property, i.e., despite their structural similarity together with their 56 % sequence identity, the oxidized form of HT is significantly more stable than that of PA, as reflected by the large difference in denaturation temperature (*T*<sub>m</sub>). Site-directed mutants of PA, for which amino acid substitutions were selected with reference to the corresponding residues in HT, exhibited thermostabilities between those of PA and HT. In this study, we elucidated the molecular mechanisms responsible for the thermostability and redox regulation of these proteins.

### Results

In order to determine the relationship between the redox property and overall protein stability, we first carried out electrochemical measurements at 25 °C using cyclic voltammetry (CV). The observed redox potentials (*E*<sup>o</sup>) were in the range of +0.2 - +0.3 V, i.e. similar to those reported for other Class I cyts *c*. The *E*<sup>o</sup> values for the PA mutants, F34Y single (sm) and F7A/V13M/F34Y/E43Y/V78I quintuple (qm), were lower than that of the wild-type, corresponding with the increase in overall protein stability. These results indicate that, upon mutation, the oxidized forms are more stabilized than the reduced ones are. Thus, the *E*<sup>o</sup> values of cyts *c* are determined by the overall stability of the oxidized forms, which is significantly affected by the mutation(s), as manifested in their *T*<sub>m</sub> values.

On electrochemical measurement at 5 - 85 °C, we observed CV responses below 70 °C and 80 °C for PA and sm, respectively. In contrast, ones for qm and HT were detected up to 85 °C. Plots of *E*<sup>o</sup> against temperature (*E*<sup>o</sup>-*T* plots) for the PA proteins (wild-type and the two mutants) gave a

straight line at lower temperatures and exhibited downward curvature with increasing temperature, while those for HT exhibited a straight line over the temperature range examined. The temperature at which the plots started to show curvature correlated with the overall protein stability. The curvature observed for the PA proteins reflects that the oxidized form becomes thermodynamically more favorable than the reduced one does as the thermal denaturation proceeds. This is because the oxidized heme becomes more favorable with removal of the hydrophobic heme environment on denaturation, as demonstrated by model studies.

From the  $E^{\circ'}-T$  plots, we estimated the enthalpic ( $\Delta H^{\circ'}$ ) and entropic ( $\Delta S^{\circ'}$ ) contributions to the  $E^{\circ'}$  value. For the PA proteins, the  $\Delta H^{\circ'}$  values increased consistently with increasing protein stability, whereas the  $\Delta S^{\circ'}$  values remained essentially unchanged. These results suggest that the effect of the mutations in the PA protein on the  $E^{\circ'}$  value is mainly an enthalpic contribution. In addition, the least negative  $\Delta H^{\circ'}$  value observed for HT was consistent with its highest stability among the oxidized forms of the proteins. Furthermore, the least negative  $\Delta S^{\circ'}$  value of HT indicates that enthalpy-entropy compensation occurs in the redox thermodynamics as found in the other electron transfer proteins such as cupredoxins and rubredoxins.

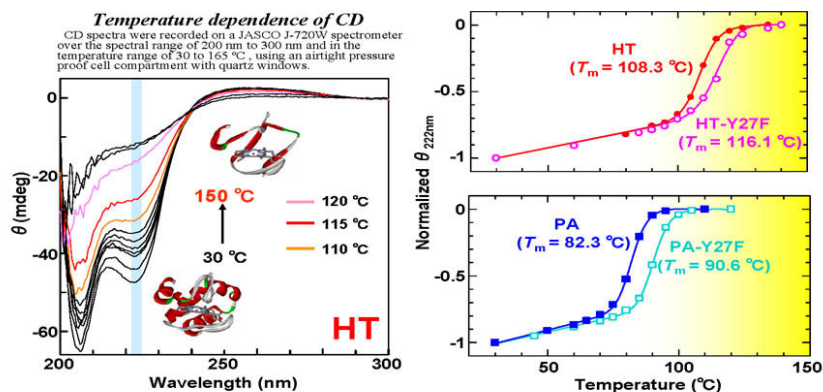
Next, we directly compared the stability of the heme active site where the electron transfer reaction occurs in cyts *c*. On  $^1\text{H}$  NMR spectroscopy, signals due to Fe-coordinated Met side chain protons are often resolved in the spectra of both the oxidized and reduced forms of cyts *c*. The temperature-dependent appearance of these signals facilitates direct comparison of the stability of the heme active site. Such Met signals were observed up to 95 °C in the spectra of the reduced forms of all the cyts *c* examined, indicating their high structure stability around the heme active site under the conditions used for the electrochemical analysis. In contrast to the reduced form, Met signals were barely observed in the NMR spectrum of the oxidized form of PA at 80 °C, suggesting that its heme active site structure was no longer retained above this temperature. Met signals could be observed up to 91 °C for sm, demonstrating that the denaturation temperature for the heme active site in sm increased by >10 °C due to the mutation, F34 to Y. The stability of the heme active site in the oxidized form of PA was further enhanced by quintuple mutations, Met signals being exhibited up to 95 °C, although the signals were not as sharp as observed for the reduced form at the same temperature. These NMR results led to two conclusions: (1) In individual proteins, the heme active site of the reduced form is more stable than that of the oxidized one. (2) The mutations that stabilize the overall protein structure of a PA protein are also responsible for the stability of the heme active site in the oxidized form. Furthermore, as previously demonstrated, the stability of the heme active site in the oxidized forms of the proteins, as determined by NMR, positively correlates with the stability of the Fe-S bond, which was inferred from the dissociation temperature obtained through variable temperature measurement of the 695-nm absorption band characteristic of the Fe-S bond in the oxidized proteins.

## Discussion & Summary

The findings described above, together with those described in our previous study, demonstrated that the overall protein stability, Fe-S bond stability, and redox property of cyt *c* are mutually related with each other. The protein conformational change induced by the mutations, which increases the overall stability of the oxidized form, happens to determine the Fe-S bond stability. Since the overall protein stability of the reduced form is less affected by the mutations than that of the oxidized one, the  $E^\circ$  values of the mutants decrease with respect to that of the wild-type PA in an enthalpic manner. Generally, oxidized heme is less favorable in the hydrophobic environment of the heme protein pocket compared with reduced heme. In the mutants, the oxidized heme is stabilized to some extent through the stronger Fe-S bond, leading to the regulation of redox property in the proteins. This is how the protein structure influences functionality of the cyts *c*.

## Figures & Tables

### Denaturation Curves



### Sequence homology 56%

PA	1	EDPEVLFRNK	11	GCVACHAIDT	21	RMVGPAYKDV	31	AKKFAGQAGA
HT		NEQLARQK		GCMACHDLKA		RVGPAYADV		AKKYAGRKDA
PA	41	EAELAQRINK	51	GSQGVWGPID	61	MPPNAVSDDE	71	AQTLAKWVLSOK
HT		VDYLAGKIRK		GGSGVWGSVP		MPPNVTDAE		AKQLAQWILSIK

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## Part II

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Reports from the Recipients of Garants  
for International Meetings  
(Fiscal Year 2005)

## 4<sup>th</sup> International Symposium on Autophagy (ISA): Exploring the Frontiers of Expanding Autophagy Research

### 1. Representative

Eiki Kominami, the organizer of the 4th International Symposium on Autophagy  
Professor of Biochemistry, Juntendo University School of Medicine, Tokyo, Japan

### 2. Opening period and Place

October 1-5, 2006; Toray Conference Center, Mishima-shi, Shizuoka-ken, Japan

### 3. Number of participants

145 persons (52 persons from foreign countries)

### 4. The total cost

15,300,000 yen

### 5. Main use of the subsidy

Support for travel expenses of invited speakers and attendees from foreign countries

### 6. Results and Impression

Autophagy is the major catabolic system responsible for the turnover of bulky cellular constituents including cell organelles, such as mitochondria, endoplasmic reticulum, and peroxisomes. Autophagy is induced under nutrient-starved conditions and positively or negatively controlled by hormones and growth factors. In starvation-induced autophagy, an isolation membrane appears in the cytosol, where it gradually elongates to enwrap cytoplasmic constituents. Subsequently, the edges of the membrane fuse together to form double-membrane vesicles termed autophagosomes. Autophagosomes rapidly fuse with lysosomes, and their luminal contents together with the inner membrane are degraded by lysosomal hydrolases. More than 20 *ATG* genes that are essential to autophagosome formation have been identified. Many of the *ATG* genes are involved in autophagy-specific ubiquitylation-like modification (*ATG* conjugation) systems, in which two modifiers, Atg12 and Atg8, are activated by a common E1-like enzyme Atg7 and transferred to two E2-like enzymes Atg10 and Atg3, respectively, to form Atg12-Atg5 and Atg8-phosphatidylethanolamine (PE).

The most important role of autophagy is the supply of amino acids under nutrient-poor conditions or fasting in eukaryotic cells from yeasts to mammals. Autophagy substantially

contributes to house keeping of the cells and cellular remodeling during differentiation and development of multicellular organisms, such as fly, worm, and slime mold. Impairment of autophagy has been implicated in the progress of diseases such as cancer and neuromuscular diseases. Recently, autophagy has also attracted increasing attention in the study of cell death mechanism. It has been also demonstrated that autophagy significantly contributes as a cell defense system against invading bacteria.

The 4th ISA, chaired by Eiki Kominami, was held at the Toray Conference Hall in Mishima-shi, Shizuoka-ken from October 1 through October 5, 2006. The symposium consists of 5 oral sessions (44 talks) and two poster sessions (53 titles).

#### Session 1: Molecular mechanism of autophagy

The session dealt with topics concerning the mechanism of *ATG* conjugation reactions, autophagosome formation, and autophagic proteolysis. There is substantial progress in structure-function relationship of Atg8; essential amino acid residues for its function (Ohsumi Y., Elazar Z.), structural comparison between Atg8 and Atg8-PE (Inagaki, F.), a new role of Atg8-PE as a phospholipid-tethering machinery (Nakatogawa H.), and so on. These results owed much to what crystal structure analyses have revealed.

Autophagy is essentially a nonselective catabolic system, but, under some particular circumstances, it is thought to function as selective degradative system. One such example is degradation of peroxisomes via autophagy. In yeast, *Pichia pastoris*, many new genes have been identified and shown to be involved in selective degradation of the organelle (Subramani, S., Sakai Y., Dunn W., Fujiki Y.). It should be also emphasized that there are growing lines of evidence for the importance of autophagy that occurs constitutively even in nutrient-rich conditions. Some presentations clearly showed that constitutive autophagy significantly contribute to maintenance of cellular homeostasis as a house keeping mechanism in neuronal tissues (Komatsu M., Bjorkoy G.).

#### Session 2: Autophagy in infection and immunity

It has been long believed that bacteria invading a cell mimic phagocytic substrates and are incorporated into phagosomes to enter host cytoplasm. Some bacteria (group A *Streptococcus*, *Streptococcus pyogenes*, *Mycobacterium tuberculosis*, etc.) in the lumen of phagosome (endosome) break through the endosomal membrane into cytoplasm and proliferate. More recently, it has been demonstrated that these bacteria in the host cytoplasm are frequently incorporated in autophagosomes and subsequently transported to lysosomes to be degraded (Yoshimori T., Deretic, V., Colombo M., Nakagawa I.). Thus, autophagy functions as a cellular innate immune system. Although autophagosomes

enwrapping bacteria are formed dependent on *ATG* genes, their size is extremely larger than that of ordinary autophagosome formed under starvation conditions. Formation of these larger autophagosomes may be controlled by distinct signaling pathway (Yoshimori T.). It is also noted that some bacteria in the cytoplasm have a mechanism to escape the surveillance of host autophagic machinery by inhibiting *ATG* conjugation reactions (Nakagawa I.).

### Session 3: Regulation of autophagy

This session mainly focused on cell signaling pathways controlling autophagy. Historically, two signaling pathways have been proposed as the major regulatory system for autophagy. One is insulin-dependent signaling cascade leading to mTor activation (Nobukuni T., Neufeld T., Meijer A.) and the other is the cascade responding to amino acid starvation (Kadowaki, M.). Researchers have paid more attention on mTor interacting proteins, such as TSC1/2-Rheb (Nobukuni T.), on one hand, and attempted to unveil the link between mTor signaling pathway and autophagy. Atg1 as an essential component connecting the two has been proposed (Neufeld T.). Also, new approach to elucidate how the cell recognizes the decrease in extracellular amino acid levels has been conducted (Mizushima, N.).

### Session 4: Autophagy and cell death

“Autophagy and cell death” is a hot subject of recent publications in this field. More and more controversies have arisen about the relationship between autophagy and cell death, such as apoptosis and necrosis. Notably, there are growing numbers of articles reporting autophagic cell death or type II cell death. Autophagy has been essentially considered as a protective mechanism necessary for cell survival, i.e., the supply of energy source and building blocks under nutrient austerity and the removal of denatured or misfolded proteins unfavorable for cell activities. However, in some particular cases, in which apoptosis is inactivated, it has been reported that cells filled with numerous autophagic vacuoles died (Uchiyama Y.). This type of cell death is called autophagic cell death. Studies using various cancer cells indicated that whether autophagy operates as a protective mechanism for cell survival or it promotes cell death is dependent on cell type and conditions used (Kondo S.). Meanwhile, inactivation of autophagy using siRNA or gene targeting techniques in the cell caused apoptosis (Codogno P.).

### Session 5: Autophagy in disease and aging

In many neurodegenerative diseases, whether they are familial or sporadic, there is a



common mechanism of disease occurrence. Conformation disorder of a certain protein causes misfolding of the protein, which results in the formation of insoluble protein aggregates. These aggregates cause inactivation of ubiquitin-proteasome. As a result, the aggregates stimulate autophagic responses and enhance autophagosome formation. However, sequestered protein aggregates cannot be degraded after maturation of autophagosome to autolysosome. Thus, impairment of autophagy is a common hallmark of many neurodegenerative diseases. Some *ATG* gene products essential for autophagy accumulate along with misfolded protein aggregates, such as Huntington with expanded poly glutamine tracts (Kopito R.). In order to prevent the outbreak of the diseases, administration of drugs, which enhance autophagy, to human may be a potential therapeutic procedure (Rubinsztein D.).

## 7. Additional description

### 7.1 Number of participating countries:

18 countries

### 7.2 Approvals:

The International Symposium on Autophagy (ISA) was originally planned to present and discuss about unpublished data to unveil the mechanism of macroautophagy, microautophagy, and pexophagy of yeasts and mammalian cells. The symposium has also focused on newly discovered functions of autophagy in diverse fields of biology and pathobiology. The 1st ISA chaired by Yosinori Ohsumi was held in 1997 at Okazaki, Japan, which was succeeded three years later by the 2nd ISA organized by Patrice Codogno at Aix-les-Bains, France. Then, the 3rd ISA was organized by Yasuo Uchiyama and held in 2002 at Osaka University Medical School. The 4th ISA chaired by Eiki Kominami was held at Toray Conference Center at Mishima, Shizuoka-ken in October 2006.

## *11th International Xenopus Conference*

### 1. Representative

Makoto Asashima, the chairman of organizing committee

### 2. Opening period and Place

September 12-16, 2006

### 3. Number of participants

314 persons (214 persons from foreign countries)

### 4. The total cost

5,591,681 Yen

### 5. Main use of the subsidy

Support for travel expenses of the special plenary lecturer (Dr. Yoshio Masui)

### 6. Result and Impression

The International Xenopus Conferences have been held biannually and alternately in the United States or Europe since April 1986 to discuss about cell and developmental biology reseach using an amphibian species *Xenopus*. *Xenopus* has been contributing to clarify molecular mechanisms of development and to establish common principles to understand how animal including human is developed. For the first time in 20 years, however, the 11th *Xenopus* Meeting was held in Kisarazu City, Japan, between September 12th and 16th 2006. We Japanese organizing committee considers that this is based on Japanese *Xenopus* researches' contribution to the community and the field is highly appreciated and thus this an extreme honor. The *Xenopus* Meeting for this year 2006 was held in the midst of tremendous progress in such epoch-making topics as genomic interpretation, genetic expression control, regeneration, transformation, organogenesis, germ cells, transgenic methods, proteomics and bioinformatics. Excellent presentations related to these themes were delivered by oral as well as poster presentations, including excellent plenary lectures "The cytoskelton of the early embryo" by Dr. Chris Wylie (Cincinnati Children's Hospital Medical Center, USA) who contributed to the *Xenopus* community not only scientifically but also by acting as the former

chief editor of a distinguished journal in developmental biology field “Development” and “Genome-wide and gene-specific analyses in *Xenopus*” by Dr. Jim Smith (Gurdon Institute, Cambridge, UK) who is one of the pioneers of the research on “embryonic inductions”. In addition, as the special plenary lecturer, Dr. Yoshio Masui who won the Lasker Prize in 1998 for his innovative contributions in understanding cell division through the discovery of maturation promoting factor, a protein that controls cell division in fertilized eggs, gave an excellent speech before banquet.

Scientific sessions were Session 1: Cell Fate Specification, Session 2: Axis determination and Pattern Formation, Session 3: Signal Transduction, Session 4: Cell Polarity and Cell Migration, Session 5: Gene Expression, Session 6: Cell Cycle, Session 7: Organogenesis and Remodeling, Session 8: Xenomics and covered most of essential topics in the field.

At the end of the conference, we all recognized that in the last few years, outstanding achievements have been made in the study of not only *Xenopus laevis* but also on *X. tropicalis*. We learned that in the next few years, a drastic and revolutionary change would happen in the field by introducing genetics with *Xenopus tropicalis*. We believe that the role of studies using *Xenopus* has been truly remarkable in the life sciences, and it was indeed meaningful at this time that the researchers who use *Xenopus* in their studies gathered from all over the world and introduced their findings and discussed their results. Beside US (83) and Japan (100), Canada (5), Asian countries include Korea (28), Singapore (3), China (1), and European countries are Germany (14), France (7), Belgium (7), Czech (2), Italy (2), Switzerland (1), Netherlands (1), Spain (1), and Israel (1). Mid- and South America are Ecuador (1), Brazil (1), and Chile (1). Especially, participation of young researchers including postdoc and graduate students from many countries, which is partly supported by the travel fellowship from the organizing committee, proved that *Xenopus* is and will continue to be an important model animal in life sciences.

Location of the conference was also splendid. Kazusa Akademia Park is situated in a quiet location not far from the Narita/Tokyo and Haneda International Airports. This somewhat isolated location enhanced intensive discussions among participants before and after meeting each day and this choice of the conference site was highly appreciated by all attendees.

Finally, we all organizing committee members would like to express our sincere thanks to all foundations including Novartis Foundation for the Promotion of

Science for their generous support and industries that had product exhibitions during the conference for their donations that made this conference possible.

## 7. Additional description

### 7.1 Number of participating countries:

32 countries and 1 area

### 7.2 Approvals:

The International Xenopus Conference was first organized by Drs. John Gurdon, Igor Dawid, and Jim Maller 20 years ago and the series of meetings have been held every 2 years in either US or in Europe by the local organizing committee. This time, 11<sup>th</sup> IXC was approved by the Japanese Society of Developmental Biologists.

### 7.3 Satellite symposium:

Prior to the main conference, on September 12, a satellite conference on Xenopus genomics “Xenomics, the next leap forward” organized by Rob Grainger and Richard Harland was held at the same conference site. Approximately 120 people attended and contributed to active discussions.

*Pre-Symposium Nagoya*  
*ICOB-5 & ISCPN-25 IUPAC International Conference*  
*on Biodiversity and Natural Products*

1 . Representative

Prof. Youji Sakagami, the chairman of organizing committee

2 . Opening period and Place

July 21–23, 2006

Hamanako Royal Hotel (4396-1 Yamazaki, Yuto-cho, Hamamatsu, Shizuoka 431-0101)

3 . Number of participants

60 persons (10 persons from foreign countries)

4 . The total cost

2,632,356 yen

5 . Main use of the subsidy

Support for travel expenses of invited speakers from foreign countries

6 . Result and Impression

This pre-symposium was held as one of six satellite symposiums of the main conference, *ICOB-5 & ISCPN-25 IUPAC International Conference on Biodiversity and Natural Products* (at Kyoto).

The big main conference is held in alternate year under auspices of IUPAC and relevant organizations of the host country. Its purpose is that researches all around the world gather to exchange and discuss the newest information and problems on biodiversity and natural products chemistry regarding isolation, chemical structural elucidation, biosynthesis, chemical biology, etc. The conference is a large-scale one, which included 17 plenary and 76 invited lectures, and approximately 600 oral and poster presentations as the scientific program, as well as 1,350 participants from all over the world.

On the other hand, unlike the main conference, this pre-symposium held at a scenic region near Nagoya is rather small so as to provide an relaxed circumstance of the academic researches and cultural exchanges not only for the invited foreign researchers but also Japanese researchers and students around Nagoya. Therefore

the participants (approximately 60) seemed to deepen exchanges each other. The scientific program of the pre-symposium, entitled ‘Bioorganic Chemistry and Chemical Biology,’ included 12 invited lectures in the fields of discovery of novel natural products, chemical biology, biosynthesis, and chemical syntheses. The titles of lectures are follows: “Chemical studies on the red sweat of the hippopotamus” (by Associate Prof. Kimiko Hashimoto at Kyoto pharmaceutical Univ.), “Bioorganic chemistry of the action plants: circadian rhythmic movement and memory in higher plants” (by Prof. Minoru Ueda at Tohoku Univ.), “Development of marine kabiramides as the new fluorescent probes for actin assay” (by Prof. Khanit Suwanborirux at Chulalongkorn Univ., Thailand), “A cyanobacterial origin of diverse biologically active Sponge and Tunicate natural products” (by Prof. William Gerwick at UC San Diego), “Chemical biology in the interaction between proteins and small molecules” (by Dr. Hiroyuki Osada at RIKEN), “Chemical genomics with natural products” (by Prof. Ho Jeong Kwon at Yonsei Univ., Korea), “Application of chiroptical spectroscopy (electronic CD and optical rotation) in natural products research” (by Prof. Nina Berova at Columbia Univ., US), “The biosynthetic potential of the myxobacterium *Chondromyces crocatus* – molecular biology and biochemistry” (by Prof. Rolf Müller at Saaland Univ., Germany), “Using synthetic organic chemistry to prove biological mechanisms” (by Prof. Dang Yang at Univ. Hong Kong, P. R. China), “Formal total synthesis of enantiopure ingenol” (by Prof. Chin-Kang Sha at National Tsing Hua Univ., Taiwan), “Synthetic studies on heterocyclic natural products” (by Prof. Marco Ciufolini at Univ. British Columbia, Canada), and “Synthesis of rare anticancer agents isolated from marine sponges” (by Prof. Ian Paterson at Cambridge Univ., UK). In addition, 18 poster presentations with a 5-minute oral presentation were performed by young researchers and graduate students. The foreign invited speakers enjoyed an excursion visiting a natural cave in the third day morning. Most participants, especially the foreign invited speakers looked satisfied with the pre-symposium. Since Nagoya is well known as an active place in the natural products chemistry field, the symposium was successful and productive.

## 7. Additional description

### 7.1 Number of participating countries:

9 countries

### 7.2 Approvals:

The international Union of Pure and Applied Chemistry (IUPAC) is a worldwide

scientific organization founded in 1919 to objectively address global issues in the chemical sciences. The co-organizers were the International Union of Pure and Applied Chemistry (IUPAC), the Chemical Society of Japan, the Pharmaceutical Society of Japan, Japan Society of Bioscience, Biotechnology, and Agrochemistry, Science Council of Japan, and the organizing committee

### 7.3 Satellite symposium:

This symposium itself is one of the satellite symposiums of the Kyoto main conference.

Follows are a list of the satellite symposiums:

Pre-symposiums were in Nagoya (July 21-23) and Tokushima (July 21-23).

Post-symposiums were in Sapporo (July 29-30), Sendai (July 30), Tokyo (July 31), and Fukuoka (July 30).

## “Functional Architecture of Cortical Microcircuits” Symposium in the 29<sup>th</sup> annual meeting of the Japan Neuroscience Society

### 1. Organizer

Yumiko Yoshimura, Nagoya University

Yoshiyuki Kubota, The National Institute for Physiological Sciences

### 2. Date

July 19-21, 2006

Kyoto International Conference Hall

Takaragaike, Sakyo-ku, Kyoto, Japan 606-0001

### 3. Number of participants at the symposium

350 persons including 50 persons from foreign countries

### 4. The total cost

1,800,000 yen

### 5. Main use of the subsidy

Support for hotel expenses of invited speakers

### 6. Result and Impression

The aim of this symposium was to promote the spread of knowledge in regard to recent advances in cortical microcircuit studies. We invited four distinguished speakers, Dr. Gabor Tamas from the University of Szeged in Hungary, Dr. Jackie Schiller from the Technion Medical School in Israel, Dr. Edward M. Callaway from the Salk Institute in the U.S.A., and Dr. Sacha B. Nelson from Brandeis University in the U.S.A. At the symposium these experts introduced recent data from cutting-edge studies on the synaptic connectivity of cortical neurons using multiple electrode and optical recording, and electron microscopic observations. A key focus of the meeting was the fundamental characteristics of neuron subtypes defined by genetic expression patterns, physiological characteristics, and *in vivo* functional architecture. All who attended the symposium enjoyed this wonderful, informative opportunity, and there was lively give and take between the expert guest speakers and the audience. I would like to express my sincere thanks to the Novartis Foundation for the Promotion of Science for the support of this symposium.



## 7. Additional description

A satellite symposium was held on July 24-26 in Okazaki.

## Dynamic Organelles in Plants

### 1. Representative

Tetsuro Mimura, the organizer

### 2. Opening period and Place

June 14 – June 17, 2006

Okazaki conference center at Aichi, Japan

### 3. Number of participants

206 persons (15 persons from foreign countries)

### 4. The total cost

c.a. 6,900,000 yen

### 5. Main use of the subsidy

Support for travel expenses of invited two foreign speakers

### 6. Result and Impression

Since plants spread their roots in the ground, they must survive in a given environment. To adapt the environment, they positively utilize the environmental changes in the life cycle as important signals that are necessary for their survival. Recent studies have shown that plant cells could induce, degenerate and differentiate their organelles to adapt the environmental changes. The flexibility of the plant organelles is the basis of the strategy for the environmental adaptation in plants.

Thus, studies on organelles dynamics are newly developing in the field of modern plant biology on the basis of molecular techniques.

This symposium intended to provide an excellent opportunity to review the recent advances on the plant organelle biology.

Many international conferences are being held on the plant organelles. However, most of those meetings are focusing on the individual organelle, such as, chloroplasts, mitochondria, golgi apparatus etc. Our conference is exceptional for focusing on the all kinds of plant organelles and their dynamics dependent on the environmental conditions.

The aim of this scientific research is to clarify the molecular mechanisms underlying induction, differentiation, and interaction of organelles, and to understand integrated function of individual plant through the organelle dynamics. We also expect that this

field will provide perspective for increase of food production, production of functional foods and phytoremediation in the future.

For these subjects, the following sessions were held; (1) Differentiation and Degradation, (2) Biogenesis and Protein transport, (3) Post-Genome approach, (4) Metabolic Regulation and Signal Transduction, (5) Integrated Functions.

Throughout 4 days, more than 200 persons took part in the conference. Many graduate students and younger staffs were also included in participants. In total, 33 oral lectures and 89 posters were presented.

In the evening session of the first day (Jun., 14), the plenary lecture by Prof. Asada who is a world-famous scientist on the photosynthesis was held. We enjoyed his interesting talk and exciting data about his main subject, "Photoreduction and Photoexcitation of Oxygen and Relaxation of Photon Excess Stress in Chloroplasts". Not only that, Prof. Asada prepared many beautiful photographs of Japanese scenery and flowers for foreign guests.

Above 5 sessions cover from molecular structure of proteins which compose organelles to physiological roles of organelles in integrated functions of a whole plant. In oral presentations, we enjoyed researches of international top scientists. If I should point out a matter on this symposium, we could not prepare enough discussion time, and English discussion was still inactive among Japanese participants. Recent younger Japanese are much better at speaking English than the elder generation. But they were very quiet during oral presentations.

Instead of that, their poster presentations were very active. On the first day, after oral presentations, poster sessions were held. Each poster presenter was given 1 minutes for explaining data in front of all participants. We call this presentation the poster marathon. All presenters from famous professors to master course students have equally shown their data for 1 minutes. Although it took longer time (two times of one hour running), it was highly reputed by foreign participants as the exciting attempt.

In the evening of the second night on Jun. 15, the banquet was held. There, all participants enjoyed in talking each other.

Throughout the conference, I was impressed that research level of organelle biology in Japan is quite high. Furthermore, the younger generation for this field are growing up very well. However, the research competition in the world is severe. We also have to make a constant challenge to progress organelle biology in plants.

## 7. Additional description

7. 1 Number of participating countries : 6 countries including Japan