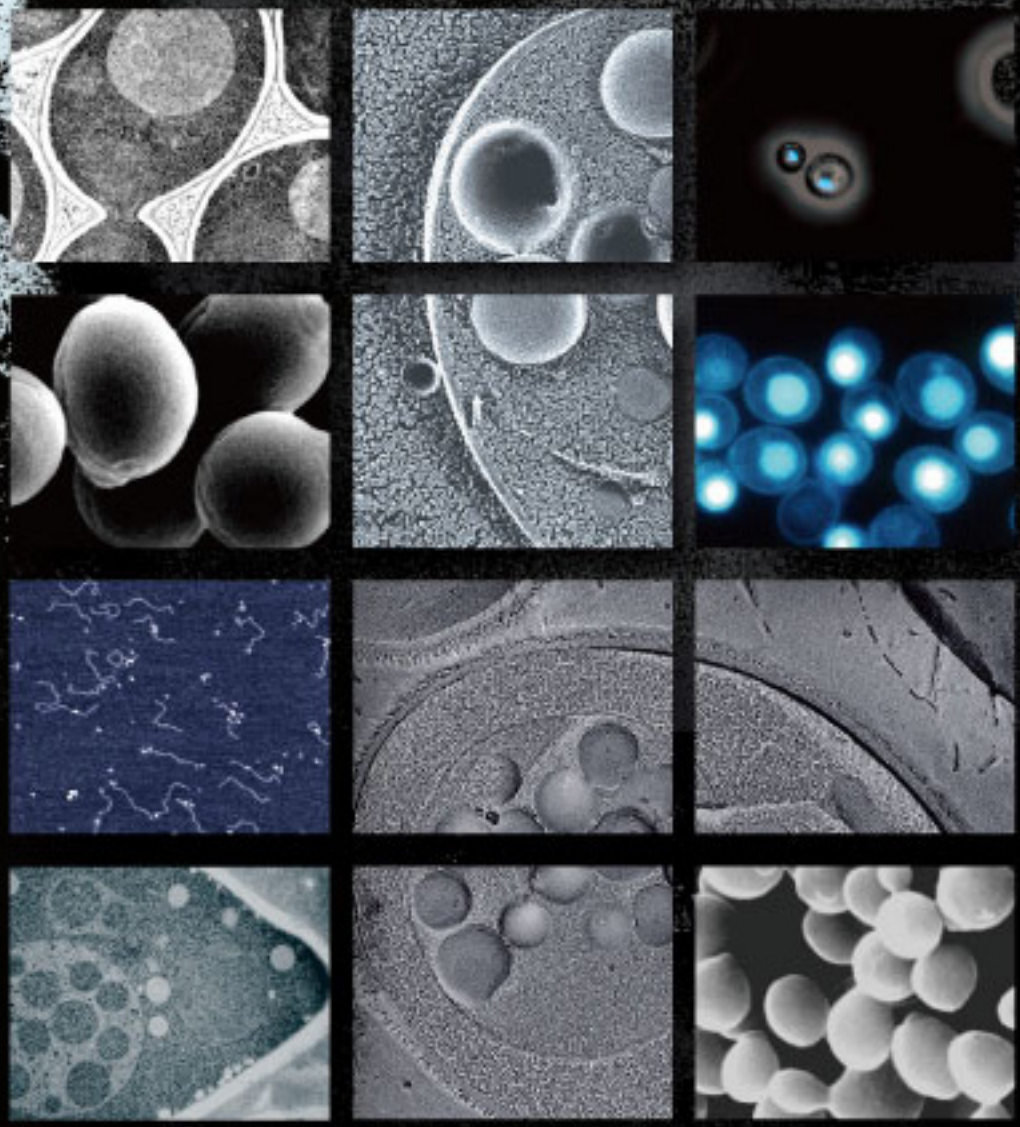


National Institute for Basic Biology

2005 ANNUAL REPORT

INTRODUCTION

The National Institute for Basic Biology (NIBB) was founded in 1977 as one of the Inter-University Research Institutes to promote and stimulate studies in the field of biology. As a center of excellence (COE), NIBB promotes not only basic biology but also the modern biological sciences by conducting first-rate research on site as well as in cooperation with national, public and private universities and research organizations. Research at the NIBB covers a wide variety of biological fields, such as cell biology, developmental biology, neurobiology, evolutionary biology, environmental biology, and theoretical biology, and is conducted to elucidate general and fundamental mechanisms underlying various biological phenomena.

In 2004, NIBB, in alliance with four other national institutes - the National Astronomical Observatory, the National Institute for Fusion Science, the National Institute for Physiological Sciences, and the Institute for Molecular Science - established the National Institutes of Natural Sciences (NINS), one of the four Inter-University Research Institute Corporations. At this time NINS began to promote collaboration among researchers of the five constituting institutes and to sponsor many projects, one of which was to hold the Imaging Science Symposium in August, in which cutting-edge imaging technology related to a wide range of basic scientific fields from astronomy to biology was discussed.

Besides being a research institute, NIBB also functions as an institution of higher education. It constitutes the Department of Basic Biology in the School of Life Science of the Graduate University for Advanced Studies (SOKENDAI). The Department offers a five-year Doctoral course (with a quota of three students) for university graduates and a three-year senior Doctoral course (with a quota of six students) for those students who have completed a Master's course.

Changes in our personnel during 2005 were as follows. Prof. Norio MURATA retired and Dr. Khoji UENO moved to Musashino University as a professor on March 31st. Dr. Tomomichi FUJITA moved to Hokkaido University as an assistant professor on June 30th. Dr. Hirokazu TSUKAYA moved to the University of Tokyo as a professor, while at the same time becoming an adjunct professor at NIBB, and Dr. Takashi KITSUKAWA moved to Osaka University as an associate professor on September 30th.

Conversely, three researchers were newly appointed: Prof. Masamitsu WADA from Tokyo Metropolitan University and former adjunct professor at NIBB, as a professor, and Dr. Hitoshi NAKATOGAWA from a JSPS Post-Doctoral fellow as a research associate, both on April 1st. Dr. Takako TANAHASHI, a visiting scientist, was appointed as a research associate on July 1st. Dr. Takehiko KOBAYASHI was promoted to an associate professor from a research associate on April 1st. In addition, Dr. Takeshi NODA returned from his stay at UC San Diego on December 12th.

NIBB has started a collaboration program with the European Molecular Biology Laboratory (EMBL) under the sponsorship of NINS. The agreement was signed on July 11th by Dr. Yoshiro SHIMURA, the president of NINS, and Dr. Iain MATTAJ, the director general of EMBL, and is effective for at least five years. The program aims to promote joint research activities between the two organizations and to prompt mutual visits by researchers and graduate students, thereby encouraging exchange of information and intimate communication, both of which are indispensable for the prosperity of joint research. Activities seen this year are as follows. A symposium on developmental and cellular biology was held in July at EMBL, Heidelberg. Seminars were held at NIBB by visiting researchers of EMBL, while NIBB researchers stayed at EMBL for the preparation of collaboration using the microscopic facility at EMBL.

The Strategic Planning Department, a new office for the promotion of collaborative activities, the management of international conferences and training courses held by NIBB, and various public relations activities such as publications, posters, and web pages, was opened in April. The office is chaired by the vice director of the institute and is expected to support the director-general in developing a future strategy for the progress of the institute.

Finally, I would like to congratulate Prof. Mitsuyasu HASEBE for winning the JSPS Prize and Japan Academy Medal in March and Prof. Yoshinori OHSUMI for winning the Fujihara Award in June.

We always welcome any questions or suggestions concerning the research activities of NIBB.

Motoya KATSUKI, D. Sc.
Director-General

ORGANIZATION OF THE NATIONAL INSTITUTE FOR BASIC BIOLOGY

The National Institute for Basic Biology (NIBB) is one of five independent institutes making up the National Institutes of Natural Sciences (NINS). NIBB, the Institute for Molecular Science (IMS) and the National Institute for Physiological Sciences (NIPS) are located on a hill overlooking the old town of Okazaki in Aichi Prefecture. NIBB was established in 1977 and its activities are supported by Monbukagaku-sho (the Ministry of Education, Culture, Sports, Science and Technology: Mext) of Japan. The Center for Integrative Bioscience (CIB) – renamed the Okazaki Institute for Integrative Bioscience on April 1st, 2004 – was established as a common facility for the three Okazaki Institutes in 2000 and opened in 2001.

Research

The NIBB conducts its research programs through 30 research units, 4 research support facilities including the Technology Department, and the Research Center for Integrative and Computational Biology. Each research unit has its own research project and is staffed (in principle) by a professor, an associate professor and two research associates. Each research unit forms an independent project team. Three of the research units are adjunct and headed by professors who hold joint appointments with other universities. Adjunct divisions have a resident research associate. This arrangement facilitates exchange in research activities in Japan. The Technical Department manages the activities of research technicians and helps to promote the research activities of each research unit and facility and to maintain the common research resources of the NIBB. The Department also undertakes the technical education of the various staffs.

Several members of the Okazaki Institute for Integrative Bioscience work jointly with the NIBB.

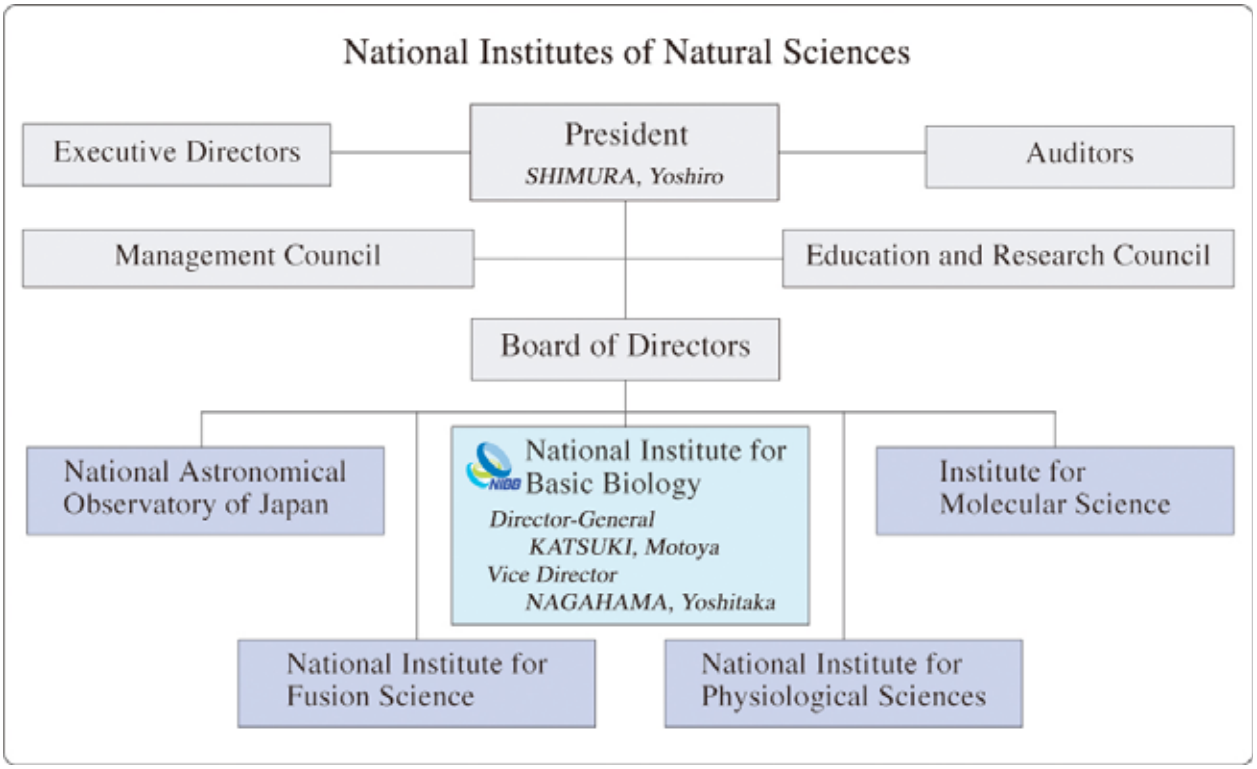
Research Support Facilities

The Research Support Facility of the NIBB consists of six sub facilities: the Large Spectrograph Laboratory, the Tissue and Cell Culture Laboratory, the Computer Laboratory, the Plant Culture Laboratory, the Plant Cell Laboratory and the Experimental Farm. The Research Center for Integrative and Computational Biology, the Center for Transgenic Animals and Plants and the Technical Division are also research support systems of the NIBB.

In addition, five facilities are operated jointly with NIPS: the Electron Microscope Center, the Center for Analytical Instruments, the Machine Shop, the Laboratory Glassware Facilities and the Low-Temperature Facilities. The Radioisotope Facilities, the Computer Center and the Animal Care Facilities are common facilities of the three Okazaki Institutes.

Campus

Together the three Okazaki Institutes cover an area of 164,783m^2 with four principal buildings. The NIBB's main research building has a floor space of 16,789m^2. Two-thirds of the space was completed in 1982 and the remaining third in June, 1983. The buildings that house the research support facilities were also completed in 1983. A building for the Laboratory of Gene Expression and Regulation (2,577m^2) was built in 1996. A building for the Center for Transgenic Animals and Plants (2,500m^2) was completed in November, 2003.



National Institute for Basic Biology

Research Unit

- **Cell Biology**
  - Division of Cell Mechanisms
  - Division of Molecular Cell Biology
  - Laboratory of Cytoskeleton
  - Laboratory of Cell Sociology
- **Developmental Biology**
  - Division of Reproductive Biology
  - Division for Sex Differentiation
  - Division for Morphogenesis
  - Division of Developmental Genetics
  - Division of Molecular and Developmental Biology
  - Laboratory of Molecular Genetics for Reproduction
- **Neurobiology**
  - Division of Molecular Neurobiology
  - Division of Brain Biology
  - Division of Behavioral Biology (adjunct)
  - Laboratory of Neurophysiology
  - Laboratory of Neurochemistry
  - Laboratory of Director General
- **Evolutionary Biology and Biodiversity**
  - Division of Molecular Genetics
  - Division of Genome Dynamics
  - Division of Evolutionary Biology
  - Division of Speciation Mechanism (adjunct)
  - Laboratory of Morphodiversity
- **Environmental Biology**
  - Division of Cellular Regulation
  - Division of Molecular Environmental Endocrinology
  - Division of Plant Developmental Genetics (adjunct)
  - Division of Photobiology (adjunct)
  - Laboratory of Photoenvironmental Biology
  - Laboratory of Stress Response
- **Theoretical Biology**
  - Division of Theoretical Biology
  - Laboratory of Genome Informatics

Research Support

- **Strategic Planning Department**
- **Research Support Facility**
  - The Large Spectrograph Laboratory
  - Tissue and Cell Culture Laboratory
  - Computer Laboratory
  - Plant Culture Laboratory
  - Experimental Farm
  - Plant Cell Laboratory
  - Laboratory of Stress-Resistant Plants
- **Center for Transgenic Animals and Plants**
- **Research Center for Integrative and Computational Biology**
- **Technical Division**

- **Okazaki Institute for Integrative Bioscience**
  - Department of Development,Differentiation and Regeneration
    - Division of Developmental Genetics
    - Division of Molecular & Developmental Biology
  - Department of Bio-Environmental Science
    - Division of Bio-Environmental Science
    - Division of Plant Developmental Genetics
- **Center for Radioisotope Facilities**
- **Center for Experimental Animal**
- **Research Center for Computational Science**

- **Center for Analytical Instruments**
  - Laboratory Glassware Facilities
  - Electron Microscope Center
  - Machine Shop
  - Low-Temperature Facilities

POLICY, DECISION MAKING, AND ADMINISTRATION

The Director-General oversees the operation of the NIBB assisted by the Advisory Committee for Programming and Management. The Advisory Committee, comprised of professors within the NIBB and an equal number of leading biologists outside the NIBB, advises the Director-General on important matters such as planning joint research programs as well as on the scientific activities of the NIBB. The Director-General Selection Committee makes a nomination for Director-General to the President after hearing the recommendation from the Advisory Committee. The Advisory Committee makes recommendations on the Director-General, faculty appointments, the NIBB's annual budget and future prospects.

Administration of the NIBB is undertaken by the Administration Bureau of the Okazaki Administration Center under the direct auspices of the Administration Office of NINS.

MEMBERS OF THE ADVISORY COMMITTEE FOR PROGRAMMING AND MANAGEMENT

Chairman

YAMAMORI, Tetsuo	Professor, National Institute for Basic Biology

Vice-Chairman

OKADA, Kiyotaka	Professor, Kyoto University
AIZAWA, Shinichi	Deputy Director & Group Director, RIKEN Center for Developmental Biology
IWASA, Yoh	Professor, Kyushu University
KOMEDA, Yoshifumi	Professor, The University of Tokyo
KONDO, Hisato	Professor, Osaka University
KUROSAWA, Yoshikazu	Professor, Fujita Health University
MACHIDA, Yasunori	Professor, Nagoya University
MURAKAMI, Fujio	Professor, Osaka University
SAGA, Yumiko	Professor, National Institute of Genetics
SEHARA, Atsuko	Professor, Kyoto University
UENO, Naoto	Professor, National Institute for Basic Biology
HASEBE, Mitsuyasu	Professor, National Institute for Basic Biology
HORIUCHI, Takashi	Professor, National Institute for Basic Biology
IIDA, Shigeru	Professor, National Institute for Basic Biology
MOROHASHI, Kenichirou	Professor, National Institute for Basic Biology
NAGAHAMA, Yoshitaka	Vice Director & Professor, National Institute for Basic Biology
NISHIMURA, Mikio	Professor, National Institute for Basic Biology
NODA, Masaharu	Professor, National Institute for Basic Biology
OHSUMI, Yoshinori	Professor, National Institute for Basic Biology
TAKADA, Shinji	Professor, Okazaki Institute for Integrative Bioscience

GRADUATE PROGRAMS

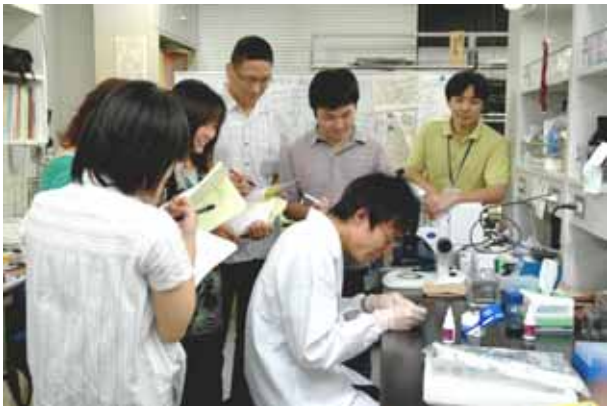
The NIBB sponsors two graduate programs.

1. Graduate University for Advanced Studies

NIBB constitutes the Department of Basic Biology in the School of Life Science of the Graduate University for Advanced Studies. The University provides a five-year Doctoral course as well as a three-year senior Doctoral course for those students who have completed a master's course (or equivalent) at any university. The Department consists of 30 Research Units (listed on page 3).

2. Graduate Student Training Program

Graduate students enrolled in other universities and institutions are eligible to conduct research for fixed periods of time under the supervision of NIBB professors.



At the 20th Bioscience Training Course in June 2005.

COOPERATIVE RESEARCH PROGRAM

The NIBB has a cooperative research program for researchers throughout Japan. Two main programs are available. The first provides laboratory space and facilities at NIBB while the second assists small groups holding research meetings at NIBB. The visiting researcher and research meeting plans are approved by the NIBB Advisory Committee.

INTERNATIONAL COLLABORATION

The NIBB hosts two international conferences, the NIBB Conference and the Okazaki Biology Conference (OBC). The NIBB Conference has been held every year since the opening of the Institute in 1977. The purpose of the NIBB Conference is to provide a limited number of active researchers with the opportunity to freely discuss current issues in various areas of biological research. The topic chosen for the 51st NIBB Conference was "New Aspects of Gene Amplification" and discussions were held on the molecular mechanisms and biological functions of gene amplification (see page 82). The OBC, initiated in 2003, has as its long-term objective the establishment of interdisciplinary networks in pursuit of the solution of major biological problems.

The NIBB performs academic exchange programs with the European Molecular Biology Laboratory (EMBL) and National Institutes of Natural Sciences (NINS) in an effort to promote intellectual, educational and technological exchange in the fields of biology and molecular biology. Academic cooperation between NIBB and ENBL includes (1) Promotion of joint research activities; (2) Invitation for faculty members and researchers to lectures, workshops, conferences, symposia, and other academic activities; (3) Exchange of graduate students for conferences and training courses; and (4) Exchange of information and academic publications.

At the 1st Okazaki Biology Conference in January 2004.

At the 1st NIBB-EMBL symposium at Heidelberg.

DIVISION OF CELL MECHANISM

Professor:	*NISHIMURA, Mikio*
Associate Professor:	*HAYASHI, Makoto*
Research Associates:	*MANO, Shoji*
	YAMADA, Kenji
Technical Staff:	*KONDO, Maki*
NIBB Research Fellows:	*KAMADA, Tomoe*
	MUTSUDA, Michinori
Postdoctoral Fellows:	*ARAI, Yuko*
	KAMIGAKI, Akane
	HATSUGAI, Noriyuki
	OIKAWA, Kazusato
Graduate Student:	*OGASAWARA, Kimi*
Visiting Scientist:	*LU, Zhongpen*
Technical Assistants:	*NAKAMORI, Chihiro*
	YAGI, Mina
	YOSHINORI, Yumi
	SUZUKI, Iku
	FUKAZAWA, Mitsue
	KATO, Kyoko
	NISHINA, Momoko
Secretaries:	*UEDA, Chizuru*
	KUBOKI, Yuko

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

The aim of this division is to clarify the molecular mechanisms underlying induction, differentiation, and interaction of organelles, and to understand the integrated function of individual plants through organelle dynamics. The Scientific Research of Priority Areas on “Organelle Differentiation as the Strategy for Environmental Adaptation in Plants” was started to clarify the molecular mechanisms underlying organelle differentiation.

I. Reversible transformation of plant peroxisomes

Dramatic metabolic changes that underlie the shift from heterotrophic to autotrophic growth occur in greening of seed germination. Accompanying these metabolic changes, many constitutive organelles are functionally transformed. Etioplasts differentiate into chloroplasts and mitochondria acquire the ability to oxidize glycine. Glyoxysomes, which are microbodies engaged in the degradation of reserve oil via β-oxidation and the glyoxylate cycle, are transformed into leaf peroxisomes that function in several crucial steps of photorespiration. After the functional transition of glyoxysomes to leaf peroxisomes during the greening of pumpkin cotyledons, the reverse transition of leaf peroxisomes to glyoxysomes occurs during senescence. Gene expression, alternative splicing, protein translocation and protein degradation control the functional transformation between glyoxysomes and leaf peroxisomes.

II. Transcriptomics, proteomics and phenomics of plant peroxisomes

Enzymes localized in plant peroxisomes are synthesized in the cytosol, and function after their post-translational transport into peroxisomes. Almost all of the peroxisomal matrix proteins are known to contain one of two targeting signals (PTS1 and PTS2) within the molecules. PTS1 is a unique tripeptide sequence found in the carboxyl terminus of the mature proteins. In contrast, PTS2 is involved in a cleavable amino terminal presequence of peroxisomal proteins that are synthesized as a precursor protein with larger molecular mass.

We identified 256 gene candidates of PTS1- and PTS2-containing proteins and another 30 genes of non-PTS-containing proteins from *Arabidopsis* genome. Custom-made DNA microarray covering all these genes was used to investigate expression profiles of the peroxisomal genes in various organs. In parallel, we made a two-dimensional protein map of glyoxysomes and leaf peroxisomes isolated from *Arabidopsis*. Peptide MS fingerprinting analyses allowed us to identify novel proteins exists in either glyoxysomes or leaf peroxisomes. Some of these proteins contain no obvious PTS1 and PTS2. Combination of the transcriptomic and proteomic analyses is providing us a new insight into plant peroxisomal functions.

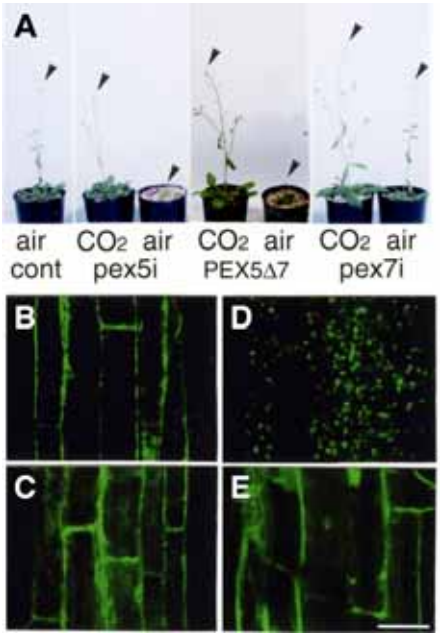


Figure 1. Differential contribution of two peroxisomal protein receptors to the maintenance of peroxisomal functions. Effect of CO_2 on the growth of pex5i, PEX5Δ7 and pex7i transgenic plants(A). Arrows indicate the top of an inflorescence apex. Import of PTS1-containing protein is inhibited in pex5i (B) but not in pex7i (D), while import of PTS2-containing protein is inhibited both in pex5i (C) and pex7i (E).

Bioinfomatic analysis of *Arabidopsis* genome predicted the presence of 15 kinds of genes for peroxisomal biogenesis factors, called *PEX* genes. We comprehensively investigated whether these predicted *PEX* genes function in peroxisome biogenesis by

generating knock-down mutants that suppress *PEX* gene expression by RNA-interference. Phenotypes of these mutants allowed us to identify the functional *PEX* genes, which can be classified into two groups, i.e. *PEX* genes regulating for peroxisomal morphology and peroxisomal protein import. These analyses revealed that PEX5, a receptor for PTS1, is involved in both lipid metabolism and photorespiration by regulating import of both PTS1- and PTS2-containing proteins (Figure 1A, 1B, 1C). In contrast, PEX7, a receptor for PTS2, is involved only in photorespiration by regulating import of PTS2-containing protein (Figure 1A, 1D, 1E).

III. Identification of novel components essential for peroxisome biogenesis

To better understand peroxisome biogenesis, we isolated a number of *Arabidopsis* mutants having aberrant peroxisome morphology (*apm* mutants) based on the different pattern of GFP fluorescence from the parent plant, GFP-PTS1, in which peroxisomes with normal size and number can be visualized with GFP.

It was revealed that one of these mutants, *apm1*, whose peroxisomes and mitochondria are long and reduced in number, is defective in DRP3A (Dynamin-related protein 3A). This finding shows that APM1/DRP3A protein is involved in both peroxisomal and mitochondrial division. We revealed that other mutants, *apm2* and *apm4*, exhibit the GFP fluorescence in not only peroxisomes but also the cytosol, and determined that both APM2 and APM4 are responsible for matrix protein transport on peroxisomal membranes. Analyses of other *apm* mutants and identification of *APM* genes will identify components necessary for peroxisome biogenesis and address the regulation of its mechanism.

IV. ER derived organelles for protein storing and defense strategy

Plant cells develop various types of endoplasmic reticulum (ER)-derived structures with specific functions. ER bodies are ER-derived compartments observed in *Arabidopsis*. They are rod-shaped structures (5 μm long and 0.5 μm wide) that are surrounded by ribosomes. ER bodies were widely distributed in the epidermal cells of whole seedlings. Rosette leaves had no ER bodies, but accumulated ER bodies after wounding or jasmonic acid treatment. This suggests that ER bodies function in the defense against herbivores. ER bodies include PYK10, a β-glucosidase with an ER retention signal, in seedlings. *Arabidopsis nai1* mutant has no ER bodies in whole plants and does not accumulate PYK10. *NAI1* encodes a transcription factor that has a basic-helix-loop-helix (bHLH) domain. Transient expression of *NAI1* induced ER bodies in the *nai1* mutant. These results provide direct evidence that NAI1 plays a role in the formation of ER bodies. We are trying to isolate additional components that are involved in ER body formation.

V. Vacuolar processing enzyme responsible for programmed cell death in plants

The vacuolar processing enzyme (VPE) belongs to the cysteine protease family found in higher plants and animals. VPE exhibits substrate specificity toward asparagine and aspartic acid residues, the amino acid well conserved at the processing sites of vacuolar proteins. Plant VPE homologues are separated into three subfamilies: seed type, vegetative type and embryogenic type. Seed type VPE is responsible for the maturation of seed storage proteins. On the other hand, the function of vegetative and embryogenic type VPEs was obscure. Recently, we revealed a novel function of VPE in various types of programmed cell death (PCD) in plants. The

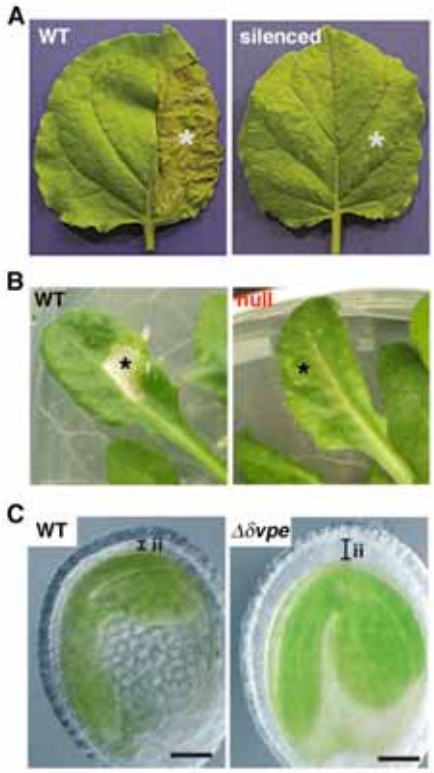


Figure 2. *VPE* deficiency suppresses various types of programmed cell death (PCD) in plants. (A) The non-silenced (WT) and *VPE*-silenced (silenced) *Nicotiana benthamiana* plants were infected with tobacco mosaic virus on halves of their leaves (indicated by asterisks). The photographs were taken after 24 hours. (B) Leaves of wild-type (WT) and VPE-null mutant (null) *Arabidopsis* plants were infiltrated with FB1, a fungal toxin (indicated by asterisks). The photographs were taken after 5 days. (C) Thickness of inner integuments (ii) of the seed coats is reduced in wild-type (WT) seed at the early stage, whereas it is not reduced in the *δvpe* mutant seed of *Arabidopsis*. PCD accompanies the shrinkage of two cell layers of the seed coat in wild type seeds.

evidence from extensive studies indicates that caspase activity is involved in plant PCD. VPE is identified as the proteinase that exhibits caspase activity in plants. The plant hypersensitive response (HR), a type of defense strategy, constitutes well-organized PCD. No HR occurs on the tobacco mosaic virus-infected leaves of *VPE*-silenced tobacco plants (Figure 2A). Fumonisin B1 (FB1), a fungal toxin, induced cell death in *Arabidopsis*. The features of FB1-induced cell death were completely abolished in the *Arabidopsis* VPE-null mutant, which

lacks all VPE genes (Figure 2B). *Arabidopsis* δVPE was recently identified as an embryogenic type VPE. δVPE specifically and transiently expressed in two cell layers (ii2-ii3) of the seed coat at an early stage of seed development. At this stage, PCD accompanying cell shrinkage occurs in ii2-ii3. In a *δvpe* mutant, shrinkage of these layers was delayed (Figure 2C). An ultrastructural analysis showed that the disintegration of the vacuolar membranes occurs before the cell death in these PCDs. These results suggest that VPE is involved in vacuolar collapse, which triggers PCD. Plants evolve a death strategy mediated by a vacuolar system, which is not seen in animals. Interestingly, a vacuolar enzyme is the key player in a plant-specific cell death system.

VI. Role of molecular chaperones on cell differentiation

Molecular chaperones are cellular proteins that function in the folding and assembly of certain other polypeptides into oligomeric structures but that are not, themselves, components of the final oligomeric structure. To clarify the roles of molecular chaperones on cell differentiation, we have purified and characterized chaperonin and Hsp70s and analyzed their roles in the translocation of proteins into chloroplasts.

Previously, we characterized a mitochondrial co-chaperonin (Cpn10), chloroplast co-chaperonins (Cpn20 and Cpn10) and a small heat shock protein from *Arabidopsis*. Recently, we started to characterize HSP90s, using a specific inhibitor of HSP90 or transgenic plants expressing mutated *Arabidopsis* HSP90. Preliminary data suggests that HSP90 is involved in various cellular signaling in *Arabidopsis*. The evolutional and functional characterization is now under experiments.

VII. Organellome database – Databases of plant organelles visualized with fluorescent protein, and protocols for functional analysis

The organellome database is a specialized database project dedicated to plant organelle research. This database is maintained by the Scientific Research of Priority Areas on “Plant Organelles”. To support this plant organelle research, we have been constructing a database consisting of three individual databases: the organellome database, the functional analysis database and external links about transcriptomics and proteomics. This database will be opened to all researchers as a public database. We expect that this database is going to be a useful analytical tool for plant organelle research.

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Original papers

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Yoshida, K., Kawachi, M., Mori, M., Maeshima, M., Kondo, M., Nishimura, M., and Kondo, T. (2005). The involvement of tonoplast proton pumps and $Na^+(K^+)/H^+$ exchangers in the change of petal color during flower-opening of morning glory, *Ipomoea tricolor* cv. Heavenly Blue. Plant Cell Physiol. *46*, 407-415.

Review articles

Mano, S., and Nishimura, M. (2005). Plant peroxisomes. In Vitamins and Hormones, Gerald Litwack ed. (Califoria, Elsevier Academic Press), pp. 111-154.

Yamada, K., Shimada, T., Nishimura, M., and Hara-Nishimura, I. (2005). A VPE family supporting various vacuolar functions in plants. Physiol. Plant. *123*, 369-375.

DIVISION OF MOLECULAR CELL BIOLOGY

Professor:	*OHSUMI, Yoshinori*
Research Associates:	*KAMADA, Yoshiaki*
	NODA, Takeshi
	SUZUKI, Kuninori
	NAKATOGAWA, Hitoshi
Technical Staff:	*KABEYA, Yukiko*
NIBB Research Fellow:	*OBARA, Keisuke*
Postdoctoral Fellows:	*ICHIMURA, Yoshinobu*
	SEKITO, Takayuki
	YOSHIMOTO, Kohki
	HANADA, Takao
	FUJIKI, Yuki
	OHNEDA, Mamoru
	OITA, Eiko
	ONODERA, Jun
	OKU, Masahide
	HARASHIMA, Toshiaki
Graduate Students:	*MATSUI, Makoto*
	KAGEYAMA, Takuya
Visiting Scientists:	*BABA, Misuzu*
	KAWAMATA, Tomoko
Technical Assistants:	*TSUKESHIBA, Kumi*
	ICHIKAWA, Rie
	KONDO, Chika
Secretary:	*HARA, Yoko*

This division aims to understand the physiological roles and molecular mechanism of autophagy in yeast and higher eukaryotes. All cellular activity is maintained by the balance between the synthesis and degradation of related proteins. It is now well known that the degradation process plays important roles in many physiological aspects. Autophagy is a bulk degradation system of cytosolic proteins and organelles in lysosome/vacuoles. Membrane dynamics during autophagy remain to be discovered.

I. Background

Upon nutrient starvation, the autophagic process starts as a building up of membrane structures (autophagosomes) in the cytoplasm. The autophagosome sequesters cytosol and organelles nonselectively. Then it is delivered to the vacuole/lysosome, and the cytoplasmic materials inside are degraded by vacuolar/lysosomal hydrolases. We discovered autophagy in a simple model organism, *Saccharomyces cerevisiae* and morphologically and genetically defined the whole process.

II. Hydrophobic residues in a ubiquitin-fold of Atg12 are important for autophagy in yeast

Atg12, a post-translational modifier, is activated and conjugated to Atg5 by a ubiquitin-like conjugation system, though it has no obvious sequence homology to ubiquitin. The carboxyl-terminal region of Atg12 is predicted to fold into a ubiquitin-like structure. We constructed amino-terminally truncated Atg12 mutants of the yeast *S. cerevisiae* according to the predicted secondary structure and showed that the ubiquitin-fold region of Atg12 is necessary and sufficient for both conjugation and autophagy. We also found that two hydrophobic residues within the ubiquitin-fold region are important for autophagy: mutations at Y149 affected conjugate formation catalyzed by Atg10, an E2-like enzyme, while mutations at F154 had no effect on Atg12-Atg5 conjugate formation but its hydrophobic nature was essential for autophagy. In the cells expressing F154 mutants, Atg8-PE conjugation, the other ubiquitin-like conjugation in autophagy, was severely reduced and autophagosome formation failed. A gel filtration analysis suggested that F154 plays a critical role in the assembly of a functional Atg12-Atg5·Atg16 complex requisite for autophagosome formation.

Crystal structure of *Arabidopsis thaliana* Atg12 homolog (AtATG12) has shown that it adopts the ubiquitin-like fold (Figure 1). Residues Y57 and F62, corresponding to Y149 and F154 in yeast, respectively, were found to be located adjacent to each other but form different hydrophobic patches, suggesting Atg12 utilizes two different hydrophobic patches for autophagy: one for conjugation reaction and the other for autophagosome formation.

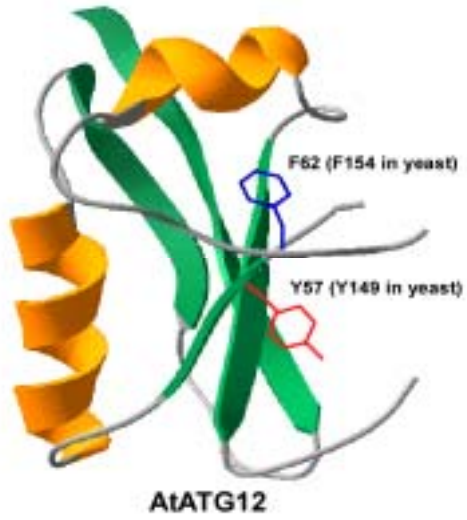

Figure 1. Two hydrophobic residues important for autophagy are located in close vicinity but form different hydrophobic patches.

III. Atg17 plays a pivotal role in autophagosome formation directed by Atg1 kinase activity

In the yeast *S. cerevisiae*, most of *ATG* genes are involved in not only the process of degradative autophagy, but also a biosynthetic process of vacuolar enzymes. In contrast, the *ATG17* gene is required specifically in autophagy. We found that the *atg17*Δ mutant under starvation condition was severely impaired in autophagosome formation. Autophagosomes with a smaller number and size were observed in the *atg17*Δ cells (Figure 2). We showed that Atg17 physically associated with an Atg1-Atg13 complex, and that it was enhanced under starvation conditions. The complex formation resulted in upregulation of an Atg1 kinase

activity, suggesting that Atg17 regulates the Atg1 kinase activity in concert with Atg13. Taken together, these results indicated that Atg17-Atg13 complex formation plays an important role in normal autophagosome formation via binding to and activating the Atg1 kinase.

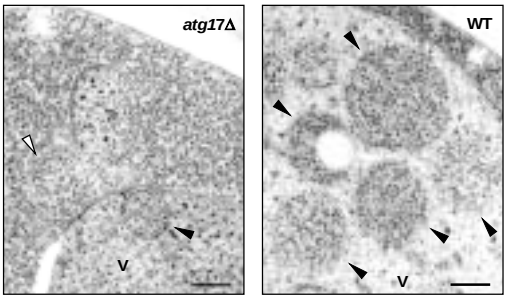

Figure 2. *ATG17* is essential for normal autophagosome formation. In a nitrogen-starvation medium, the *atg17*Δ mutant generates fewer and smaller autophagosomes (arrowheads) than those in the wild-type. Bar: 200 nm. V, vacuole.

IV. Identification and characterization of a novel autophagy-specific gene, *ATG29*

At present, at least 16 autophagy-related genes (*ATG*) have been identified. To search for novel genes involved in autophagy, we performed a genome-wide screen using a collection of ~4,500 yeast single-gene deletion mutants and identified a novel ORF, *ATG29*/*YPL166w*. *atg29*Δ cells were sensitive to starvation, and induction of autophagy was severely retarded. However, the Cvt (Cytoplasm to vacuole transport) pathway, which shares mechanistic components with autophagy, operated normally. Therefore, Atg29 is the second protein specifically required for autophagy. Furthermore we observed that an Atg29-GFP fusion protein was localized to the PAS, at which most Atg proteins are colocalized (Figure 3). From these results, we propose that Atg29 functions in autophagosome formation at the PAS in collaboration with other Atg proteins.

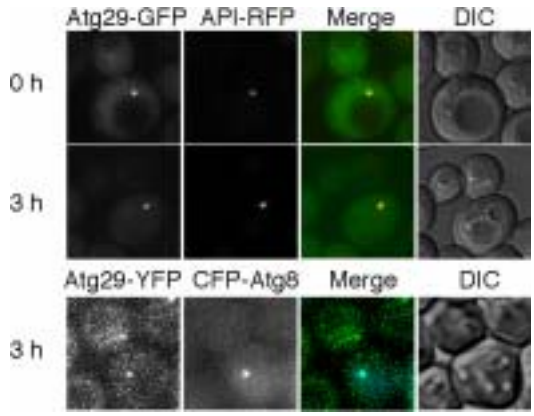

Figure 3. Localization of Atg29 at the PAS. Growing (0 h) and rapamycin treated (3 h) cells were observed by fluorescence microscopy. Atg8 and API were used as markers for the PAS.

V. Autophagy is essential for protein synthesis under nitrogen starvation

Autophagy is involved in degradation of cytoplasmic components including a significant amount of proteins, rRNA and phospholipids of organelle membranes. So far, the precise fate and the physiological importance of each degradation products generated by autophagy had been unclear. Recently, we examined changes in protein synthesis after long term nitrogen starvation and found that a heat shock protein (Hsp26p) and an enzyme involved in an amino acid biosynthesis (Arg1p) were up-regulated by nitrogen starvation in wild-type cells, but these changes did not occur in *atg* mutant cells (Figure 4A). The mRNA levels in the *atg* mutant cells were as high as those in the wild-type cells under nitrogen starvation (Figure 4B). Thus, it was suggested that synthesis of these proteins was inhibited at the translational step. We also found that bulk protein synthesis was substantially reduced in the *atg* mutant cells under nitrogen starvation compared with the wild-type cells. The wild-type cells maintained amino acid levels to survive under starvation conditions. In contrast, the total intracellular amino acid pool was reduced in the *atg* mutant cells, and the levels of several amino acids fell below critical values. These results indicate that autophagy is required to maintain physiological amino acid levels under nitrogen starvation conditions, and inability to synthesize proteins newly may explain most phenotypes in autophagy-defective mutants (e.g. inability of spore formation and so on).

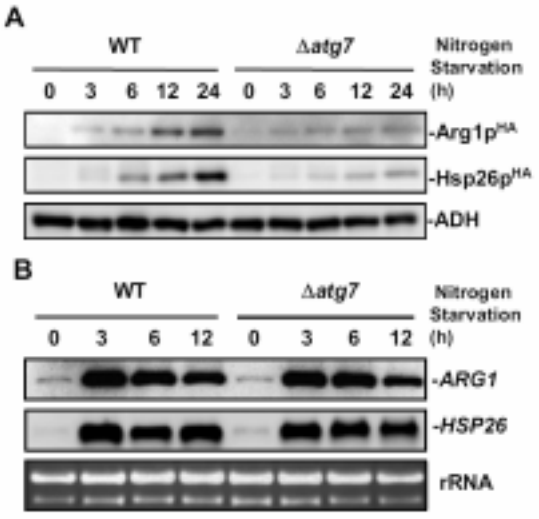

Figure 4. Autophagy is required for the expression of nitrogen starvation-induced proteins at the translational level. (A) Immuno-blot analysis of starvation-induced proteins, Arg1p and Hsp26p. (B) Northern-blot analysis of starvation-induced gene, *ARG1* and *HSP26*. ADH and rRNA were used as loading controls.

VI. Autophagy plays important roles in various aspects of plant life

By establishing an autophagy-monitoring system in a whole plant we were able show that *atatg* mutants were defective in autophagy. Our recent studies indicated that the autophagy defective mutants exhibited a reduction in the growth rate of roots under nitrogen-starved conditions and early senescence phenotype even in nutrient-rich conditions. In addition, hypersensitive response cell death was accelerated in the mutants during the plant innate immune response. These results indicate that, regardless of nutrient conditions, autophagy plays important roles in various aspects in plant life.

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Original papers

Hamasaki, M., Noda, T., Baba, M., and Ohsumi, Y. (2005). Starvation triggers the delivery of the endoplasmic reticulum to the vacuole via autophagy in yeast. Traffic *6*, 56-65.

Hanada, T., and Ohsumi, Y. (2005). Structure-Function relationship of Atg12, a ubiquitin-like modifier essential for autophagy. Autophagy *1*, 110-118.

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Komatsu, M., Waguri, S., Ueno, T., Iwata, J., Murata, S., Tanida, I., Ezaki, J., Mizushima, N., Ohsumi, Y., Uchiyama, Y., Kominami, E., Tanaka, K., and Chiba, T. (2005). Impairment of starvation-induced and constitutive autophagy in Atg7-deficient mice. J. Cell Biol. *169*, 425-434.

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Onodera, J., and Ohsumi, Y. (2005). Autophagy is required for maintenance of amino acids levels and protein synthesis under nitrogen starvation. J. Biol. Chem. *280*, 31582-31586.

Pyo, J.O., Jang, M.H., Kwon, Y.K., Lee, H.J., Jun, J.I., Woo, H.N., Cho, D.H., Choi, B., Lee, H., Kim, J.H., Mizushima, N., Ohsumi, Y., and Jung, Y.K. (2005). Essential roles of Atg5 and FADD in autophagic cell death: dissection of autophagic cell death into vacuole formation and cell death. J. Biol. Chem. *280*, 20722-20729.

Sugawara, K., Suzuki, N.N., Fujioka, Y., Mizushima, N., Ohsumi, Y., and Inagaki, F. (2005). Structural basis for the specificity and catalysis of human Atg4B responsible for mammalian autophagy. J. Biol. Chem., in press.

Suzuki, N.N., Yoshimoto, K., Fujioka, Y., Ohsumi, Y., and Inagaki, F. (2005). The crystal structure of plant ATG12 and its biological implication in autophagy. Autophagy *1*, 119-126.

Review article

Bassham, D.C., Laporte, M., Marty, F., Moriyasu, Y., Ohsumi, Y., Olsen, L.J., and Yoshimoto, K. (2005). Autophagy in development and stress responses of plants. Autophagy, *2*, in press.

DIVISION OF Cell Structure

Associate Professor: *OGAWA, Kazuo*

Dynein is a molecular motor that carries cargoes to the direction of centriole from the cell periphery along the microtubules in a cell. It is composed of three domains: stem, motor, and stalk. Targeting of dynein to specific sites of cargoes may be related to the NH_2-terminal one-thirds of molecule constituting stem where amino acid sequence diversity was found between dynein superfamily (cytoplasmic and axonemal dynein), while the COOH-terminal two-thirds of molecule showing sequence conservation in distantly related species constitutes motor. Having interrupted long heptapeptide repeats in the sequence, the stalk takes an extended flexible structure and binds to microtubule in an ATP-dependent manner. Thus, dynein binds to microtubule at stalk domain of itself and cargoes at stem domain of itself.

The mechanism how cytoplasmic and axonemal dyneins target the cargoes has been gradually made clear in terms of molecules participated in. Cytoplasmic dynein is linked with dynactin complex containing at least 10 proteins. It is via dynactin complex that dynein targets to a receptor of cargo membranes.

In flagellar and ciliary movement, outer and inner dynein arms are projected from the A-subfiber of peripheral doublet microtubule of axoneme corresponding to the cargo. They bind to the B-subfiber of neighboring doublet microtubule in an ATP-dependent manner. Axonemal dynein is hardly detached from the A-subfiber being the cargo, while the cargoes of cytoplasmic dynein are thought to be detached from the motor after arrival to cell center to recruit the motor. Thus, there may be different targeting mechanism for dynein superfamily to specific sites of the cargoes.

When Triton-model sperm were exposed to hard condition such as 0.5 M KCl or NaCl (so called chemical dissection), outer dynein arm was detached from the A-subfiber. Triton-sperm losing outer dynein arm swam with a half beat frequency of control sperm. Re-binding of outer dynein arm onto the A-subfiber was possible by remixing the extracted Triton-sperm with the extract in a low salt concentration solution. Recovered Triton-model sperm swam with a normal beat frequency. Thus, a high salt extract might contain some proteins necessary for correct positioning of outer dynein arm and scaffold proteins to mediate binding of outer arm dynein onto the A-subfiber. During the course of characterizing proteins containing in the extract, we found a novel protein with molecular mass of 58 kDa designated as ap58. Immuno-electron microscopy using antibodies raised against recombinant ap58 shows that gold-particles are found at 25 nm repeat along the length of axoneme coinciding with the repeat of outer dynein arm (longitudinal sections of Figure 1). Thus, we conclude that ap58 is binding to in situ outer dynein arm.

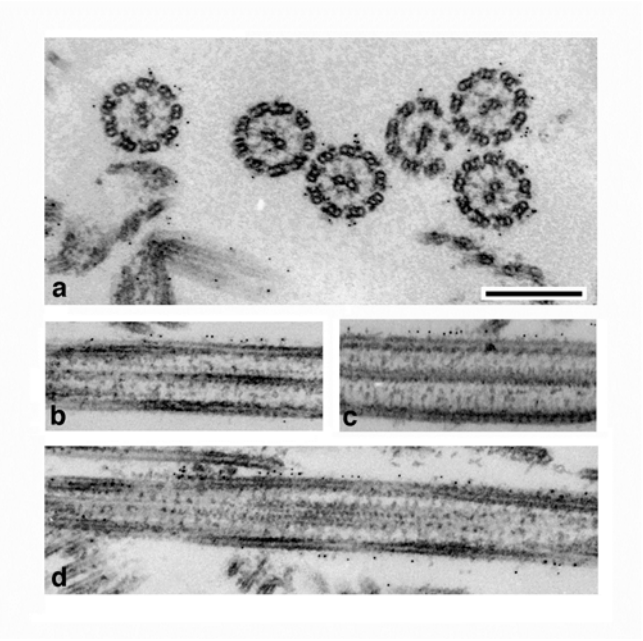

Figure 1. Immuno-electron micrographs to show the localization of ap58 within the sea urchin sperm axonemes. a: cross section; b-d: longitudinal sections (Ogawa and Inaba).

Publication List:

Original paper

Morii, H., Shimizu, T., Mizuno, N., Edamatsu, M., Ogawa, K., Shimizu, Y., and Toyoshima, Y.Y. (2005). Removal of tightly bound ADP induces distinct structural changes of the two tryptophan-containing regions of the ncd motor domain. J. Biochem. *138*, 95-104.

LABORATORY OF CELL SOCIOLOGY

Research Associate: *HAMADA, Yoshio*

Animal organs consist of several types of cells. They are organized in an ordered fashion wherein the proportion of each cell type is constantly maintained. The ordered cell arrangement and proportion are built up during organogenesis by cell-cell interactions. Since *Notch* has been postulated to play a role in cell fate decision by mediating cell-cell interactions, we are trying to find out cellular and molecular mechanisms working in organogenesis by studying the function of *Notch*.

Organogenesis of the mouse placenta occurs during early pregnancy, embryonic days 7-9, before the establishment of molecular transport mechanisms in the definitive placenta takes place. Trophoblasts not adjacent to the inner cell mass differentiate into trophoblast giant cells and lie at the outside, forming interface with maternal deciduas. The polar trophectoderm gives rise to cells of the chorion, and ectoplacental cone. They produce labyrinthine and spongiotrophoblast layer, respectively. While maternal red blood cells begin to perfuse into trophoblast cell layers and reach at labyrinthine layer by E9.5, invasion of embryonic allantoic mesenchyme into labyrinthine layer and differentiation of fetal red blood and endothelial cells which line fetal capillary takes place around E9.5.

The mutation in the ankyrin repeats of mouse *Notch2* results in embryonic lethality by embryonic day 11.5 due to a poor maternal vascular beds formation. The mutant placenta showed an early invasion of angiogenic allantoic mesenchyme followed by premature formation of fetal blood vessels in the mutant placentas as early as E9.0. However, specification of trophoblast subtypes appeared not to be drastically disturbed. Thus, in the developing mouse placenta, *Notch2* is unlikely involved in cell fate decision, but rather participates in a formation of circulatory systems in the labyrinth layer where the expression of *Notch2* was detected (Figure 1). Although inadequate formation of maternal vascular beds was partially restored by aggregating mutant diploid embryos with wild type tetraploid embryos (Figure 2), networks of the mutant fetal vasculatures and maternal blood spaces appeared still comprised in the 4N chimeric placenta. These results indicate that *Notch2* works promotive in vasculogenesis. Thus, *Notch2* is not cell autonomously required for the early cell fate determination of subtypes of trophoblast cells, but plays an indispensable role in coordinated maternal and fetal vasculogenesis in the developing mouse placenta.

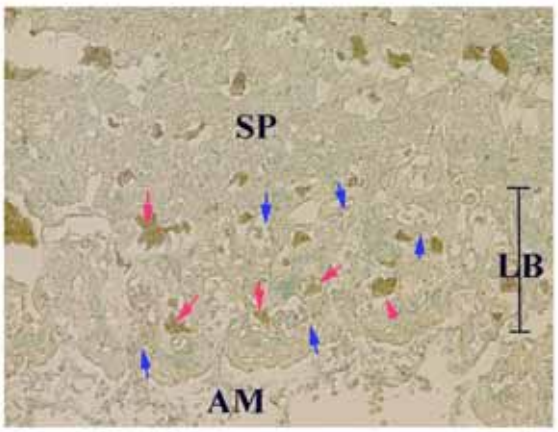

Figure 1. Expression of *Notch2* gene in developing mouse placenta. A cryo-section of *Notch2*$^{+/LocZ}$ placenta at E10.0 was incubated in X-gal staining solution. Trophoblast in maternal vascular bed side expresses the gene, but not in allantoic mesenchyme (AM) side in labyrinth layer (LB). The expression also occurs in the mesenchyme. Enucleated maternal red blood cells are indicated by red arrow heads. Nucleated fetal red blood cells are by blue arrow heads**.**

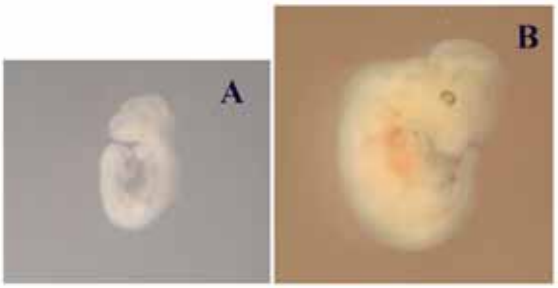

Figure 2. Survival of *Notch2* mutant embryo in a chimera with tetraploid wild type embryo. The mutant obtained in the intercrosses by the heterozygotic mice shows developmental retardation at E10.5 (A) and die before E11.5. The mutant is able to survive in a chimera with wild type tetraploid embryo until E13.5 (B).

Publication List:

Original papers

Gotoh, N., Manova, K., Tanaka, S., Murohashi, M.Hadari, Y., Lee, A., Hamada, Y., Hiroe, T., Ito, M., Kurihara, T., Nakazato, H., Shibuya, M., Lax, I., Lacy, E., and Schlessinger, J. (2005). The docking protein FRS2alpha is an essential component of multiple fibroblast growth factor responses during early mouse development. Mol. Cell. Biol. *25*, 4105-4116.

Nakamura, Y., Hamada, Y., Fujiwara, T., Enomoto, H., Hiroe, T., Tanaka, S., Nose, M., Nakahara, M., Yoshida, N., Takenawa, T., and Fukami, K. (2005). Phospholipase C–δ1 and –δ3 are essential in trophoblast for placental development. Mol. Cell. Biol. *25*, 10979-10988.

DIVISION OF REPRODUCTIVE BIOLOGY

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	OHNO, Kaoru
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	CHAUBE, Radha
	GUAN, Guijun
	IJIRI, Shigeho
	KANEKO, Hiroyo
	LAU, En-Lieng
	OHTA, Kohei
	PAUL, Bindhu
	SAKAI, Fumie
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	ZHOU, Linyan
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Technical Assistants:	*HARA, Ikuyo*
	HAYAKAWA, Rie
	HIRAKAWA, Eri
	SHIBATA, Emiko
	TAKAKI, Chikako
Secretary:	*SHIMADA, Yu*

Our research focuses on (1) the identification of regulators and steroidal mediators involved in sex determination, gonadal sex differentiation and gametogenesis, and (2) the mechanisms of synthesis and action of these mediators.

I. Molecular mechanisms of sex determination and gonadal sex differentiation

Fish have a range of gonadal differentiation types including gonochoristic species as well as hermaphroditic species. We have been using two fish species to investigate the molecular mechanisms of sex determination (medaka, *Oryzias latipes*) and gonadal sex differentiation (Nile tilapia, *Oreochromis niloticus*).

Medaka possesses a stable genetic XX/XY sex determining system. Using positional cloning and detailed sequence analysis of BAC clones by shotgun sequencing, we identified *DMY* (DM domain gene on the Y chromosome) as a strong candidate for the sex-determining gene of medaka. *DMY* encodes a protein of 267 amino acids including the highly conserved DM domain. Our loss- (natural mutants analysis and knock-down) and gain- (over-expression using transgenic techniques) of-function studies indicate that *DMY* is the sex-determining gene of medaka. *DMY* provides the first example of a sex-determining gene in non-mammalian vertebrates.

In tilapia, all genetic female (XX) or male (XY) broods can be obtained by artificial fertilization of normal eggs (XX) and sex-reversed, pseudo male sperm (XX), or normal eggs (XX) and super male sperm (YY), respectively. Steroidal enzymes P450scc, 3β-HSD, and P450c17 are found at high levels in female gonadal anlargen of tilapia at 7-10 days posthatching, but are only seen weakly in males and not until 30 days posthatching. Further, the enzyme aromatase is only detected in ovaries. Treatment of XX fry with fadrozole (aromatase inhibitor) or tamoxifen (estrogen receptor antagonist) caused complete sex reversal to functional males. These results suggest that endogenous estrogens are critical for directing initial ovarian differentiation in tilapia. We recently showed that Foxl2 and Ad4BP/SF-1 play important roles in the differential expression of aromatase in XX tilapia gonads during sex differentiation. The critical role of Foxl2 in ovarian differentiation was confirmed by male sex reversal of XX transgenic tilapia carrying a dominant-negative mutant of *Foxl2*. In XY fry, *DMRT1* gene is expressed male-specifically in testicular Sertoli cells prior to and during sex differentiation. XX tilapia carrying extra copies of tilapia *DMRT1* as a transgene induced various degrees of gonadal changes including complete transformation to testis. These results suggest an important role for DMRT1 in testicular differentiation in tilapia.

II. Molecular mechanisms of sex change

The gobiid fish, *Trimma okinawae*, possesses ovarian and testicular tissues simultaneously in its gonad and is able to change sex repeatedly in both directions depending on its social surroundings, thus providing an excellent animal model to investigate molecular mechanisms of sex change. Aquarium experiments were carried out in the laboratory. Two males (M/M) or two females (F/F) were kept in separate tanks. In the M/M aquarium, the smaller male changes its sex to female. On the contrary, the larger female changes its sex to male in the F/F aquarium. Behavioral changes occurred within 30 minutes of social manipulation (Figure 1). After pairing, the larger male or female attacked the smaller fish, which fled and often hid in a nest. After 30 min., however, the larger fish began to court the smaller fish. These results suggest that the brain sex of a sequential hermaphrodite is determined independently of gonadal effect and is induced only by social cues.

Figure 1. Sexual behavior of *Trimma okinawae* immediately after pairing

The involvement of gonadotropins in sex change was examined by determining the changes in gonadotropin (FSH and LH) receptor gene expression in gonads during the onset of sex changes from female to male and male to female. Expression appears to be related to sexual phase with quick location switching of the two genes after social manipulation to stimulate sex change. This differential expression of the two gonadotropin receptor genes is an earlier event occurring in gonads after pairing and plays a critical role in the sex change.

III. Embryonic development of gonadotropin-releasing hormone (GnRH) neurons

Neurons that synthesize and release GnRH are essential for the central regulation of reproduction. X-linked Kallmann syndrome (X-KS), characterized by failed gonadal function, is caused by a mutation in *KAL1*, which is suggested to regulate the development of GnRH neurons. Since rodents lack *Kal1* in their genome, the pathogenesis of X-KS has been difficult to study. We identified a *KAL1* ortholog in the medaka. Antisense knockdown of the *KAL1* ortholog in the transgenic medaka in which GnRH neurons were visualized with GFP led us to observe the inappropriate accumulation of GnRH neurons in the olfactory compartment and loss of their ability to migrate into the forebrain (Figure 2). This result was consistent with that reported in a fetus with X-KS. Thus, our data demonstrate that X-KS can be phenocopied by antisense knockdown of *kal1* and can be directly monitored in the transgenic medaka. Taken together, the medaka system provides a useful in vivo model for studying disorders of GnRH deficiency including X-KS.

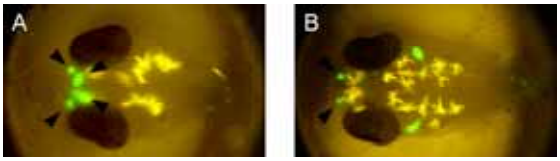

Figure 2. Knockdown of *kal1* in the transgenic medaka that express GFP in gnrh1 (panel A) and gnrh3 (panel B) neurons resulted in the deficient migration of GnRH neurons (arrowheads).

IV. Endocrine regulation of oocyte maturation

The process of oocyte maturation is the time period between the resumption of meiosis and the second meiotic metaphase, and thus, is a prerequisite for successful fertilization. It has been well established that hormones play an important role in inducing oocyte maturation in invertebrates as well as vertebrates. Our studies using vertebrate (fish) and invertebrate (starfish) models have revealed that the basic mechanisms involved in oocyte maturation are the same in these two species, despite the differing chemical nature of the hormonal agents involved. In both species, three major mediators have been shown to be involved: GSS, 1-methyladenine (maturation-inducing hormone, MIH), and maturation-promoting factor (MPF) in starfish, and LH, 17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-DP) (MIH), and MPF in fish (Figure 3).

The primary hormone involved in starfish reproduction has been called the gonad-stimulating hormone (GSS), which is a peptide hormone produced in the radial nerves. Although the hormonal activity of GSS has been known for a long time, only recently has it been possible to determine its primary structure. GSS has been purified from the radial nerves of starfish (*Asterina pectinifera*), and the amino acid sequence was determined. GSS is a heterodimeric peptide with a molecular weight of 4737, consisting of A and B chains; the A chain contains 24 residues and the B chain 19 residues. Chemically synthesized GSS is as active as native GSS in the homologous *in vitro* GVBD assay.

In fish, LH acts on ovarian follicle cells to produce fish MIH (17α, 20β-DP). 17α, 20β-DP is synthesized by a two-step process involving two ovarian cell layers, the thecal and granulosa cells. Unlike other steroid hormones, 17α, 20β-DP binds to a novel, G-protein-coupled membrane progestin receptor (non-genomic action), leading to the *de novo* synthesis of cyclin B, the regulatory component of MPF, which activates a preexisting 35-kDa cdc2 kinase via phosphorylation of its threonine 161 by a threonine kinase, thus producing the 34 kDa active cdc2. Upon egg activation, MPF is inactivated by degradation of cyclin B. We showed that the 26S proteasome initiates cyclin B degradation through the first cut of its NH_2 terminus at lysine 57.

An endocrine-disrupting chemical, diethylstilbestrol (DES), a nonsteroidal estrogen, triggers oocyte maturation in goldfish (*Carassius auratus*) and zebrafish (*Danio rerio*). The morphology (the time course of the change in germinal vesicle breakdown) and an intracellular molecular event (the *de novo* synthesis of cyclin B) induced by DES are indistinguishable from those induced by 17α, 20β-DP. Both 17α, 20β-DP- and DES-induced oocyte maturation is inhibited by an antibody against the membrane progestin receptor. These results suggest that DES may act on the membrane progestin receptor as an agonist of 17α, 20β-DP.

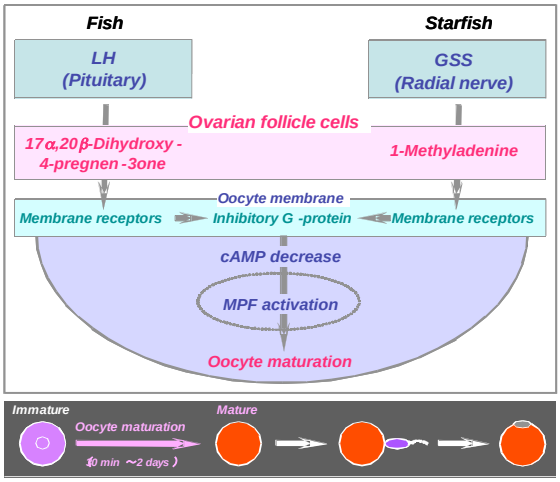

Figure 3. Regulation of oocyte maturation in fish and starfish

Publication List:

Original papers

Bhandari, R.K., Nakamura, M., Kobayashi, T., and Nagahama, Y. (2005). Suppression of steroidogenic enzyme expression during androgen-induced sex reversal in Nile tilapia (*Oreochromis niloticus*). Gen. Comp. Endocrinol., in press.

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Sexual dimorphism manifests most obviously in the gonads (testis and ovary), and is thereafter observed in other parts of the body such as the external genitalia, muscle, and brain. This process of sex differentiation is divided into three steps. The first step occurs at fertilization, during which the sexes of fertilized eggs are determined genetically according to a combination of sex chromosomes. During the second step, individuals carrying XY and XX sex chromosomes develop the testis and ovary, respectively, in the case of mammals. This gonadal sex differentiation usually proceeds during fetal stages, and subsequently sex steroids synthesized in the sexually differentiated gonads control the sexes of the other tissues. Therefore, the gonadal sexes are quite important for the sex differentiation of animals.

A number of transcription factors are known to play crucial roles in the process of gonadal differentiation. Some of these genes such as *SRY*, *WT1*, *DAX-1*, *SOX9* and *ARX* were identified as the genes responsible for human diseases that display structural and functional defects in the gonads. Functions of the other genes such as *Ad4BP/SF-1*, *Emx2*, *M33*, and *Lhx9* were elucidated by the phenotypes of the gene-disrupted mice. In addition, their expression profiles in the sexually differentiating gonad strongly suggested their functional significance at the early stage of gonadal differentiation. However, it remains to be elucidated how the genes are expressed by upstream regulators. Studies from the aspect above are quite important to define the gene regulatory cascade and the molecular mechanisms mediating sex differentiation of the gonad.

Tentatively, we have hypothesized that the sexually indifferent gonads determine their sexes under the control of two opposite signals, one of which is the signal for male (testicular) differentiation and the other is the signal for female (ovarian) differentiation. Possibly the nature of the signals is transcriptional activities of the transcription factors expressed in the sexually differentiating gonads, or otherwise activities of certain types of growth factors. The study in this division has been performed mainly from the aspect of the transcriptional control of the genes implicated in gonadal sex differentiation.

I. Boundary of *Ad4BP/SF-1* gene locus

The spatial and temporal control of gene expression is essential for establishment of cell fates in multicellular organisms and the transcriptional control requires appropriate interaction between enhancers and basal promoter. In certain genes, this interaction is enhanced by additional regulatory elements such as the locus control region (LCR).

The LCR at the *β-globin* locus is one of the most intensively studied elements, and in the case of the chicken *β-globin* LCR consists of four DNase I hypersensitive (HS) sites, suggesting an open chromatin structure that, unlike closed chromatin, can be easily accessed by transcription factors. Because many regulatory fragments including insulators show nuclease hypersensitivity, we examined whether DNase I HS sites are present between *GCNF* and *Ad4BP/SF-1* gene loci. Nuclei prepared from Y-1 cells, which are derived from mouse adrenal cortex and have robust expression of Ad4BP/SF-1, were treated with DNase I, and three DNase I HS sites (adHS1, 2, and 3) were found between the last exon of *GCNF* and the first exon of *Ad4BP/SF-1* (Figure 1). CTCF, a ubiquitous nuclear protein with multiple zinc finger motifs, is regarded as an insulator-binding protein solely known to date in vertebrates. In order to examine whether CTCF contributed to the nuclease hypersensitivity on adHS sites, we performed chromatin immunoprecipitation (ChIP) assays using anti-CTCF antibody. CTCF-attached DNA fragments were amplified by PCR using primers designed against regions around the adHS sites. Interestingly, CTCF was found to bind with only a region located downstream of adHS1 but not located in the proximity of adHS2 and 3 (CTCF in Figure 1). These data suggest that the DNase I hypersensitivity of adHS1 is most likely due to CTCF-mediated changes in chromatin architecture while the hypersensitivity at adHS2 and 3 results from chromatin structures generated by proteins other than CTCF.

The nuclear matrix is the fraction that is not solubilized by treatment with high salt buffer, and is thought to comprise the nuclear architecture that provides a platform for nuclear events such as transcription and have suggested that the nuclear matrix defines a transcriptional unit by establishing a chromatin loop. In addition, a collection of DNA fragments known as a matrix attachment region (MAR) is reported to be able to protect a transgene from position effects. It is believed that this phenomenon results from insulation by the MAR to block expansion of silent heterochromatin. We therefore examined whether a MAR exists around the adHS sites for the insulator activity on *Ad4BP/SF-1* locus. Interestingly, a fragment corresponding to adHS2 and adHS3 exclusively bound to nuclear matrix (MAR in Figure 1).

Posttranslational modifications of amino terminal tails of histones are thought to be epigenetic marks governing control of gene expression. Acetylation at lysine residues of the tail is known to correlate with activation of transcription, and chromatin associated with an active promoter contains mostly acetylated histones. ChIP assays with anti-AcH3 and AcH4 demonstrated that acetylated boundary was formed around adHS1 and proximal to the transcription start site (AcH3/H4 boundary in Figure 1).

Taken together, we have demonstrated currently the existence of an insulator defining the transcriptional boundary between *Ad4BP/SF-1* and *GCNF* gene loci by DNase I hypersensitive assays. Our further characterization indicated that these HS sites correspond to regions containing potential binding sites for CTCF and nuclear matrix. Importantly, these sites are almost coincident with a divergence of acetylation pattern of histone H3 and H4 tails and constitute a boundary between active and inactive chromatin domains. The correlation between the binding of CTCF and nuclear matrix attachment sites to a functional boundary for transcription is consistent with previous reports that CTCF and the nuclear matrix are required for insulator activities.

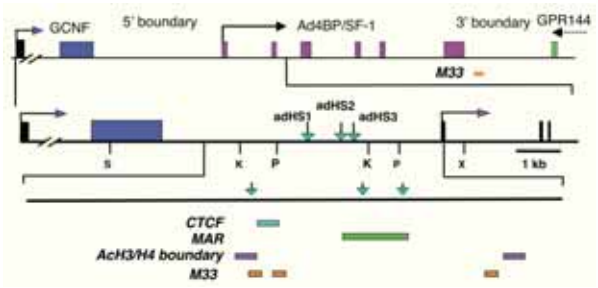

Figure 1. Structure of boundary between *Ad4BP/SF-1* and *GCNF* gene loci. *Ad4BP/SF-1* gene is localized at the downstream of another nuclear receptor gene, *GCNF*, and at the upstream of uncharacterized *GPR144* gene. DNase I hypersensitive sites, adHS1, adHS2, and adHS3, CTCF binding site, nuclear matrix binding site (MAR), acetyl histone H3 and H4, and M33 binding sites are schematically presented. All these sites are localized between *GCNF* and *Ad4BP/SF-1* gene.

II. Function of M33 as a factor bound to the boundary of *Ad4BP/SF-1* locus

In mammals, multiple orthologues of *PcG* components members have been identified. *Polycomb (Pc)* has three mouse counterparts, *M33 (Mpc1)*, *Mpc2*, and *Mpc3*. Likewise, other *PcG* members have multiple counterparts. Therefore, mammalian PcG complexes are thought to be comprised of distinct sets of constituent group members. In fact, mammalian PcG complexes were recently sub-divided into two distinct types according to their biochemical and functional properties. The first complex, containing Eed and Eenhancer of Zeste (Enx1 and Enx2), appears to be required to initiate repression of target gene expression in early development, whereas a second complex containing M33, Mpc2, rae28/Mph1, Bmi1, and Mel18 appears to be required for stable maintenance of the repression state.

Previously we showed that disruption of the murine *M33* gene, a *PcG* member, displayed posterior transformation of the vertebral columns and sternal ribs. In addition, hypoplasia and sex-reversal of the gonads has been observed. In the current study, we identified defects in the splenic and adrenal formation of *M33*-knockout (KO) mice on a C57BL/6 genetic background. Histological examination revealed that the vascular endothelium and its surrounding structures were disorganized. Immunohistochemical analyses demonstrated disturbances in vascular formation and colonization of immature hematopoietic cells. These splenic phenotypes observed in the *M33*-KO mice were quite similar to those seen in *Ad4BP/SF-1* knockouts. Moreover, we observed that the adrenal gland of *M33*-KO and *Ad4BP/SF-1* heterozygous KO mice are smaller than that of wild-type. These phenotypic similarities strongly suggested the presence of genetic and functional correlations between the two genes. In fact, western-blot, immunohistochemistry, and RT-PCR analyses of the *M33* knockouts all indicated significantly decreased expression of Ad4BP/SF-1, indicating that M33 is an essential upstream regulator of *Ad4BP/SF-1* gene.

Upstream stimulatory factors 1 (USF-1 and USF-2), Sox9, and Wt1(-KTS form) have all been identified as direct-acting positive regulators of *Ad4BP/SF-1* gene transcription. These factors bind recognition sites in the 5' upstream region of the *Ad4BP/SF-1* gene and thereby activate gene transcription. However, the present genetic analyses could not distinguish whether *Ad4BP/SF-1* is regulated directly or indirectly by M33. Therefore, we tested whether M33 directly binds to *Ad4BP/SF-1* gene locus by ChIP assay using antibody to M33. Interestingly, the assays with Y-1 adrenocotical cells revealed that target sites of M33-containing PcG complex are present at the upstream region of the first exon and the immediately downstream region of the last exon of *Ad4BP/SF-1* gene (Figure 1). As described above, our recent study demonstrated that the intergenic region between *Ad4BP/SF-1* and *GCNF* gene contains functional architectures such as DNase I hypersensitive sites, a nuclear matrix attachment region (MAR), and CTCF binding sites. Moreover, ChIP assays showed a discontinuous pattern of histone H3 and H4 acetylation over the intergenic region. These observations strongly suggested that this region forms a boundary, the so-called insulator, between the two transcriptional units, *Ad4BP/SF-1* and *GCNF*. This assumption was consistent with a previous observation that CTCF and nuclear matrix are required for insulator activity. In this regard, it seemed interesting to examine whether M33-containing PcG complex binds to this intergenic region because PcG complex is known to function in formation of inactive chromatin arrays. Expectedly, our ChIP assays demonstrated that M33-containing PcG complex binds to sites adjacent to and overlapped with the CTCF binding region and MAR, respectively. Similarly, it is noteworthy

that M33-containing PcG complex bound to region adjacent to the last exon of *Ad4BP/SF-1* gene. Although further examination is required, it is likely that M33-containing PcG complex is implicated in formation of intergenic boundary. This presumptive function of PcG complex is supported by previous *Drosophila* studies. Mihaly *et al.* (1997) showed that a *cis*-regulatory element, Fab-7, consists of a boundary element and PRE, both of which are implicated in regulation of parasegmental-specific expression of bithorax genes. In the case of *gypsy* insulator, Gerasimova and Corces (1998) demonstrated that a *gypsy* insulator binding protein, mod(mdg4), requires Pc, a PcG component, for the insulator function. Considering these observations, the current studies strongly suggested that PcG complex together with CTCF and nuclear matrix forms intergenic boundary between *Ad4BP/SF-1* and *GCNF* genes, although functional and physical correlation of PcG complex with CTCF and/or nuclear matrix remains to be elucidated (Figure 2).

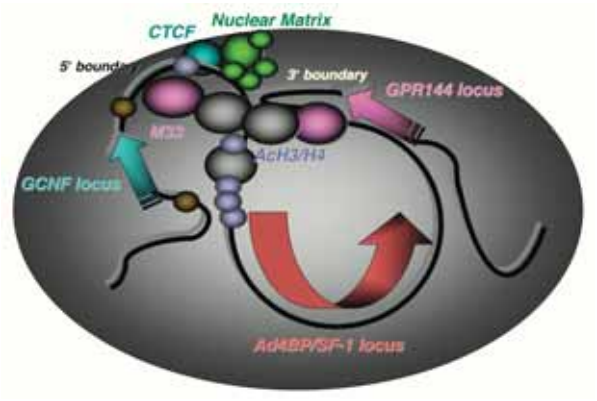

Figure 2. Intranuclear structure of *Ad4BP/SF-1* gene locus. Through binding of the multiple factors at the upstream and downstream of *Ad4BP/SF-1* gene, the gene locus appears forms structurally and functionally a single transcription unit.

It has been well accepted that PcG complex directly binds to target gene loci to keep silent chromatin conformation. Nevertheless, in our current study, multiple binding regions of the M33-containing PcG complex were found to lie at the 5' boundary region of the transcriptionaly active *Ad4BP/SF-1* gene. Although our findings seemed contradictory to the accepted notion, recent ChIP analyses with antibodies to PcG components have accumulated observations similar to the present study, showing that PcG complex binds to not only inactive but active gene loci. Accordingly, it is reasonable to assume that PcG complex is implicated in the formation of transcriptionaly active chromatin unit. Based on these observations together with our current results, *Ad4BP/SF-1* gene is thought to form a transcriptional unit through forming complexes M33 containing polycomb, CTCF, and nuclear matrix. Considering that the complex formation is tightly coupled with the gene transcriptional regulation, it might be noteworthy that structural and thus functional properties of this region might be differentially modified according to developmental stages, tissues and possibly sexes.

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The complex morphogenesis of organisms is achieved by consecutive cell-to-cell interactions during development. Recent studies suggest that growth factors play crucial roles in controlling such intercellular communications in a variety of organisms. In addition to secretory factors that trigger intracellular signaling, transcription factors that act in the nucleus to regulate gene expression are thought to be essential for the determination of cell fates. Our main interest is to know how pattern formation and morphogenesis during development is regulated by these growth factors and transcription factors. We address this problem using several model animals, including frog, fly and ascidian, employing embryology, genetics, molecular and cellular biology, and biochemistry. In addition, we have recently introduced genomics technologies to elucidate the precise genetic programs controlling early development.

I. Molecular and cellular mechanism of vertebrate gastrulation

Gastrulation is one of the most important processes during morphogenesis of early embryo, involving dynamic cell migration and change in embryo shape. Almost all animals undergo gastrulation to form the gut. In spite of its importance, the mechanism underlying the event has just begun to be studied at molecular level. During *Xenopus* gastrulation, mesodermal cells migrate to the inside of the embryo and move on the blastocoel roof. One of the important mechanisms for this process is the cell movement called “convergent extension (CE)”. As convergent extension begins, cells are polarized and aligned mediolaterally, followed by the mutual intercalation of the polarized cells. In the regulation of convergent extension, several growth factors are implicated. Recent studies revealed that one of the Wnt signaling pathways, called Wnt/JNK (c-Jun N-terminal kinase) pathway, is shown to be important for the regulation of convergent extension. The pathway is highly conserved among species and initially found to be essential for the establishment of planar cell polarity (PCP) of *Drosophila* wing hair and often called Wnt/PCP pathway.

To clarify the molecular mechanism of gastrulation, we have been attempting to identify novel regulatory components controlling gastrulation cell movements by an expression cloning based on morphology of dorsal explant (Keller’s explant) and of embryo. One of identified genes was found to encode a member of the family of ArfGAP, GTPase activating protein (GAP) for ADP rybosylation factors (ARFs), which we named XGAP. Further functional analyses of XGAP revealed that XGAP is required for gastrulation cell movements, particularly CE and the establishment of cell polarity which is manifested by the localization of cellular protrusions such as lamellipodia. In addition, we found that XGAP is required for the proper localization of PAR proteins that are highly conserved proteins essential for establishing cell polarity in a wide range of organisms from *C. elegans* to humans. Studies on the precise molecular mechanism of PAR proteins’ regulation by XGAP are currently undertaken.

Aside from the molecular mechanism, we conducted experiments to ask how and when cell polarity is established in the cells participating in CE. To grasp cellular events triggered upon the cell polarization, we focused on microtubule (MT) formation. Based on the assumption that MT formation and direction of MT extension are closely related to cell polarity, we observed MT formation using GFP-fused EB1/3 proteins that are known to bind the plus-end of MTs. The time-lapse recording of the GFP fluorescence shows clear bidirectional extension of MTs in cells of Keller’s explant participating in CE, while MTs are radially extended in ectoderm cells. We are investigating what makes the difference in MT dynamics and how it is related to cell morphology and polarity formation.

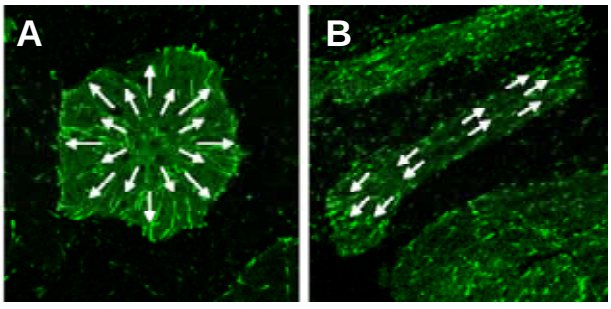

Figure 1. Microtubule dynamics in animal cap and dorsal mesoderm cells revealed by EB3 movement. In animal cap cells, the direction of EB3 movements are random (A), while the EB3 moves towards both poles of cells in dorsal mesoderm (B). Green comets: EB3-GFP, white arrows: the direction of EB3 movements.

II. Roles of membrane trafficking in the regulation of gastrulation movements

Membrane trafficking has been implicated in signal transduction, cell polarity control and cell movements. To investigate the role of membrane trafficking in the regulation of gastrulation movements in *Xenopus* embryos, we focused on Rab GTPases, key regulators for many steps of membrane trafficking. We searched for Rab GTPases that function in gastrulation by overexpressing dominant-negative Rab mutants and identified four Rabs that inhibited gastrulation movements. Loss-of-function of one of these Rabs by the specific morpholino oligonucleotide (Mo) also perturbed normal gastrulation, suggesting that it plays an essential role in this process. Interestingly, the Rab Mo inhibited the non-canonical Wnt pathway which has been implicated in the regulation of convergent extension movements. In the Mo-injected cells, the Wnt ligand and its receptor Frizzled were localized normally, but they were not able to activate the downstream signal in the cells. Dishevelled, which plays the pivotal role in the Wnt signal pathway, is known to be translocated from the cytoplasm to the plasma membrane in response to the Wnt signal activation. The Rab Mo inhibited its translocation, suggesting that membrane trafficking might be important for signaling from Wnt/Frizzled to Dishevelled in this pathway.

III. Notochord-derived fibrinogen-like protein regulates a long-range patterning of nervous system in chordate embryos

The dorsal nervous system (CNS) and the notochord underneath CNS are two major organs characteristic to chordate body plans. Experimental embryology demonstrated that the notochord play a critical role in the patterning of CNS during vertebrate embryogenesis, but little is known about genes or molecules involved in this interaction. In vertebrates, a T-box gene, *Brachyury* (*Bra*), plays a pivotal role in the formation of notochord. This is the case of urochordate ascidian; *Bra* is expressed exclusively in primodial notochord cells and its role is essential for the notochord cell differentiation. We have already isolated nearly 40 genes that are direct or indirect targets of *Ci-Bra* of *Ciona intestinalis*. An ascidian homolog (Ci-scale) of the *Drosophila Scabrous* gene encodes a fibrinogen-like protein is specifically expressed in notochord cells, its protein product is not refined to notochord but distributed underneath the CNS with fibril-like protrusions or vesicles. Knockdown of Ci-Scale function resulted in failure of convergent extention of notochord cells and differentiation of neuronal cells and axon guidance. A proper distribution of Ci-Scale proteins is dependent on Notch signaling delivered by the CNS. These results suggest that cooperative function of Ci-Scale and Notch is essential for the patterning of CNS and axon guidance in chordate embryos.

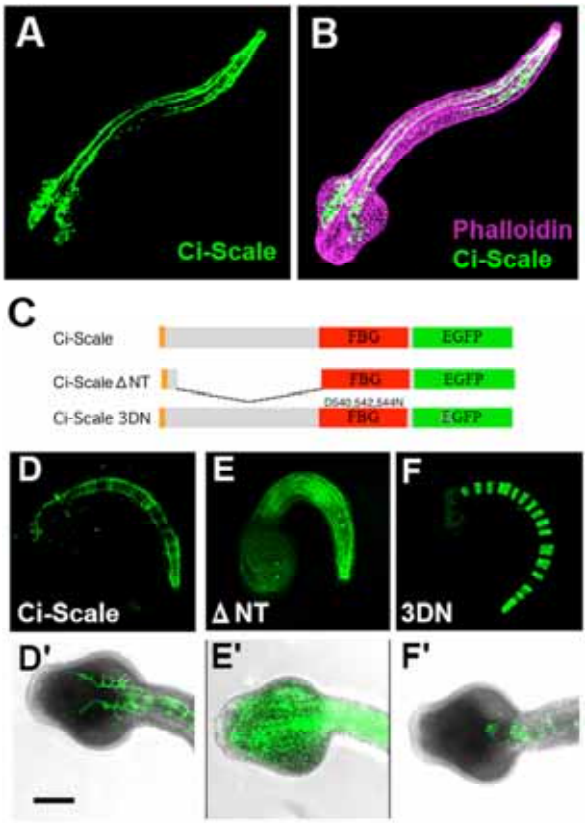

Figure 2. Distribution of Ci-Scale protein in *Ciona intestinalis* embryos. Staining of tailbud stage embryos with an anti-Ci-Scale antibody shown in green and staining with phalloidin in magenta (A, B). Fusion gene constructs tagged with EGFP to examine the role of Ci-Scale components in its extracellular distribution. dNT mutation lacks the N-terminal half, and 3DN was a mutation in which the three conserved D residues in the DXDXD motif are changed to N residues (C). Distribution of EGFP in embryos electropolated with (D) Ci-Scale (control), (E) dNT or (F) 3DN; side view of the tailbud embryos, and (D'-F') dorsal view of the head region of embryos. EGFP in a dNT mutated embryo was seen disused distribution while 3DNmutant was seen only in the cytoplasm of notochord cells, suggesting the NT domain and the FBG domain, especially the DXDXD motif, is necessary for its proper distribution. Scale bar, 50 μm.

IV. Trithorax group components *tonalli* and vertebrate homolog TONAS proteins participate roles in protein SUMO modification and epigenetic regulation

Drosophila tonalli (*tna*) mutant is a previously reported mutant that is involved in epigenetic regulation. The *tna* mutant phenotype mimics the homeotic loss-of-function phenotype and this mutant shows striking genetic interaction with the mutants of *trithorax group* genes. We isolated two mutant alleles of *tna* as a putative downstream target of the DPP (corresponding to vertebrate BMP) signaling. We also isolated vertebrate homologues of *tna* and named as *tonalli* related SP-RING finger protein, TONAS-1 and TONAS-2. The role of

Tna/TONAS proteins in the epigenetic regulation and also in Trithorax group function is totally unknown. The most characteristic feature of Tna/TONAS proteins is the existence of a single SP-RING finger motif in the middle. The SP-RING motif was originally found in the PIAS family SUMO-E3 ligase proteins. We have addressed TONAS SUMO E3 ligase activity by in vitro experiments as well as cultured cell over-expression study. TONAS itself is a good substrate for SUMO-2/3 modification and the SP-RING motif is essential for this activity. Other SUMOylation targets of TONAS proteins are currently unknown. We isolated TONAS binding partners from FLAG-TONAS expressing HEK293T cell-extract by protein affinity purification and LC-MS-MS analysis. Some of the candidate interactors are known to be substrates of SUMO-modification. We will confirm whether these proteins are specific TONAS SUMOylation targets by further analysis.

V. *Xenopus* functional genomics (Xenomics)

Using *Xenopus laevis* as a model, we have been attempting to reveal the complex regulatory network controlling the morphological processes in early embryogenesis through the clarification of gene function and their interactions in the system.

We constructed the comprehensive database XDB3 that stores EST sequences, assembled sequences, full insert sequences and WISH (whole-mount *in situ* hybridization) data with the descriptions of predicted gene function. We added a new browsing method of WISH data as 3D animation model, a method developed under collaboration with Drs. P. Vize and V. Gerth (University of Calgary).

We have also created several transgenic *X. laevis* lines that are labeled with fluorescent proteins (EGFP, Venus, or RFP) localized in subcellular components such as nucleus, nuclear membrane, mitochondria, Golgi, and ER. These frogs will be very powerful tools for the functional analysis of genes in the post genome era.

Figure 3. Xenopus Data Base 3 (http://xenopus.nibb.ac.jp).

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DIVISION OF DEVELOPMENTAL GENETICS

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Germ cells are the specialized cells that can transmit the genetic materials from one generation to the next in sexual reproduction. All the other cells of the body are somatic cells. This separation of germ and somatic cells is one of the oldest problems in developmental biology. In many animal groups, a specialized portion of egg cytoplasm, or germ plasm, is inherited by the cell lineage which gives rise to germ cells. This cell lineage is called germline. The germline progenitors eventually migrate into the gonads, where they differentiate as germ cells when the organisms are physically matured. Earlier investigators have demonstrated that germ plasm contains maternal factors required and sufficient for germline formation. In *Drosophila*, this cytoplasm is histologically marked by the presence of polar granules, which act as a repository for the maternal factor required for germline formation. Our molecular screens have identified several factors stored in the polar granules.

I. The role of mitochondrial ribosomal RNAs in pole cell formation

Ultrastructural studies have shown that the germ plasm is basically composed of polar granules and mitochondria. While the primary roles of the mitochondria are oxidative phosphorylation and biosynthesis of many metabolites, it has now become evident that they are also involved in the formation of the germline progenitors, or pole cells. In *Drosophila*, pole cell formation requires the function of mitochondrial ribosomal RNA in germ plasm. We have previously reported that mitochondrial large rRNA (mtlrRNA) and small rRNA (mtsrRNA) are both transported from mitochondria to polar granules. This transportation occurs during early embryogenesis, when mitochondria are tightly associated with polar granules in germ plasm. Mitochondrial rRNAs remain on the polar granules until pole cell formation and are no longer discernible on the granules within pole cells. Reduction of the extra-mitochondrial mtlrRNA amount results in the failure to form pole cells and injection of mtlrRNA is able to induce pole cells in embryos whose ability to form these cells has been abolished by uv-irradiation. These observations clearly show that the extra-mitochondrial mtlrRNA on polar granules has an essential role in pole cell formation, cooperating with mtsrRNA (Figure 1).

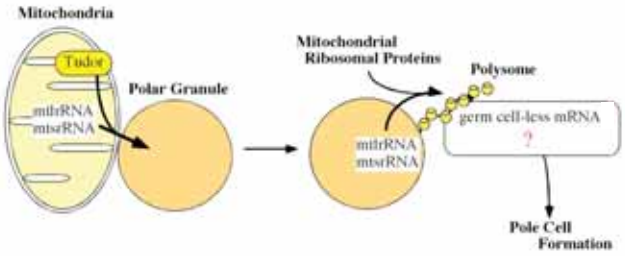

Figure 1. Role of mitochondrial rRNAs in pole cell formation

Recently, we have found that injection of kasugamycin (KA) and chloramphenicol (CH), inhibitors for prokaryotic-type translation, disrupt pole cell formation in early embryos. The number of mitochondrial-type ribosomes on polar granules is significantly decreased by KA treatment, as shown by electron microscopy (Figure 2). In contrast, ribosomes in the mitochondria and mitochondrial activity are unaffected by KA and CH. Furthermore, injection of KA and CH impairs production of Germ cell-less (Gcl) protein, which is required for pole cell formation. The above observations suggest that mitochondrial-type translation is required for pole cell formation, and Gcl is a probable candidate for the protein produced by this translation system.

II. The role of maternal Nanos protein

Among the maternal components of germ plasm, Nanos (Nos) is essential for the germline-specific events occurring in pole cells. Nos mRNA is localized in the germ plasm during oogenesis, and is translated in situ to produce Nos protein after fertilization. Nos is only transiently present in the posterior half of embryos during the preblastoderm stage, and is required there for posterior somatic patterning. Nos in the germ plasm is more stably inherited into the pole cells at the blastoderm stage, remaining detectable in these cells throughout embryogenesis. Pole cells that lack Nos (*nos*$^-$ pole cells) are unable to follow normal germline development; they fail to migrate properly into the embryonic gonads. Nos represses translation of mRNAs with discrete RNA sequences called Nos response elements (NREs). In the pathway leading to posterior somatic patterning, Nos acts together with unlocalized Pumilio (Pum) protein to repress translation of maternal *hunchback* (*hb*) mRNA. This translational repression is mediated by binding of Pum to NREs in the 3'-untranslated region (UTR) of hb mRNA. In pole cells, Nos also acts with Pum to regulate germline-specific events. Pum, like Nos, is required in pole cells for their migration to the gonads.

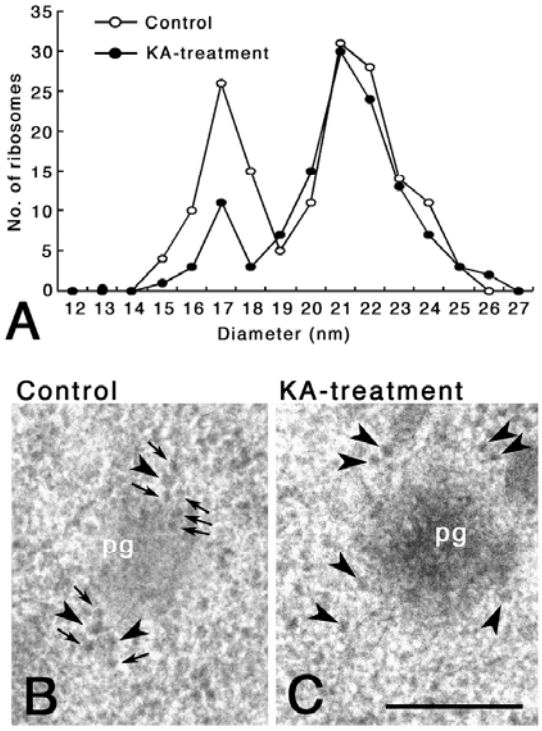

Figure 2. Injection of KA reduces the number of mitochondrial-type ribosomes around polar granules. A, The average number of ribosomes in an unit area (8.4×10^3 nm^2) is plotted against their diameter. The diameters of ribosomes around polar granules were measured in stage 2 embryos injected with DW (control) and KA (KA-treatment). The number of ribosomes smaller than 20 nm in diameter was significantly decreased by KA treatment. B, C, Electron micrographs of sections through polar granules in stage 2 embryos. B, In a control embryo injected with DW, there were two types of ribosomes around the polar granules. C, In an embryo injected with KA, the smaller ribosome was indiscernible around polar granules. Arrows and arrowheads point to the smaller (mitochondrial-type) and the larger (cytosolic-type) ribosomes, respectively. pg, polar granules; Scale bar = 0.2 μm.

Our unpublished observations suggest that one of the targets of Nos and Pum is *head involution defective* (*hid*) mRNA, which also contains an NRE in its 3'UTR and encodes a protein required for the induction of apoptosis. In the absence of Nos or Pum, migrating pole cells are eliminated by an apoptotic mechanism which is initiated at stage 9/10 in the developing embryo (Figure 3). We have also found that *Df(3L)H99* (H99), a small deletion within the genomic region that includes the *hid* gene, suppresses apoptosis in *nos* pole cells. In embryos lacking both maternal Nos and zygotic H99 activity (*nos*-H99 embryo), there is no apoptotic death of any pole cells. In addition, *nos*-H99 pole cells have the ability to migrate into the gonads when transplanted into normal host embryos. Therefore, the ability of *nos* pole cells to migrate into the gonads is fully restored by the suppression of apoptosis in our transplantation experiments. This clearly demonstrates that Nos inhibits the apoptotic response in pole cells to permit their proper migration into the gonads.

The above observations suggest that pole cells have the potential to enter into apoptosis, which somewhat contradicts the notion that the germline is fundamentally immortal as it is required for the propagation of any given species. We speculate, however, that this apoptotic pathway may be part of a mechanism that eliminates "aberrant pole cells" that have inherited an insufficient quantity of germ plasm components, such as maternal Nos protein.

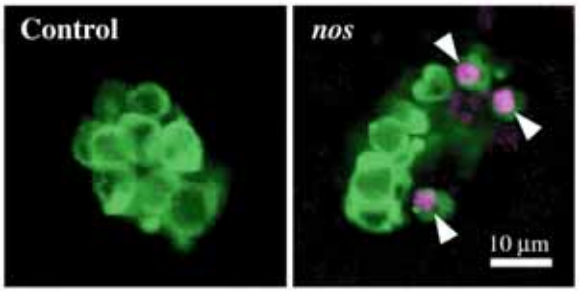

Figure 3. Nanos prevents apoptosis in pole cells. Confocal images of the pole cells in control (left) and *nos* (right) embryos at stage 13, stained with TUNEL labeling (magenta) and an antibody against Vas (green). Arrowheads show TUNEL-positive pole cells.

III. The role of maternal Sva53 in meiosis

Meiosis is an obligatory step to produce haploid gametes that can transmit the genetic materials from one generation to the next. However, little is known about how the germline progenitors acquire the ability to undergo meiosis. We have found that a novel maternal factor, SVA53 is essential for meiosis. SVA53 is a member of widely conserved BTB/POZ-zinc finger proteins, which are known to function as transcriptional regulators by altering chromatin structures. Maternal SVA53 is enriched in pole cells, and a reduction in its activity prevents meiosis. We propose that SVA53 is the first maternal molecule that regulates a genetic pathway leading to meiosis. Thus, our current findings provide the basis for the understanding of a novel epigenetic mechanism that regulates the meiotic cell cycle in *Drosophila* and in a variety of other animal groups.

IV. Expression of meiotic genes in pole cells

In *Drosophila*, genetic screens have identified many genes required for meiotic division. However, it remains elusive as to when and how these meiotic genes are activated during germline development. To obtain insights into their regulatory mechanisms, we examined the expression of 38 meiotic genes in pole cells during embryogenesis. We have found that the transcripts of 12 meiotic genes were enriched in pole cells within the embryonic gonads. Among them, *bag of marbles* (*bam*), *benign gonial cell neoplasia* (*bgcn*), *deadhead* (*dhd*), *matotopetli* (*topi*) and *twine* (*twe*) were activated only in

pole cells within the gonads, whereas the transcripts from *grapes* (*grp*), *Kinesin-like protein at 3A* (*Klp3A*), *pavarotti* (*pav*), *lesswright* (*lwr*), *mei-P26*, *Topoisomerase 2* (*Top2*) and *out at first* (*oaf*) were distributed ubiquitously in early embryos and then became restricted to pole cells and to a subset of somatic tissues at later embryonic stages. These observations suggest that pole cells have already acquired the potential to express several meiotic genes. Our data will thus provide a useful basis for analyzing how the germline acquires a potential to execute meiosis.

V. Microarray analysis of pole cells

To explore the regulatory mechanism of germline specification, we attempted to identify genes expressed in pole cells during embryogenesis. From the embryos carrying EGFP-vasa transgene that express GFP only in pole cells, we isolated pole cells by using fluorescence-activated cell sorting (FACS), and they were used for hybridization of microarray that contains probes for all predicted genes in *Drosophila* genome. Our microarray analysis has identified approximately 300 maternal and 200 zygotic transcripts enriched in polar plasm and pole cells. The functional analysis of these transcripts is now ongoing.

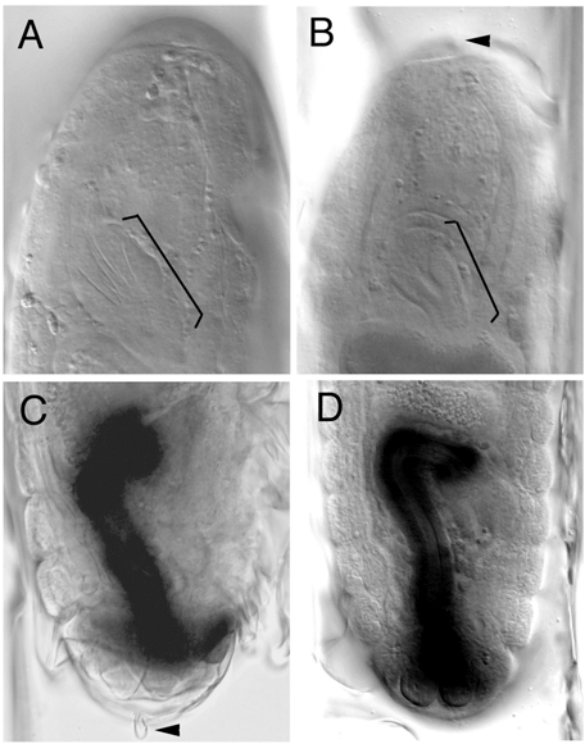

Figure 4. LR asymmetries of the proventriculus and the hindgut in embryos with fully reversed AP polarities. Dorsal views of the anterior (C, B) and the posterior region (A, D) of normal embryos (B, D) and A/P-reversed embryos (A, C) at stage 15-16. A, B, Brackets mark the proventriculi. Note that the posterior of the proventriculi are oriented to the right side of the embryos. C, D, The hindguts are stained. The anterior of the hindguts are oriented to the right. Arrowheads point to the micropyles, a morphological marker for the anterior pole of the eggs.

VI. Left-right asymmetry in embryos

In many animal groups, left-right (LR) asymmetry within the body is observed. The left and right sides of the body are generally defined with reference to the anterior-posterior (AP) and dorsal-ventral (DV) axes. We investigated whether LR asymmetry is solely dependent on the AP and DV polarities in *Drosophila* embryos. We focused on the proventriculus, a posterior part of the foregut, and the hindgut because LR asymmetry in these body parts is highly stable in normal embryos. In embryos with a fully reversed AP polarity, LR asymmetry in both the proventriculus and the hindgut was re-oriented in relation to the reversed AP polarity (Figure 4). This demonstrates that inversion of AP polarity does not affect LR asymmetry of these tissues, and implies that LR asymmetry is specified in relation to the AP and DV polarities. Our findings were not consistent with the alternative hypothesis that LR asymmetry is pre-determined by maternal signals that localize asymmetrically along the LR axis in the oocyte and/or early embryo. This work was supported by the Super Science High school (SSH) program.

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Review article

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DIVISION OF MOLECULAR AND DEVELOPMENTAL BIOLOGY

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During morphogenesis of vertebrates, the embryonic body is gradually divided into sub-regions that are specified to give rise to functional units. Most of the processes of regionalization and specification are regulated by cell signaling molecules. So far, spatial distribution of secreted signals, called morphogens, has been considered to regulate regionalization and specification during embryogenesis. However, the molecular basis to generate a variety of function of secreted signals, including specific interaction between secreted signals and their receptors and specific activation of their target genes in particular cells, has not yet been discovered. To better understand this molecular basis, we are currently examining biochemical characteristics of Wnt proteins and the molecular mechanism to activate specific targets.

In contrast, some regionalization processes have also been shown to be regulated by different manners. One of the typical examples is the segmentation of somites. Somites are the morphologically distinct segmental units that are transiently formed during early vertebrate development and subsequently give rise to metameric and fundamental structures such as the vertebrae of the axial skeleton, their associated muscles, and tendons. The molecular mechanism underlying the periodical formation of somites is coupled to an internal oscillator, referred to as the “segmentation clock”. To gain insight into molecular mechanism underlying this specific regionalization, we are also characterizing genes involved in somite segmentation.

I. Specificity between Wnt ligands and their receptors

In vertebrates, Wnt proteins constitute a large family of cysteine-rich secreted glycoproteins. Secreted Wnt proteins exert their effects on neighboring cells by binding to 7-transmembrane Frizzled (Fz) family receptors, as well as to LDL receptor-related family members, LRP5 and 6, which act as co-receptors. The Fz receptor family also consists of many members, each of which shows a distinct expression pattern during embryogenesis. However, the receptor specificity of each Wnt ligand has been poorly understood.

To investigate systematically the combinatorial effects of Wnts and various Fzs on canonical Wnt/β-catenin signaling, we analyzed the ability of these Wnt proteins to increase stability of armadillo/β-catenin proteins in Drosophila S2 cells expressing vertebrate Fzs (Takada *et al.* 2005). Wnt-3a increases the amount of armadillo proteins in cells expressing Fzs 4, 5, and 8, but not Fzs 3 and 6; whereas Wnt-5a does not increase it in any cell line. In contrast, both Wnt-3a and Wnt-5a increase the phosphorylation of Dsh in combination with most of the Fzs. This Dsh phosphorylation is abrogated by decreasing the levels of casein kinase I α by double-stranded RNA-mediated translational interference. These observations indicate that both Wnt proteins can interact with the majority of Fz receptors and elicit signaling reactions exemplified by Dsh phosphorylation but that the stabilization of β-catenin/armadillo proteins in the Wnt/β-catenin signaling occurs only when specific combinations of Wnt and Fz meet.

II. Identification and functional characteristics of Wnt target genes during embryogenesis

To identify Wnt-target genes during mouse development, we used the induction gene-trap approach (Yamaguchi *et al.* 2005). We screened 794 trapped ES lines and recovered 2 ES cell lines that contained trapped genes responsive to WNT-3A protein. One gene was mainly expressed in the ductal epithelium of several developing organs, including the kidney and the salivary glands, and the other gene was expressed in neural crest cells and the telencephalic flexure. The spatial and temporal expression of these two genes coincided well with that of several *Wnt* genes. Furthermore, the expression of these two genes was significantly decreased in embryos deficient for *Wnts* or in cultures of embryonic tissues treated with a Wnt signal inhibitor. These results indicate that the gene trap is an effective method for systematic identification of Wnt-responsive genes during embryogenesis.

We have further analyzed the developmental role of the former gene by generating mutant embryos defective in its function from the gene-trapped ES cell line. Our results indicated that this gene plays essential roles in differentiation of ductal epithelial cells in the kidney and salivary glands.

III. Identification and characterization of genes required for somite segmentation

3-1 Hairy/Enhancer of split protein, Her13.2, links FGF signaling to cyclic gene expression in the periodic segmentation of somites

The somites are subdivided from the anterior end of the

unsegmented paraxial mesoderm, called the presomitic mesoderm (PSM), and sequentially generated in an anterior to posterior direction in a rhythmic fashion at regular spatiotemporal intervals. The molecular mechanism underlying the periodical formation of somites is coupled to an internal oscillator, referred to as the "segmentation clock", which has been evidenced by the cyclic expression of genes in the PSM.

Most genes that exhibit a cyclic expression pattern in the PSM are involved in the Notch signaling pathway. For instance, various *hairy/Enhancer of split* (*Espl*)-related bHLH genes, including *her1* and *her7* in the zebrafish, are transcriptional targets of the Notch signaling and are expressed in a dynamic pattern of stripes across the PSM in a posterior to anterior direction. These cyclic genes, as well as other components of the Notch signaling pathway, were shown to be required for the proper somite segmentation in mice and zebrafish. Furthermore, the cyclic expression of *her1*, and *her7* requires their own activity, suggesting that a negative feedback loop involving these genes is a critical component of the oscillation machinery.

The oscillating and anteriorly propagating wave of gene expression, which is maintained in the posterior PSM, becomes fixed to cause segmentation in the anterior PSM. The activity gradient of fibroblast growth factor (FGF) is proposed to regulate the differentiation of PSM cells along the anteroposterior axis from a state permitting the oscillating gene expression to a state driving the segmentation program. However, the molecular mechanism by which FGF signaling permits the cyclic gene expression in the posterior PSM has been totally unknown.

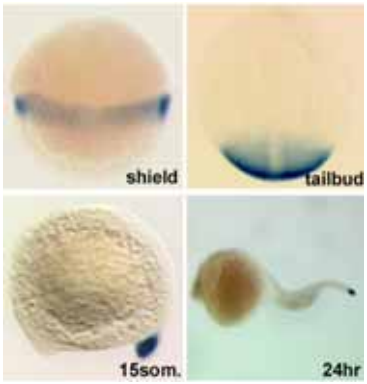

Figure 1. Expression of *her13.2* in zebrafish development. Expression of zebrafish *her13.2* is first observed at shield stage. During the stages when somites are generated, its expression is localized in the posterior PSM and the tailbud (15som. and 24hr). This expression is induced by FGF signaling to allow the cyclic gene expression in the posterior PSM (see Kawamura *et al.* 2005a).

To gain insight into the molecular mechanism underlying the segmentation process, we sought to identify genes specifically expressed in the PSM and tailbud by performing an *in situ* hybridization screening using zebrafish embryos (Figure 1). We show that a gene identified by this screening, *Hes6*-related *hairy/Enhancer of split*-related gene, *her13.2*, links FGF signaling to the Notch-regulated oscillation machinery in zebrafish (Kawamura *et al.* 2005a). Expression of *her13.2* is induced by FGF-soaked beads and decreased by an FGF signaling inhibitor. *her13.2* is required for periodic repression of the Notch-regulated genes, *her1* and *her7*, and for proper somite segmentation. Furthermore, Her13.2 augments auto-repression of *her1* in association with Her1 protein. Therefore, FGF signaling appears to maintain the oscillation machinery by supplying a binding partner, Her13.2, for Her1.

This result also provides a novel molecular mechanism whereby Notch signaling, which activates the expression of *her1* and *her7*, cooperates with FGF signaling. This molecular mechanism may be widely employed in regulation of developmental process where Notch signaling cooperates with FGF signaling.

3-2 Groucho-associated transcriptional repressor Ripply1 is required for proper transition from the presomitic mesoderm to somites

Prior to morphological segmentation, which is a process including inter-somitic boundary formation and mesenchymal-epithelial transition, a segmental pre-pattern, characterized by segmental gene expression, is established in the anterior PSM. The establishment of the segmental pre-pattern in the anterior PSM has been revealed to require a number of processes regulated by many transcription factors and signaling molecules. Concomitant with the transition from the anterior PSM to somites, the characteristic gene expression in the PSM is translated into the segmental structure. However, most of the events accompanying the transition from the anterior PSM to somites have remained obscure.

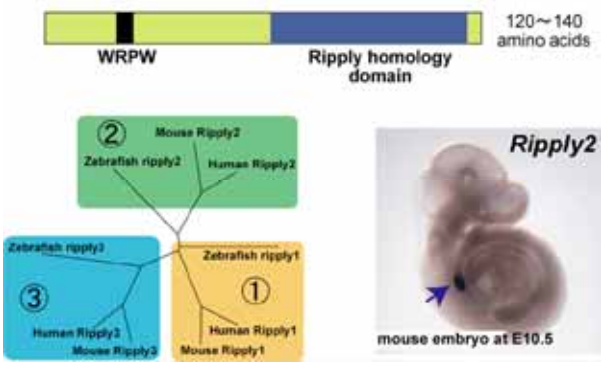

Figure 2. The structure and expression of *ripply* family proteins. Three *ripply* genes exist commonly in zebrafish, human and mouse genomes. All Ripply proteins possess the WRPW motifs and conserved sequences at the carboxyl terminus (Ripply homology domain). A typical example of *ripply* gene (mouse *ripply2*) expression is shown. *Ripply1* and *ripply2* are specifically expressed in the PSM and/or somites, suggesting that they function during somitogenesis (see Kawamura *et al.* 2005b).

We show that another gene identified by our *in situ* hybridization screening, *ripply1*, encoding a nuclear protein associated with the transcriptional co-repressor Groucho, is required for this transition (Figure 2,

Kawamura *et al*. 2005b). Zebrafish *ripply1* is expressed in the anterior PSM and in several newly formed somites. Ripply1 represses specific gene expression in the PSM through a Groucho-interacting motif. In *ripply1*-deficient embryos, somite boundaries do not form, the characteristic gene expression in the PSM is not properly terminated, and the initially established rostrocaudal polarity in the segmental unit is not maintained, whereas paraxial mesoderm cells become differentiated. Thus, *ripply1* plays two key roles in the transition from the PSM to somites: termination of the segmentation program in the PSM and maintenance of the rostrocaudal polarity.

3-3 Integrinα5-dependent Fibronectin assembly is required for maintenance of somite boundaries.

The segmental pre-pattern established in the anterior PSM lead to morphological segmentation. Boundary formation and epithelialization are crucial processes in the morphological segmentation of vertebrate somites, but the molecular mechanisms underlying these processes are not yet clearly understood.

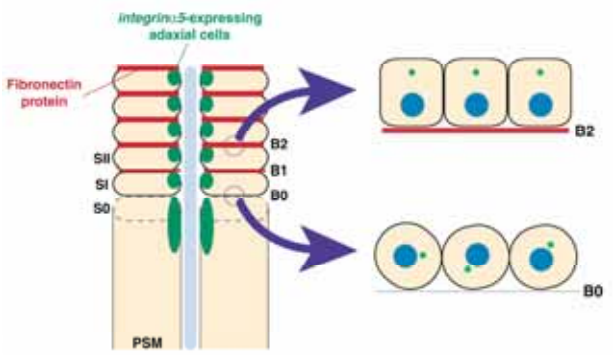

Figure 3. The role of Fibronectin assembly in somite boundary formation. Gradual accumulation of Fibronecin proteins (red) at somite boundaries is required for maintenance of the boundaries and for polarization of epithelial cells. Integrinα5 (green) is required for this accumulation (see Koshida *et al.* 2005).

To gain insight into the mechanism underlying somite development, we performed an ENU mutagenesis screening of zebrafish, in addition to *in situ* hybridization screening described above. We found that *integrinα5* and *fibronectin* were mutated in embryos showing defective boundary formation in their anterior somites (Koshida *et al.* 2005). Fibronectin proteins accumulate at somite boundaries in accordance with epithelialization of the somites. Both Fibronectin accumulation and the epithelialization are dependent on *itga5*, which is expressed in the most medial part of somites. While somite boundaries are initially formed, but not maintained, in the anterior trunk of the mutant embryos deficient in either gene, their maintenance is defective at all axial levels of embryos deficient for both of these genes. Therefore, Integrinα5-directed assembly of Fibronectin appears critical for epithelialization and boundary maintenance of somites (Figure 3). Furthermore, with an additional deficiency in *ephrin-B2a*, the segmental defect in *itga5* or *fn* mutant embryos is expanded posteriorly, indicating that both Integrin-Fibronectin and Eph-Ephrin systems function cooperatively in maintaining somite boundaries.

The results shown in “3-1” to “3-3” indicate that our strategies are effective for identification of genes involved in the somite segmentation process. We are further searching for other genes involved in this process by both the expression screening and the mutagenesis screening methods. This systematic screening should reveal another interesting mechanism underlying somite segmentation.

Publication List:

Original papers

Kassai, Y., Munne, P., Hotta, Y., Penttila, E., Kavanagh, K., Ohbayashi, N., Takada, S., Thesleff, I., Jemvall, J., and Itoh, N. (2005). Regulation of mammalian tooth cusp patterning by ectodin. Science *309*, 2067-2070.

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Kawamura, A., Koshida, S., Hijikata, H., Ohbayashi, A., Kondoh, H., and Takada, S. (2005b). Groucho-associated transcriptional repressor Ripply1 is required for proper transition from the presomitic mesoderm to somites. Dev. Cell *9*, 735-744.

Koshida, S., Kishimoto, Y., Ustumi, H., Shimizu, T., Furutani-Seiki, M., Kondoh, H., and Takada, S. (2005). Integrinα5-dependent fibronectin accumulation for maintenance of somite boundaries in zebrafish embryos. Dev. Cell *8*, 587-598.

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LABORATORY OF MOLECULAR GENETICS FOR REPRODUCTION

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Laboratory Scope

Sexually dimorphic gonads mainly consist of two different cell lineages, somatic gonadal cells and germ cells. During the course of development, the gonadal precursor cells should be specified through the process of mesoderm patterning, which subsequently associates primordial germ cells to form the indifferent gonad. Once indifferent gonad is formed in the gonadal area, sex determination gene is expressed in the gonadal mesoderm and organogenesis of the gonads (sex differentiation) initiates in mice.

Since the sex determination gene in mice was identified in 1990, much research has been intensively focused on the testis development. When considering, however, the differentiation of the precursor cells determining sex, the interaction of somatic cells and germ cells, the differentiation of primordial germ cells (PGCs) to germ cells having the capability to undergo meiosis, and so on, there remain many important and fascinating problems to be solved.

Our laboratory aims to reveal the molecular mechanisms of the formation of gonads and the sex differentiation, with an emphasis on visualizing specific cell lineages in living medaka embryos and by applying molecular genetics to medaka embryos.

I. Analysis of movement of primordial germ cells by timelapse movies

One of the advantages using medaka is that the embryos are transparent and the development can be seen from outside. This encouraged us to generate transgenic medaka exhibiting GFP exclusively in germline and succesfully established the trangenic lines (*olvas*-transgenic medaka). On the other hand, we have been screening medaka genes expressing in germline and found that some of the 3'UTRs are critical for maintenance and translation of their own transcripts specifically in germline. This translational mechanism also allows to visualize the germline by injecting GFP-3'UTR chimeric RNA into fertilized eggs.

With the two means mentioned above, we have been monitoring the origin and the mode of movement of medaka primordial germ cells (PGCs) during embryogenesis. We revealed that presumptive PGCs are already present by early gastrulation stage by analyzing the expression of one of the germline-expressing genes and that PGCs exhibit three different modes of migration to reach the gonadal area. After appearance of PGCs at early gastrulation stage around the animal pole, timelapse analyses show that PGCs actively move between epiblasts and hypoblasts towards the marginal region (Figure 1). This movement is dependent on the activity of the chemo-attractant, CXCR4 (the first mode). PGCs then move medially towards the embryonic axis with lateral somatic cells undergoing convergent movement (the second mode). After the bilateral alignment of PGCs along the embryonic axis, the PGCs actively resume directional movement towards the region of a gonadal field in the posterior lateral plate mesoderm. SDF-1a and HMGCoA reductase are independently involved in this posterior migration (the third mode) (Kurokawa *et al*. 2006).

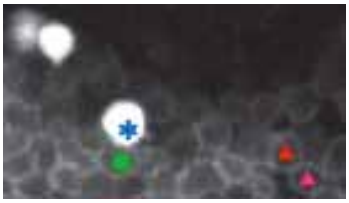

Figure 1. PGCs (asterisk) migrate between epiblasts (square) and hypoblasts (triangles) during epiboly.

II. Origin of gonadal mesoderm

Much research has focused on the mechanisms of sex differentiation. However, the origin and the lineages of the gonadal precursor cells have remained to be elucidated in vertebrates.

We have successfully demonstrated the presence of the gonadal field where the precursors of gonadal mesoderm arise. This region is located at the most posterior end of *sdf-1a* expression domain. It is known that *sdf-1a* functions as a guidance cue for PGCs to migrate to the gonadal area and the expression becomes restricted to the posterior lateral mesoderm. Our result indicates that the most posterior end of *sdf-1a* expression domain is the place that coordinates PGC migration and development of the gonadal mesoderm. We further demonstrated that

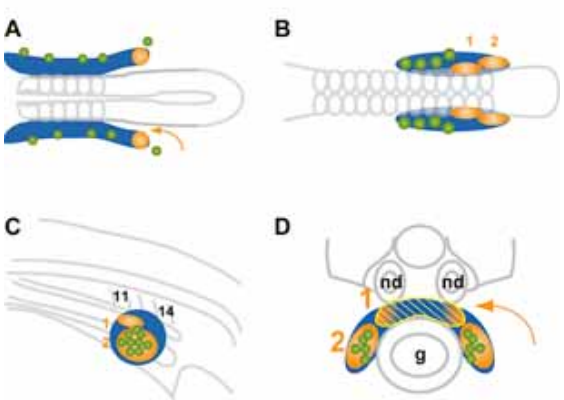

Figure 2. The two different precursors of gonadal mesoderm arise from the gonadal field (orange). *sdf-1a* expression domain (blue). PGCs (green).

two different populations with distinct gene expression are spatially organized from the gonadal field along the embryonic axis and are specified before the gonadal primordium forms (before the sex is determined) (Nakamura *et al*. 2006) (Figure 2).

Generally, in vertebrates, the presence and characteristics of gonadal precursors before sex determination are poorly understood. Cellular interaction among the different cell precursors, which brings about development of the supporting cells such as Sertoli cells and granulosa cells, also remains to be elucidated.

We have generated medaka embryos that completely lack germ cells in the gonadal primordium by impairment of PGC migration and have confirmed the biased ratio of phenotypic sex in the germ cell-less adult medaka. The morphology of the gonad in the germ cell-less medaka exhibits the appearance of neither testis nor ovary. We are currently investigating the involvement of germ cells in the development of the gonadal supporting cells.

III. Generation of transgenic medaka visualizing different cell lineages of the supporting cells in the gonad

To clarify how many kinds of supporting cells appear during the formation of gonadal primordium and the sex differentiation, we are systematically identifying the different types of supporting cells by *in situ* hybridization and generating the transgenic medaka that visualizes each type of the supporting cells. Currently we are trying to monitor different cells with GFP fluorescence driven by the regulatory regions of several different genes. One line has been established as a transgenic line that allows us to observe somatic cells in gonadal primordium (Figure 3). The transgenic medaka suggests that several

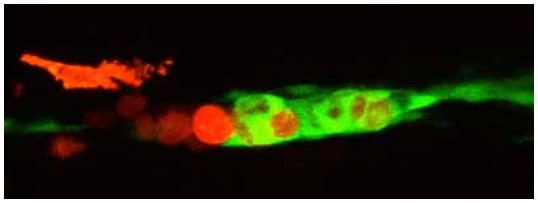

Figure 3. Somatic cells (green) surround germ cells (red) in the gonadal primordium (from in *vivo* timelapse movie).

different types of supporting cells have already been differentiated before sexual differentiation (organogenesis of ovary or testis) occurs.

The attempt to monitor the process of development of each lineage has also been made in living embryos and larva by timelapse movies. In order to solve the difficulties in visualizing the cells located in the deep positions in the embryos and larva, the confocal microscopy and SPIM have been applied to the transgenic embryos and larva. This attempt is still in progress in collaboration with Jochen Wittbrodt Lab. in EMBL.

IV. Analysis of mutant medaka affecting formation of gonad

Medaka is a small vertebrate and produces next generations in 3 months after hatching. These characteristics are suitable for applying molecular genetics to this small animal.

In collaboration with ERATO Kondoh differentiation signaling project, we have been screening mutants affecting the development of primordial germ cells and the formation of gonads. The screening has been performed in such a way that particular attention is paid to the presence, the number or the distribution pattern of germ cells at a somitogenesis stage and at ten days post hatching (10 dph). Nine mutants (19 alleles) and twelve mutants (14 alleles) were identified for PGCs and gonads, respectively.

One mutant, *totoro*, is very interesting in that the phenotype has not been described before in other animals. *totoro* has a swollen large abdomen filled with a gonad. Our analyses show that the phenotype is semidominantly inherited. Gonads are sex-reversed in genetic males and fullfilled with numerous oocytes regardless of its genetic sex. The positional cloning is ongoing with the ERATO project and three genes are identified as the possible candidate genes responsible for the phenotype.

Figure 4. Blue staining shows PGCs in the gonad. Left: *zenzai* mutant that cannot maintain germ cells. Middle: wild type. Right: *totoro* mutant that shows overproliferation of germ cells.

Another mutant, *zenzai*, is a good contrast with the *totoro* mutant and is unique in that germ cells are not maintained in the gonad (Figure 4). Inheritance of the phenotype indicates that the allele is recessive. The characterization and the positional cloning are also in progress. We are narrowing down the genomic region responsible for *zenzai* phenotype to approximately 110 Kb.

We are also characterizing other mutants in another category, irregular distribution of germ cells in gonads. These mutants include *hadare*, *mizore*, *hyou* and *arare*.

Publication List:

Original paper

Hano, T., Oshima, Y., Oe, T., Kinoshita, M., Tanaka, M., Wakamatsu, Y., Ozato, K., and Honjo, H. (2005). Quantitiative Bio-imaging analysis for evaluation of sexual differentiation in germ cells of *olvas*-GFP/STII YI medaka (*Oryzias latipes*) nanoinjected in ovo with ethinylestradiol. Environ. Toxicol. Chem. *24*, 70-77.

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We have been studying the molecular and cellular mechanisms underlying the development of the vertebrate central nervous system, mainly using the visual system of the chick and mouse. This research covers many developmental events including the patterning of the retina, axonal navigation, branching and targeting, synapse formation, refinement and plasticity, and axonal regeneration. The scope of our interests also encompasses mechanisms for various functions of the mature brain, including sensation, emotion, behavior, learning and memory.

I. Mechanisms for the regional specification in the developing retina

Topographic maps are a fundamental feature of neural networks in the nervous system. Understanding the molecular mechanisms by which topographically ordered neuronal connections are established during development has long been a major challenge in developmental neurobiology. The retinotectal projection of lower vertebrates including birds has been used as a readily accessible model system. In this projection, the temporal (posterior) retina is connected to the rostral (anterior) part of the contralateral optic tectum, the nasal (anterior) retina to the caudal (posterior) tectum, and likewise the dorsal and ventral retina to the ventral and dorsal tectum, respectively. Thus, images received by the retina are precisely projected onto the tectum in a reversed manner.

Regional specification along the nasotemporal and dorsoventral axes precedes the topographic retinotectal projection in the developing retina. To understand the molecular basis of topographic retinotectal projection, an overall view of the asymmetrically expressed molecules in the developing retinas is needed. In the past ten years, we have been devoting our efforts to searching for molecules with asymmetrical distribution in the embryonic chick retina, and to characterization of their roles in the topographic retinotectal projection.

We performed a large-scale screening using differential hybridization and restriction landmark cDNA scanning (RLCS) on the embryonic day 8 (E8) chick retina. RLCS is a cDNA display system, in which a large number of cDNA species are displayed as two-dimensional spots with intensities reflecting their expression levels in mRNA. We detected ~200 spots that gave different signal intensities between the nasal and temporal retinas or between the dorsal and ventral retinas. The asymmetric expression of each gene was verified by Northern blotting and *in situ* hybridization. By subsequent analyses using molecular cloning, DNA sequencing, and database searching, 33 asymmetric molecules along the nasotemporal (N-T) axis and 20 along the dorsoventral (D-V) axis were finally identified.

Their expression profiles revealed by *in situ* hybridization are highly diverse and individual. Moreover, some of them begin to be expressed in the retina from the early developmental stages (peaking before E6), suggesting that they are implicated in the establishment (and maintenance) of regional specificity in the developing retina. We have already described some novel molecules in published papers, but the study to understand the functions of the remaining molecules and the overall hierarchical order to establish the regional specificity in the retina is currently in progress.

II. Mechanisms for the topographic retinotectal projection

In the chick embryos, the first retinal ganglion cells become postmitotic at day E2 and their axons leave the retina at E3. The earliest axons arrive at the most anterior part of the tectum at day E6 and advance over its surface in the posterior direction. These retinal axons form the stratum opticum (SO), which covers the entire tectum at E12.

At the onset, the chick retinotectal projection has a vague topographic order, and axonal sprouting begin predominantly at the vicinity and towards the site of normal terminal zone (TZ). In all vertebrates studied, however, the developing axons make trial branches, many of which are not entirely within the retinotopic area, and elimination of ectopic branches and elaboration of appropriate branches is shaped in an activity-dependent manner (Figure 1).

We have already identified a battery of molecules in the region-specific molecules in the retina (peaking after E8) that show abnormal axonal targeting, branching or synaptic arborization, when their expression was experimentally enhanced or suppressed *in vivo*. We expect that molecular mechanisms underlying the branching, pruning, and synapse formation of retinal axons shall be revealed through studies on these molecules.

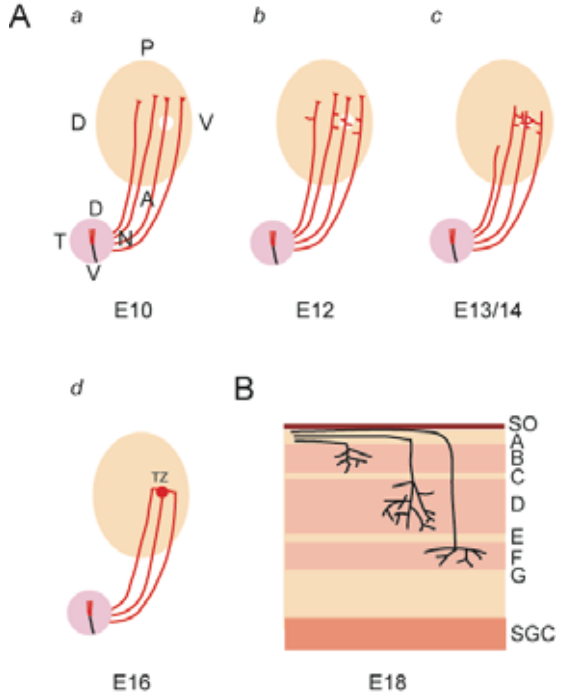

Figure 1. Development of retinotectal projection. A, Developmental processes of the topographic projection of dorsal retinal axons. TZ, terminal zone. B, Layer-specific synapse formation of retinal axons in the tectum.

III. Physiological roles of protein tyrosine phosphatase receptor type Z (Ptprz)

Protein tyrosine phosphorylation plays crucial roles in various biological aspects including brain development and brain functions. PTPζ/RPTPβ/Ptprz, a nervous system-rich receptor-type PTP, is expressed as a chondroitin sulfate proteoglycan in the brain from the early developmental stage to adulthood in neurons as well as astrocytes. This suggests that this molecule plays variegated roles in the brain development and brain function.

Ptprz binds various cell adhesion molecules (Nr-CAM, L1, contactin, NCAM and TAG-1) and extracellular matrix molecules (tenascin-C/R). In addition to these molecules, we revealed that Ptprz binds pleiotrophin (PTN)/HB-GAM and midkine (MK), closely related heparin-binding growth factors which share many biological activities. The chondroitin sulfate portion of Ptprz is essential for the high affinity binding to these growth factors.

We recently developed a genetic method named “yeast substrate-trapping system” to screen for substrates of PTPs. This method carries the advantage that not only substrates but also continuously interacting molecules are capable of being identified at the same time. By this screening, we identified Rho-family GTPase related molecules like p190 RhoGAP, Git1 (ArfGAP) and Pist as substrates, and PDZ-domain containing molecules like PSD-95 as continuously interacting proteins. The expression profile and interacting molecules suggested that Ptprz is implicated in synaptic plasticity in the hippocampus.

We then examined phenotypes of mutant mice deficient in *Ptprz* using electrophysiological, pharmacological, and behavioral approaches. Mutant mice exhibit enhanced long-term potentiation (LTP) in the CA1 region of hippocampal slices and impaired spatial learning abilities in an age-dependent manner (Figure 2): Young adult (<10 weeks old) mutant mice show normal LTP and learning abilities in the Morris water maze task, whereas adult (>13 weeks old) mutant mice exhibit enhanced LTP and impairment in the task.

The enhanced LTP is specifically canceled out by pharmacological inhibition of Rho-associated kinase (ROCK), a major downstream effector of Rho. These findings suggest that the lack of *Ptprz* leads to aberrant activation of ROCK and resultantly to enhanced LTP in the slice and learning impairments in the animal.

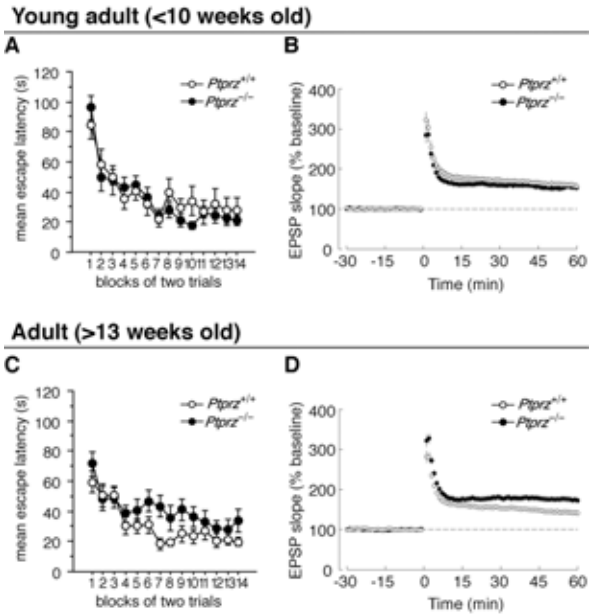

Figure 2. Age-dependent impairment of spatial learning and enhancement of hippocampal long-term potentiation (LTP) in *Ptprz*-deficient mice. A and C, Hidden-platform task of Morris water maze test. B and D, The time course of LTP in CA1 region of hippocampal slices. Tetanic stimulation (100 Hz for 1 s) was applied at 0 min. *Ptprz*-deficient mice exhibited a slower rate of spatial learning specifically at the age of >13 weeks, but not at the age of <10 weeks, which was in accordance with the age-dependent enhancement of LTP.

IV. Mechanisms for Na^+-level sensing in the brain and body fluid homeostasis

Dehydration causes an increase in the sodium (Na) concentration and osmolarity of body fluids in mammals. For Na homeostasis of the body, controls of Na and water intake and excretion are of prime importance. However, the system for sensing the Na level within the brain that is responsible for the control of Na- and water-intake behavior has not been fully elucidated.

We reported previously that the Na_x channel is preferentially expressed in the circumventricular organs

(CVOs) in the brain, and that *Na*$_x$ knock-out mice do not stop ingestion of saline under dehydrated conditions. Subsequently, we demonstrated that Na_x is a Na-level-sensitive Na channel. Last year, we showed that the subfornical organ (SFO), one of the CVOs, is the center of the control of salt-intake behavior in the brain, where the Na-level-sensitive Na_x channel is involved in sensing the physiological increase in the Na level of body fluids.

This year, we dissected the subcellular localization of Na_x. Double-immunostaining and immunoelectron microscopic analyses revealed that Na_x is exclusively localized to perineuronal lamellate processes extended from ependymal cells and astrocytes in the organs. In addition, glial cells isolated from the SFO were sensitive to an increase in the extracellular sodium level, as analyzed by an ion-imaging method (Figure 3). Glial cells bearing Na_x channel are thus the first to sense a physiological increase in the level of sodium in the body fluid, and regulate the neural activity of the CVOs by enveloping neurons.

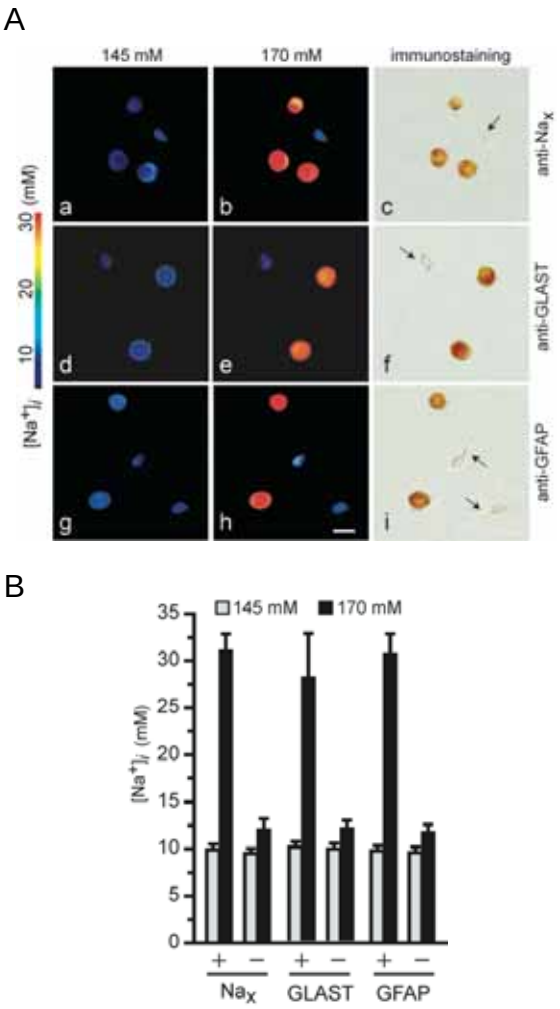

Figure 3. A, Glial cells isolated from the SFO express Na_x channel and show sensitivity to the extracellular sodium level. Pseudocolor images of the intracellular sodium concentration ($[Na^+]_i$) of SFO cells in the control solution and in the high sodium solution. A-*b*, -*e*, -*h* are images 20 min after stimulation with the hypertonic 170 mM $[Na^+]$ solution. After sodium-image recordings, cells were fixed and stained with anti-Na_x (A-*c*), anti-GLAST (A-*f*) or anti-GFAP (A-*i*) antibodies. All the sodium-sensitive cells are immunopositive for Na_x, GLAST and GFAP, indicating that these cells are glial cells. Small cells bearing short neurites (arrows) in A-*c*, -*f* and -*i* appear to be neurons, which are all insensitive to the extracellular sodium increase. Scale bar: 20 μm. B, Quantified intracellular sodium-ion concentrations before (open bars) and after (filled bars) the stimulation in Na_x-positive (+) or -negative (-) cells, in GLAST-positive (+) or -negative (-) cells, and in GFAP-positive (+) or -negative (-) cells. Data represent mean and SE (*n*=20, each).

Publication List:

Original papers

Fukada, M., Kawachi, H., Fujikawa, A., and Noda, M. (2005). Yeast substrate-trapping system for isolating substrates of protein tyrosine phosphatases: Isolation of substrates for protein tyrosine phosphatase receptor type z. Methods *35*, 54-63.

Niisato, K., Fujikawa, A., Komai, S., Shintani, T., Watanabe, E., Sakaguchi, G., Katsuura, G., Manabe, T., and Noda, M. (2005). Age-dependent enhancement of hippocampal long-term potentiation and impairment of spatial learning through the Rho-associated kinase pathway in protein tyrosine phosphatase receptor type Z-deficient mice. J. Neurosci. *25*, 1081-1088.

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Watanabe, E., Hiyama, T.Y., Shimizu, H., Kodama, R., Hayashi, N., Miyata, S., Yanagawa, Y., Obata, K., and Noda, M. (2005). Sodium-level-sensitive sodium channel Na_x is expressed in glial laminate processes in the sensory circumventricular organs. Am. J. Physiol. – Regul. Integr. Comp. Physiol. *Epub ahead of print*, PMID: 16223844.

Review articles

Fukada, M., and Noda, M. (2005). Yeast substrate-trapping system for isolating substrates of protein tyrosine phosphatases. Methods in Mol. Biol., in press.

Noda, M. (2005). The subfornical organ, a specialized sodium channel, and the sensing of sodium levels in the brain. The Neuroscientist, in press.

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Our research is focused on understanding the formation and evolution of the brain and on the mechanisms underlying memory.Our approach is threefold. Firstly, we are studying the genes that are expressed in specific areas of the primate neocortex. We have obtained genes that show marked differences within primate neocortical areas. Secondly, we are studying the mechanisms underlying learning behaviors by examining gene expression. Thirdly, we are studying a new transcriptional factor in which both sense and antisense RNA are expressed in the brain.

I. Genes expressed in specific areas of the neocortex

The neocortex is most remarkably evolved in the primate and plays the major role in higher brain functions. It is divided into distinct functional and anatomical areas and it has been a matter of debate what extent areas of the neocortex are genetically and environmentally determined. It is also puzzling why, during the evolution of mammals, the neocortex was markedly expanded while the number of the genes in the mammal was little changed. In order to elucidate these questions, we studied gene expression within different areas of the neocortex. In the last several years, we reported the following findings.

1) Examining 1088 genes by microarray analysis, most genes showed only less two fold difference in their expressions among the three neocortical (frontal, motor and visual) areas. Only one gene showed more than three fold difference and another one was between two and three fold difference within the three areas (Watakabe *et al.* Mol. Brain Res. *88*, 74-82, 2001). These results suggest that the genes that are expressed among the different areas of the human neocortex are very similar. However, the question remained whether there are any genes that show marked neocortical area difference.

2) In order to answer this question, we have employed differential display methods and found three genes that indicated area specific expressions.

i) One gene, designated *occ1*, is specifically expressed in the occipital cortex, particularly in V1 area, in the primate brain. We also demonstrated that *occ1* expression was markedly increased postnataly in V1.

ii) The other gene that showed marked difference within the neocortex, is *gdf7*, a member of BMP/TGF-β family, which is specifically expressed in the motor cortex of the African green monkey (Watakabe *et al*. J. Neurochem. *76*, 1455-1464, 2001).

iii) *Rbp* (retinol-binding protein) is preferentially expressed in association and higher areas in the neocortex (Komatsu *et al.* 2005). *Rbp* also shows characteristic features. a) Its expression is high in sensory association and higher association areas and limbic areas, but low in the primary sensory areas. Expression is complementary to that of *occ1* and to parvalbumin immunoreactivity (PV-IR) in primary sensory areas. b) In early sensory pathways, the expression is limited to superficial layers only (in particular, layer 2). With progression into higher sensory areas, the expression is expanded into layers 3 and then 5. c) In higher-order association areas, *Rbp* is expressed throughout all layers except layer 4. d) This characteristic distribution of *Rbp* is mainly formed during postnatal development. *Rbp* probably regulates the concentration of retinoic acid (RA) by the delivery of retinol, which is converted into RA in cells. Although the role of RA in the mature brain is not yet known, the characteristic expression of *Rbp* within association areas may provide a clue to the molecular basis of the formation and function of the association areas.

In this year [2005], we also reported on the features of *occ1* expression in mammalian neocortices. Although *occ1* is abundantly expressed in the primary visual cortex, we found that there is another mode of expression of *occ1*: sparsely expressed throughout the entire neocortex. Therefore, there are two different modes of *occ1* expression (Figure1). One mode is the expression in excitatory cells in the primary sonsory areas, particularly in visual cortex, which receive thalamocortical projections. The other mode is the expression in inhibitory GABAergic cells that are spread throughout all layers of entire neocortex. We found that among inhibitory neurons only Parvalbumin (PV) positive neurons expressed *occ1*.

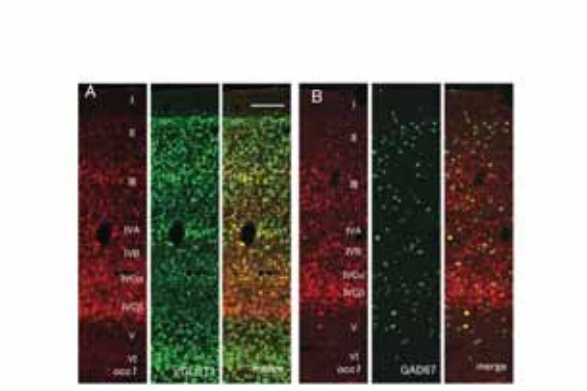

Figure 1. Expression pattern of *occ1*. *occ1* RNA is markedly expressed in the visual cortex and expressed in both excitatory (A:vGlut positive) and inhibitory (B:GAD) neurons (Takahata *et al*. Cerebral Cortex, 2005 Sep 8; [Epub ahead of print]).

As previously reported, *occ1* expression in the primary visual cortex is activity-dependent. In monkeys whose eyes are monocularly deprived by TTX injection, the *occ1* expression is markedly decreased in the monocularly deprived column. We then examined which mode of *occ1* expression is activity-dependent. *occ1* expression in excitatory neurons is decreased in the monocularly deprived column but not in inhibitory interneurons. Since primate brains have distinct features from other mammalian brains, we thought the expression profiles in primates might be different from those of other mammals. The most striking difference was observed in the expression in excitatory neurons in primaly sensory areas, particularly in the visual cortex. No clear border was observed in V1 and V2 in mammals (mice, rabbits, ferrets) other than those of macaques and marmosets. These results suggest the *occ1* expression in excitatory neurons was aquired in a specific step of evolution from an ancestor to the primate.

II. Gene expression under a declarative and a non-declarative memory

In order to study informational processing underlying the declarative and non-declarative memory at molecular and cellular levels in the brain, we established two behavioral systems.

1) We have been collaborating with professor Yoshio Sakurai (Kyoto University) who developed an audio-visual discrimination task (AVD-task) system. In this task, a rat was asked to choose either an audio cue (a high tone or low tone) or a visual cue (a light from the right or the left) to obtain a food pellet. We found that the visual and audio tasks enhanced the specific expression of c-Fos in the visual and audio cortices, respectively. Among the early visual and auditory pathways examined, c-Fos was specifically induced in the cortices but not in the earlier pathways, suggesting the neural modulation of the neocortex depending on the types of the tasks. Interestingly, the task-dependent Fos expression was only observed in excitatory neurons in the relevant sensory cortices.

Although this AVD task system is quite powerful to analyze the problem described above and useful for studying underlying molecular and cellular mechanisms because of the advantages of using rodents, one concern was that the auditory stimuli and visual stimuli were in different positions. Thus, we could not exclude the possibility that the difference between the auditory task and the visual task may not completely depend on the modality (visual Vs auditory) difference.

We wanted to solve this problem by placing auditory and visual stimuli in the same position. We also use nose-poking to measure the reaction time in which a rat responds to stimuli. By using this behavioral system, we were able to confirm amodal recognition of space which means that a rat can respond to a different modality (visual or auditory) if the stimuli are in the same position as previously reported in other systems. We also confirmed multisensory enhancement is indeed observed in rats. These results suggest that this new modified AVD system can be used to explore the molecular and cellular mechanisms underlying multisensory processing in rats (Sakata *et al*. Exp. Brain Res.,*159*, 409-417, 2004).

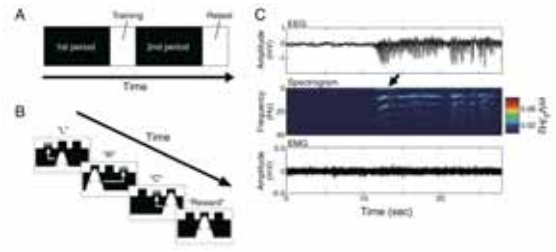

Figure 2. Properties of 7-12 Hz oscillations.(A) Schedule for experiments under task conditions of EEG and EMG recordings. (B) Illustration of one-trial schedule in sequential nose-poking task. In this task, when rats were responded to a predetermined sequence of the direction of lights (e.g., L-R-C), they were rewarded by a food pellet. (C) Top panel: an EEG signal from a frontal region during 7-12 Hz oscillations. Middle panel: an interpolated spectrogram of the EEG signal. These oscillations consist of basic frequences (7-12 Hz) with their harmonics. An arrow indicates slow-wave activities (<4Hz) observed in the initial stage. Bottom: a stimultaneous EMG signal. (These data are shown in Sakata *et al*. Neuroscience *134*, 1099-1111, 2005)

Various oscillations are observed depending on brain-states. Spike wave complexes (Sws), 7-12 Hz cortical oscillations with harmonics in awake but immobile rats, have been widely regarded as a model of paroxysmal activities in absence epilepsy. However, several studies have suggested that SWs in the primary somatosensory cortex are analogous to human mu rhythms. Because SWs have been frequently observed depending on vigilance levels, SWs in rats might represent normal brain-states related to the sleep-waking cycle. To elucidate behavioral contexts to induce SWs and temporal relations between SWs and neuronal ensemble activities, we recorded local field potentials (LFP) and multi-unit activities (MUAs) in the medial prefrontal cortex and electroencephalogram (EEG) in the bilateral regions of rats. Long-term recordings of EEG revealed that SWs were prominently generated in frontal and parietal regions and that SWs frequently followed non-REM sleeps. Occurrence probabilities of SWs significantly increased after the rats performed cognitive tasks. Our results suggest that SWs are one of the brain-state-specific oscillations rather than pathological activities. We also observed that MUAs were organized into phase-locked patterns in cycles of these oscillations. MUAs recorded from electrodes apart to each other were synchronized during SWs (Sakata *et al.* 2005a).

2) The other task we developed is a wheel running system in which a water-deprived mouse is made to run to obtain water because the wheel with the pegs is turning tothe other direction (Kitsukawa *et al*. SFN Meeting, 2002). The pegs can be changed with [?] various patterns as desired. The task required of the mouse thus can be regarded as representing a procedural learning. We examined various areas of mouse brain following

changes to the peg pattern. Among the areas examined, we found marked c-Fos expression in the striatum, cerebral cortex. The striatum, which is composed of projection neurons and several distinguished types of interneurons, is known to play an important role in reward-based learning. The characterization of these subtypes of interneurons has been progressed. However, their roles in behavioral tasks have remained obscure. We are currently examining the altered behavior under the pharmacological treatments that affect the metabolism of the interneurons in the striatum.

III. A transcriptional factor (zfh-5) both of which sense and antisense RNA are expressed in the brain

Antisense RNAs have recently been reported to be expressed much more than previously thought. However, the roles of antisense RNAs have been widely unknown, particularly in the brain. We found that the antisense strand of zfh-5, the gene for a novel transcription factor that we identified, was also transcribed in the developing mouse brain, in a manner complementary to the expression of zfh-5 mRNA (Figure3; Komine *et al*. 2005). Using gene-targeting approach, we showed that the expression of zfh-5 mRNA was negatively regulated by this antisense RNA. Our observations suggest that the suppression mechanism by the zfh-5 antisense RNA differs from those by previously known antisense RNAs.

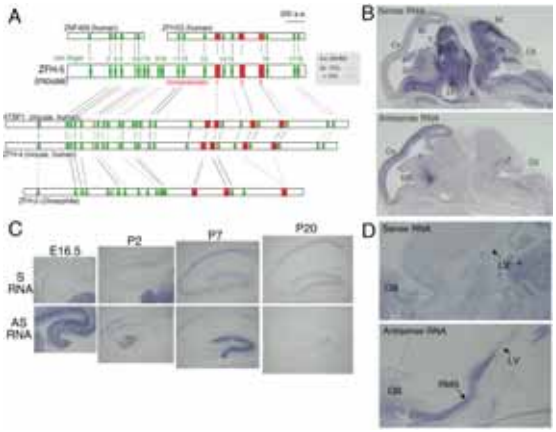

Figure 3. *zfh-5* expression in *ΔASC* mutant. (**A**) A pair of wild-type (+/+) and homozygous (*ΔASC/ΔASC: deleted in antisence control region*) littermates was used. In the *ΔASC/ΔASC* embryo (E16.5), the antisense RNA was almost depleted and the amount of the sense RNA increased in various regions of the brain compared with the same age of +/+ embryo. Arrows: pontine nuclei. (**B**) In the *ΔASC/ΔASC* mutant mice (P8), the ectopic expression of the sense RNA was prominent. (**C**) Two sets of RNAs (each lane contains 1 μg of polyA$^+$ RNA) from wild-type (+/+), hetero- (+/*Δ*) and homozygous (*Δ*/*Δ*) mice were electrophoresed and each set was hybridized with a strand-specific probe. As the amount of the antisense RNA decreased (+/+>+/*Δ*>*Δ*/*Δ*), that of the sense RNA including the full-length mRNA (indicated by arrowheads) increased (D) The expression patterns of sense and antisense RNA in RMSs (Rostal migratony stream) (The figure is shown in Komine *et al*. Mol. Cell Neurosci. 2005 Oct 27; [Epub ahead of print])

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Komatsu, Y., Watakabe, A., Hashikawa, T., Tochitani, S., and Yamamori, T. (2005). Retinol-binding protein gene is highly expressed in higher-order association areas of the primate neocortex. Cereb. Cortex *15*, 96-108.

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DIVISION OF BEHAVIORAL BIOLOGY (ADJUNCT)

Professor (Adjunct): *MORI, Yuji*
Associate Professor (Adjunct): *TSUKAMURA, Hiroko*
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NIBB Research Fellow: *IKEMURA, Ryota*
Visiting Scientists: *TAKEUCHI, Motoki*
SATO, Hiroaki

In mammals, several social behaviors are dependent on sex. The sex-dependent patterns of behaviors must be acquired through highly irreversible processes during development. We hypothesize that the long-term effects of sex steroids at the perinatal stage on behaviors after puberty are somehow marked at the genome level. We are currently investigating the epigenetic status of the critical brain subareas responsible for the sex difference of the behaviors.

I. DNA methylation of nuclear receptors

In mammals, DNA methylation, mainly occurring on CG dinucleotides, is a fundamental mechanism that differentiates the gene expression pattern in respective cell. DNA methylation is a restraint of the pluripotency because once the pattern is established during development it is maintained through cell division. On the other hand, for example, some fish that contain much less methylation activity are found to easily and reversibly change the sex status according to the environmental context. In rodents, endocrine disturbance at the fetal and/or postnatal stage irreversibly changes the behaviors such as the lordosis (in females) and the mounting (in males) after the pubertal stage that are normally dependent on genetic sex. In some cases, lordosis can be observed even in males and *vice versa*. These clearly indicate that sex-dependent patterns of behaviors are not directly dependent on "sex-specific" genes but rather established through epigenetic processes. We have found the sex-dependent DNA methylation pattern of steroid receptor genes (estrogen receptor α, ERα; androgen receptor, AR; progesterone receptor, PR) in the male and female rat brain subareas. In this year of 2005, we report herein the DNA methylation analyses of the steroid receptor genes and identification of the naturally occurring antisense RNAs as potent and specific epigenetic regulators in rats and mice.

1-1 Sex-dependent DNA methylation

In general, the binding affinity of the transcription factors is reduced by hypermethylation that are frequently observed on promoter region of either ERα, AR or PR in tumor cells. We analyzed the DNA methylation status of their 5'-flanking regions in the rat using the bisulfite sequencing method. DNA methylation patterns differed depending on brain subarea. For ERα, no differences were found within male rat brain subareas examined. In contrast, hypermethylation was specifically observed in the bed nucleus of the stria terminalis (BNST) of female rats. The sex-dependent DNA methylation was also found in the mouse BNST. It was to be noted that methylation pattern differed depending on brain subarea examined even in male mice. These suggested the species difference of the transcriptional regulation of the ERα. Nonetheless, sex-dependent difference was specifically observed in the BNST, suggesting that the BNST is an area important for the sexual differentiation of the brain in rats and mice.

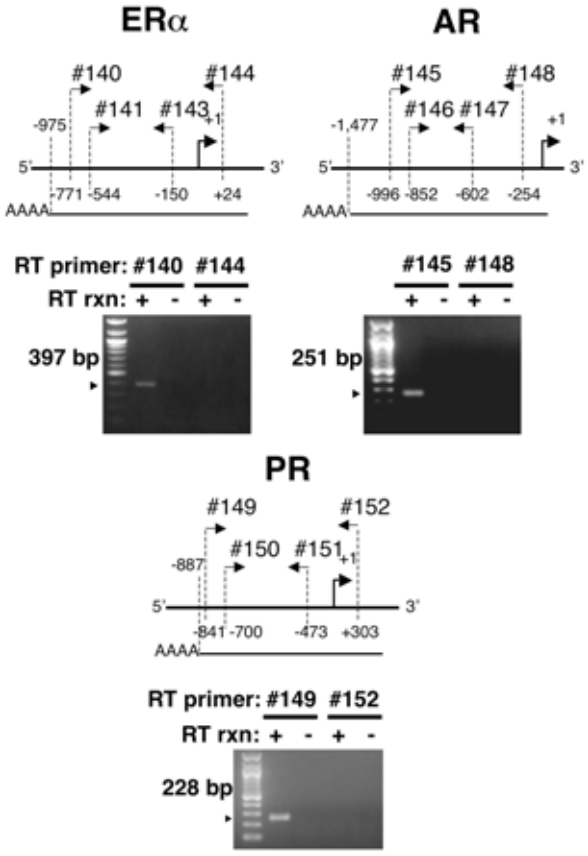

Figure 1. Detection of ERα-as, AR-as and PR-as. Each upper panel shows the genomic structure of ERα, AR, or PR. Arrows denote the primers used for the PCR amplification. Each lower panel shows the strand-specific RT-PCR. Note that specific bands were observed when sense primers were used for RT reaction.

1-2 Identification of naturally occurring antisense RNAs

The number of mammalian non-coding RNA genes is rapidly expanding. We found naturally occurring antisense RNAs for rat and mouse ERα, AR, and PR, tentatively named ERα-as, AR-as, and PR-as, respectively (Figure 1). Neither of the antisense RNAs showed obvious open reading frames, and possible amino acid motifs, raising the possibility that these function without translation. Each RNA overlapped with the respective promoter region including the sex-dependent differentially methylated region. In adult tissues, expression patterns were tissue- and/or sex-specific. For example, ERα-as and PR-as were predominantly expressed in the ovary. In the testis, these expressions were under the detectable level. In contrast, AR-as was highly expressed in the testis, but not in the ovary. It was to be noted that expressions in the brain were also area-

and/or sex-dependent. These structural features led us to investigate the possible involvement of these non-coding RNAs on the establishment of the specific DNA methylation pattern.

We used a rat pheochromocytoma cell line, PC12, that differentiates in response to NGF, as a model system (Figure 2). Most of the CG sites located on the sex-dependent differentially methylated region of ERα became methylated after the NGF treatment, confirming that the examined region was methylatable according to the cellular status. We introduced an expression vector driving a fragment of rat ERα into PC12 cells by transient transfection. Overexpression of a fragment of ERα-as containing the differentially methylated region dramatically diminished genomic methylation at the corresponding CG sites. This effect was gene-specific, because a PR-as fragment did not reverse the DNA methylation of ERα. We are currently investigating the expression profile of the novel antisense RNAs and their potential use for DNA methylation manipulation at the perinatal stage, that is thought critical period of sexual differentiation of the brain.

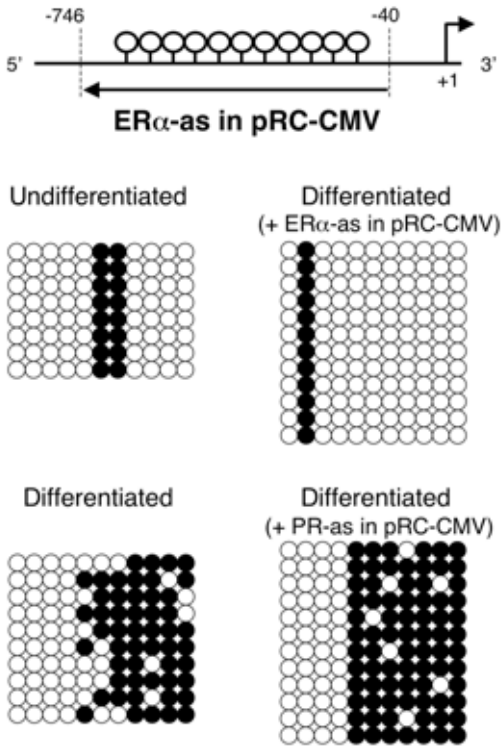

Figure 2. Methylation analysis of the ERα promoter in PC12 cells. Upper panel shows the location of CG sites and the fragment introduced into the cells. In the lower panel, each row of circles represents a single cloned allele with open circles for non-methylated cytosines and filled circles for methylated cytosines.

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Momozawa, Y., Takeuchi, Y., Tozaki, T., Kikusui, T., Hasegawa, T., Raudsepp, T., Chowdhary, B. P., Kusunose, R., and Mori, Y. (2005). Sequence, detection of polymorphisms and radiation hybrid mapping of the equine catechol-o-methyltransferase gene. Anim. Genet. *36*, 190.

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LABORATORY OF NEUROPHYSIOLOGY

Associate Professor: *WATANABE, Eiji*
NIBB Research Fellow: *YAMADA, Misuzu*
Technical Assistant: *TAKEUCHI, Kazumi*

When the correct balance between water and sodium level in the body fluid has been broken, terrestrial animals feel water and salt appetite or satiety, and these perceptions subsequently induce the animal behaviors referred to as ingestion or aversion. Our research is focused on understanding the molecular and neural mechanisms underlying the animal behaviors essential to homeostasis of the body fluid.

To explain the properly regulated animal behaviors, neurobiologists have postulated the existence of both osmoreceptors and specific sodium receptors in the brain. However, the molecular entities of these receptors have not been found for a long time. In 2000, by using the gene-targeting technology, we first clarified that Na_x sodium channel is a probable candidate for the specific sodium receptor in the brain.

Na_x had long been classified as a subfamily of voltage-gated sodium channels (NaChs) that serve to generate action potentials in electrically excitable cells such as neuronal and muscle cells. Compared to the other NaChs, however, Na_x has unique amino acid sequences in the regions, which are known to be involved in voltage-dependent activation and inactivation, suggesting that it must have specific functional properties.

To clarify the functional role of Na_x channel, *Na_x*-gene deficient mice were generated by gene-targeting technique and the physiological phenotypes have been examined. Behavioral studies suggested that the Na_x channel plays an important role in the central sensing of body-fluid sodium level and regulation of salt intake behavior. Na_x-deficient mice ingested hypertonic sodium chloride solution in excess in comparison with wild type-mice. LacZ reporter gene knocked into *Na_x*-gene locus revealed that *Na_x* gene is expressed in the circumventricular organs, which are the specialized central organs involved in sensing of sodium concentration and osmosity in the body fluids.

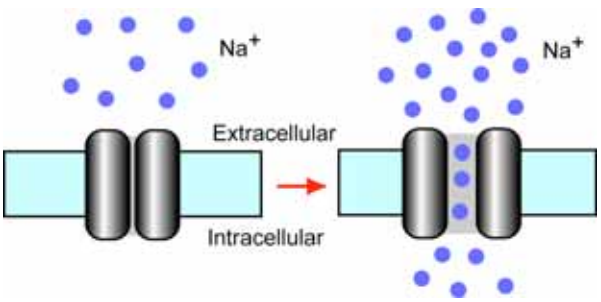

Figure 1. Na_x is a sodium channel sensitive to extracellular sodium level. When the extracellular sodium concentration increases, Na_x channel opens the gate pore and generates the sodium ion influx into the cells. This view was hypothesized by ion-imaging studies.

In 2002, sodium ion imaging and electrophysiological studies using cultured cells derived from the subfornical organs demonstrated that Na_x channel is an extracellular sodium-level sensitive sodium channel (Figure 1). Further, we found that Na_x channel is expressed in non-myelinating Schwann cells and alveolar type II cells in addition to the cells in the circumventricular organs. Na_x channel is thus likely to be involved in reception of sodium-level in the body fluids at the circumventricular organs and sodium absorption in the visceral nervous system and in the lung.

In 2003, we found in collaboration with Prof. Yamamoto's group at Osaka University that the peripheral nervous system has only subtle effects on the higher preference for sodium chloride as observed in the mutant mice. The results suggest that the mutant phenotype is mainly due to the lack of Na_x channel in the central nervous system.

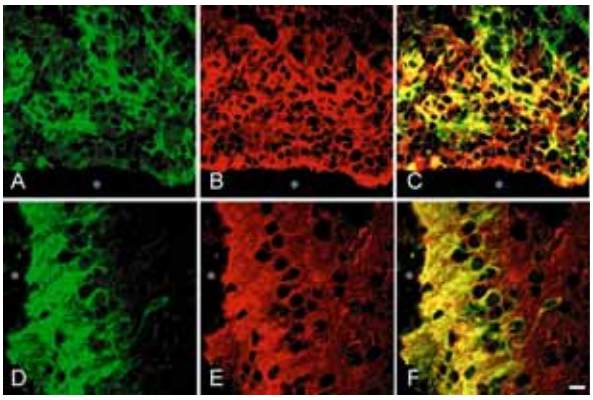

Figure 2. The Na_x channel is co-localized with a glia-specific glutamate transporter GLAST in the SFO and OVLT. Coronal tissue sections of the SFO (*A-C*) and OVLT (*D-F*) were double-stained with anti-Na_x (*A, D*) and GLAST (*B, E*) antibodies. Right panels (*C, F*) are merged images of the left (*A, D*) and middle (*B, E*) panels. Asterisks indicate the ventricles. A large number of round GLAST- and Na_x-negative black holes represent neuronal cell bodies. Scale bar: 10 μm.

In 2004, we developed an automatic measurement equipment for intake volume of drinking solutions. Using this equipment, we showed that the subfornical organ is the principal site for the control of salt-intake behavior, where the Na_x channel is the sodium-level sensor. Infusion of a hypertonic sodium solution into the cerebral ventricle induced extensive water intake and aversion to saline in wild-type animals but not in the knockout mice. Importantly, the aversion to salt was not induced by the infusion of a hyperosmotic mannitol solution with physiological sodium concentration in either genotype of mice. When Na_x cDNA was introduced into the brain of the knockout mice with an adenoviral expression vector, only animals which received a transduction of the *Na_x* gene into the subfornical organ among the circumventricular organs recovered salt-avoiding behavior under dehydrated conditions.

These results clearly show that the subfornical organ is the center of the control of salt-intake behavior in the brain, where the sodium-level-sensitive Na_x channel is involved in sensing the physiological increase in the sodium level of body fluids.

In this year of 2005, in order to understand how the circumventricular organ translates extracellular sodium-level sensed by Na_x channel to the neural activities, we identified subcellular localization of Na_x channel in the organs. Double immunostaining (Figure 2) and immuno-electronmicroscopic (Figure 3) studies clearly showed that Na_x channel was exclusively localized to perineuronal lamellar processes extended from astrocytes and tanycytes in the organs. Importantly, glial cells derived from the organs were capable of sensing extracellular sodium-level, as analysed by ion-imaging method. In addition, we found that the Na_x-expressing glial cells enveloped multiple kinds of neurons including GABAergic interneurons in the organs. Finally, in the organs, neuronal population activated by water deprivation was different from GABAergic interneurons, as monitored by Fos immunoreactivity. Together with previous observation that the organs of *Na_x* knockout mice is hyperactive under water deprivation, these results indicate that the glial Na_x channel senses increased sodium-level in the body fluid and controls the neuronal activity through glial cells.

Since we first reported aberrant behaviors found in *Na_x* knockout mice, a series of our studies have clarified that Na_x channel is a sodium-level sensitive sodium channel playing an essential role in the sodium-sensing of the circumventricular organs and in the control of salt-intake regulation. These works identified the molecular entity of the brain sodium sensor, which has long been hypothesized as one of the important physiological issues. In this year, we newly demonstrated that the primary subcellular locus sensing sodium-level is perineuronal glial processes. This finding suggests that neuron-glia complex plays a key role on the sodium sensing in the circumventricular organs.

Currently, we are now trying to construct functional expression systems of Na_x sodium channel using various heterologous cell lines. The heterologous expression system will provide us useful information on the channel characters. Furthermore, we are studying the involvement of Na_x sodium channel in the regulation of hormone release, using neurohypophyseal vasopressin system. The posterior pituitary is one of simple model systems for research of Na_x sodium channel, since there are only two kinds of cellular components, the nerve terminals releasing neurohypophyseal hormones and glial cells expressing Na_x sodium channel. The model system will also provide us useful information on the physiological function of Na_x channel.

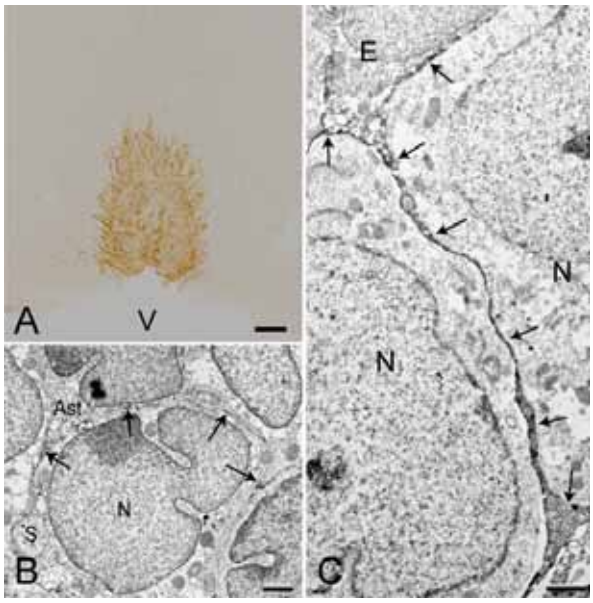

Figure 3. The Na_x channel is localized to glial processes enveloping neurons in the OVLT. *A*, A coronal tissue section of the OVLT stained with anti-Na_x antibody. Fiber-like structures radiating out from the midline and ventricle were immunopositive. *B*, *C*, Immunoelectron microscopy using anti-Na_x antibody. In *B*, the core region of the OVLT is shown. Neurons and their processes are surrounded by immunopositive thin processes of astrocytes. In *C*, a ventricular region in the OVLT is shown. Ventricular side towards the upper side. Neurons are covered by extremely thin immunopositive processes of ependymal cells. Arrows in *B* and *C* indicate immunopositive signals. V, ventricle; N, neuron; S, synapse; E, ependymal cell; Ast, astrocyte. Scale bars: 50 μm for *A*, and 1 μm for *B* and *C*.

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Original papers

Niisato, K., Fujikawa, A., Komai, S., Shintani, T., Watanabe, E., Sakaguchi, G., Katsuura, G., Manabe, T., and Noda, M. (2005). Age-dependent enhancement of hippocampal LTP and impairment of spatial learning through the ROCK pathway in protein tyrosine phosphatase receptor type Z-deficient mice. J. Neurosci. *25*, 1081-1088.

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LABORATORY OF NEUROCHEMISTRY

Associate Professor: *SASAOKA, Toshikuni*

Our major research interest is to understand the physiological role of dopaminergic system in animal behavior, especially locomotion and eating behavior, using genetically altered mice, both transgenic and gene knockout mice. In addition, we have developed a novel method of conditional mutagenesis in mice in order to analyze the function of the gene of interest in detail. We analyze function of neurotransmitter receptor complex by using biochemical analysis of the dystrophin complex on the skeletal muscle membrane.

I. Role of dopaminergic transmission in locomotion and eating behavior

The dopaminergic system is implicated in the regulation of the several peptide hormones in the pituitary, the modulation of locomotor activity, the modulation of synaptic plasticity and the development of neuron. The dopaminergic system is also implicated in control of emotion, motivation and cognition. Dysfunction of dopaminergic system can result in several neurological and psychiatric disorders such as Parkinson's disease and schizophrenia.

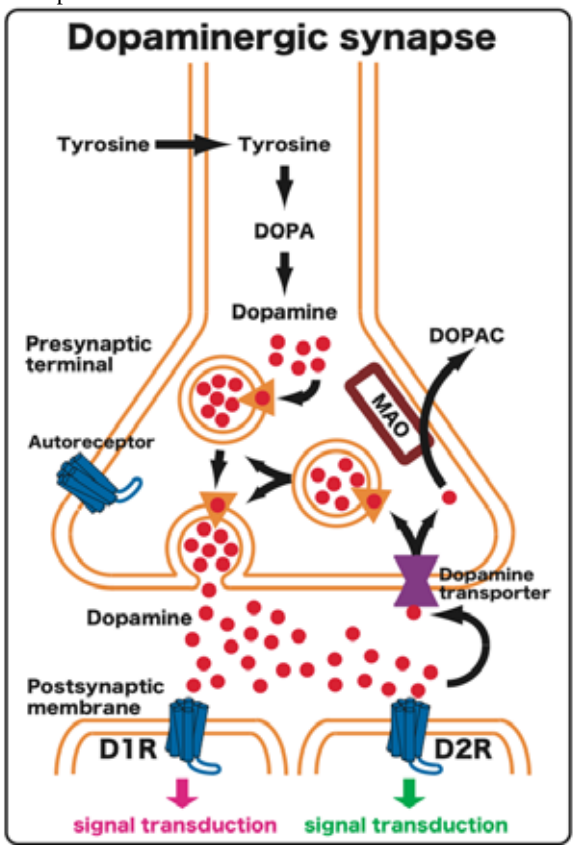

Figure 1. Schematic drawing of dopaminergic synapse

In mammals five subtypes of dopamine receptor (D1R, D2R, D3R, D4R and D5R) are identified and divided into two subgroups referred to as D1-like (D1R, D5R) and D2-like (D2R, D3R and D4R) receptors on the basis of their gene structure and their pharmacological and transductional properties. D1R and D2R are most abundantly and widely expressed in the brain and often play a role synergistically. D1R has an opposite property to D2R with respect to the intracellular signal transduction.

We have been investigating the involvement of dopaminergic transmission via D1R and D2R in the regulation of locomotion and eating behavior in collaboration with the Laboratory of Director General. We generated *D1R/D2R* double knockout (DKO) mice by crossing *D1R* knockout (KO) with *D2R* KO mice, and observed that *D1R/D2R* DKO mice exhibited impairment in locomotion and eating behavior and died prematurely. To investigate molecular mechanism of regulation in locomotion and eating behavior, we generated transgenic mice harboring tetracycline-regulated expression of the *D1R* gene on the *D1R/D2R* DKO background. Several transgenic mouse lines successfully rescued lethal phenotype of the *D1R/D2R* DKO mice and showed doxycycline (Dox) controllable expression of transgenic *D1R* gene (named as *D1R/D2R* DKO-*D1R* rescued mice). The *D1R/D2R* DKO-*D1R* rescued mice exhibited decrease in locomotion and food/water intake as well as decrease in amount of transgene expression after Dox administration (Figure 2).

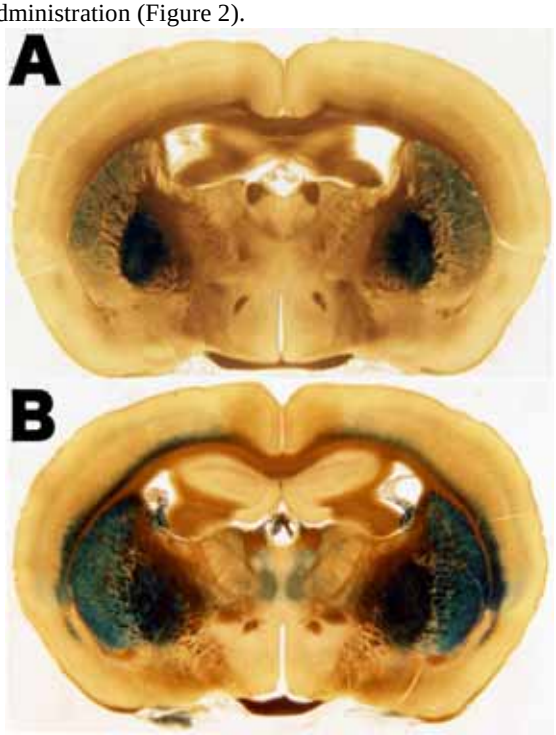

Figure 2. (A) The amount of transgene expression was suppressed in the striatum of the *D1R/D2R* DKO-*D1R* rescued mice by doxycycline (Dox) administration. (B) Before Dox administration the intensive expression of transgene was seen in the striatum. Frontal sections of mouse brains with X-gal staining were shown.

We are attempting to identify areas in which the *D1R* expression was suppressed by Dox administration in order to point out areas responsible for regulation of locomotion and eating/drinking behavior. In addition we are investigating whether or not there is critical period in

development for regulation of locomotion and eating behavior by dopaminergic transmission.

II. Developing a novel conditional mutagenesis method in mice

In order to overcome the limitations of the conventional mouse molecular genetic approach in the functional analysis of target genes, we substituted one critical amino acid residue of N-methyl-D-aspartate receptor (NMDAR), leading to NMDAR activation. The NMDARs are widely expressed in the nervous system, fundamental to excitatory neurotransmission, and play a number of important roles at different brain loci and time points. The NMDARs act as a coincidence detector and are not only important for neuronal differentiation, migration, and survival but are also critical for activity dependent synapse formation. It is suggested that the aberrant activation of NMDAR causes excitotoxicity, leading to neuronal death in various neurological diseases.

That the Ca^{2+} permeability through NMDAR is blocked by magnesium (Mg^{2+}) in a voltage-dependent manner indicates an essential role of NMDAR as a coincidence detector. Functional NMDARs consist of NMDAR1 (NR1) subunit and at least one subunit of NMDAR2A-2D (NR2A-NR2D). It has been shown that the NR1/NR2A complex expressed in cultured cell is highly sensitive to the voltage-dependent Mg^{2+} block and that the substitution of asparagine (Asp595) by glutamine (Gln595) in the second transmembrane domain of the NR2A subunit results in a reduction of the Mg^{2+} block of the NR1/NR2A complex.

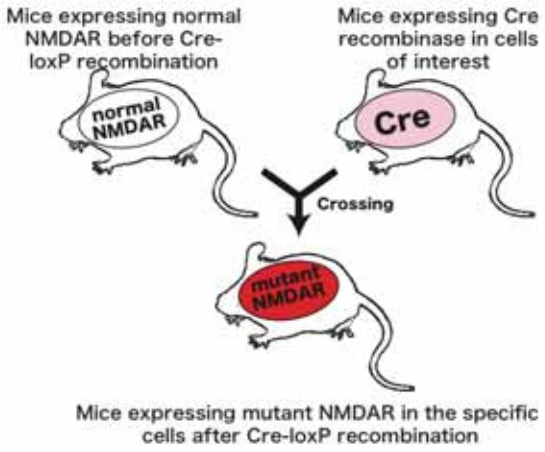

Figure 3. Conditional mutagenesis in mice. First, mutant mice expressing normal NMDAR molecule before Cre-loxP recombination were generated. Second, transgenic mice expressing Cre recombinase in cells of interest were generated. Third, these two mouse lines were crossed to generated mice expressing mutant NMDAR molecule in the cells in which Cre-loxP recombination was executed.

However, the role of Asp595 of the NR2A subunit and the effects of substitution of it with Gln595 on the function of NMDAR *in vivo* remain to be clarified.

We develop conditional mutagenesis method in mice using Cre-loxP recombination (Figure 3). By our method, we accomplished conditional substitution of the amino acid in mice and our mutant mice exhibited aberrant NMDAR activation and a neurological phenotype, similar to that of mouse models of neurological disorders. Interestingly, the phenotype of the mice was completely suppressed by administration of NMDAR antagonists. This clearly indicates that the NMDAR activation by the critical amino acid substitution leads to the neurological phenotype.

Our method is vastly applicable to functional analysis of any desired gene and should contribute to studies on the structural and functional relationships of relevant genes.

III. The molecular architecture and the physiological role of the sarcoglycan complex (SGC)

Sarcoglycans (SGs) are trans-sarcolemmal glycoproteins that associate together to form SGC and are present in the sarcolemma. SGC, together with dystrophin and the dystroglycan complex, comprises the dystrophin complex, which is considered as the mechanical link between the basement membrane and the intracellular cytoskeleton for protecting the sarcolemma from mechanical stress during muscle contraction. Each of four SG subunits (α-, β-, γ- and δ-SG) is responsible for four respective forms of SG-deficient muscular dystrophy, sarcoglycanopathy (SGP). All of the SGs and sarcospan are absent in the sarcolemma in any form of SGP, suggesting that the SGC is not assembled if a single subunit of the SGC is absent.

To analyze the function of the SGC, we generated the β-SG KO and γ-SG KO mice. These KO mice developed progressive muscular dystrophy and all SGs and sarcospan were absent in the sarcolemma. The dystrophin complex isolated from the SG-deficient skeletal muscles was biochemically unstable. This indicates that SGC and sarcospan play an important role in stabilizing the dystrophin complex connecting the basement membrane and the cytoskeleton.

We have generated the KO mice of the other subunit of the dystrophin complex to analyze the physiological role of the subunit.

Publication:

Original paper

Tanaka, T., Watanabe, N., and Sasaoka, T. (2005). Unidirectional subcloning to generate more than 10^9 transformants from 1 microgram of vector DNA. The Nihon University Journal of Medicine, in press.

Review article

Ozawa, E., Mizuno, Y., Hagiwara, Y., Sasaoka, T., and Yoshida, M. (2005). Molecular and cell biology of the sarcoglycan complex. Muscle and Nerve. *32*, 563-576.

LABORATORY OF DIRECTOR GENERAL

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One of the largest themes of the 21st century is to promote brain research in an attempt to understand the "mind." There are many approaches to elucidating the "mind," including research into intelligence, memory, cognition, emotion and volition. Research on developing an excellent artificial system for information processing, research on the ontogeny and the development of the brain, and research on protecting the brain from aging, neurological and psychiatric disorders are also important themes.

Our approach to understanding the brain is to explore the molecular mechanism of higher brain function by employing genetic modification of living organisms. Major research interests of the laboratory are to elucidate a physiological role of dopamine receptors in animal behavior, an implication of N-methyl-D-aspartate receptors (NMDARs) in psychiatric disorders and the roles of the *ras* family in the brain by producing genetically altered mice, both gene targeted and transgenic mice.

I. Dopamine regulates locomotion and eating behavior

The dopaminergic system is considered to be involved in locomotor control, emotional behavior, reward, motivation and thought process. Hypoactivity or hyperactivity of dopaminergic system can result in neurological and psychiatric disorders such as Parkinson's disease and schizophrenia. In mammals five subtypes of dopamine receptors (D1R-D5R) are identified and classified into two major groups, D1-like (D1R, D5R) and D2-like (D2R, D3R, D4R) receptors on the basis of the gene structure and the pharmacological and intracellular signaling properties. The contribution of D1-like and D2-like receptors to behaviors is determined pharmacologically.

We generated knockout (KO) mice lacking each of five dopamine receptors and multiple KO mice lacking more than one dopamine receptor simultaneously. We focused on D1R and D2R, major subtypes of D1-like and D2-like receptors, respectively, which are most widely and abundantly expressed. We found the *D1R/D2R* double knockout (DKO) mice showed severe impairment in locomotion and feeding that was not observed in *D1R* or *D2R* KO mice. Although the *D1R/D2R* DKO mice were born normally and showed suckling behavior, the *D1R/D2R* DKO mice exhibited rapid decrease in locomotion and no initiation of eating and eventually died [of starvation?] by the third postnatal week. These findings suggest that dopaminergic transmission via D1R or D2R is involved in neural development of the areas that are implicated in the regulation of locomotion and eating.

To examine the involvement of [the?] dopaminergic system in the regulation of locomotion and eating we generated mutant mice in which dopaminergic transmission can be shut off at a time point of interest. We utilized tetracycline controllable expression system and generated transgenic mice harboring conditional *D1R* expression on the *D1R/D2R* DKO background (Figure 1).

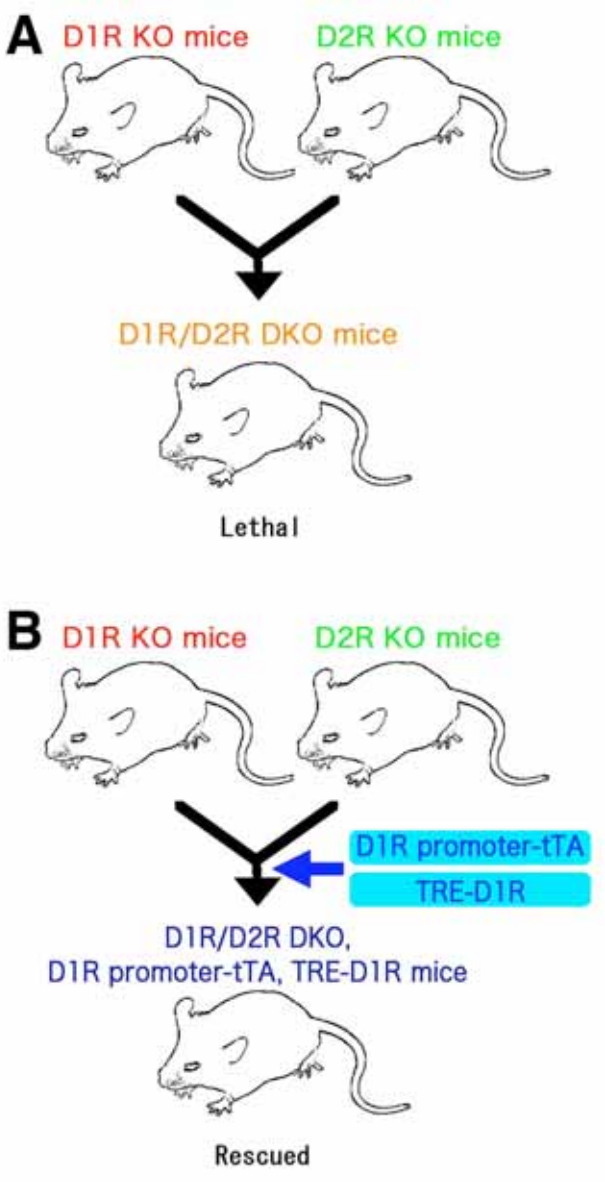

Figure 1. *D1R/D2R* DKO mice were rescued by conditional *D1R* expression. (A) *D1R/D2R* DKO mice showed impairment in locomotion and feeding and died prematurely. (B) To rescue *D1R/D2R* DKO mice, transgenic mice harboring conditional *D1R* expression on the *D1R/D2R* DKO background were generated by tetracycline controllable expression system.

We obtained several transgenic mouse lines rescuing lethal phenotype of the *D1R/D2R* DKO mice (*D1R/D2R* DKO-*D1R* rescued mice). The *D1R/D2R* DKO-*D1R* rescued mice exhibited decrease in expression level of transgene in the striatum and decrease in locomotion and food/water intake by doxycycline (Dox) administration (Figure 2). These results indicate that areas harboring Dox-controllable *D1R* expression are responsible for regulation of locomotion and eating behavior.

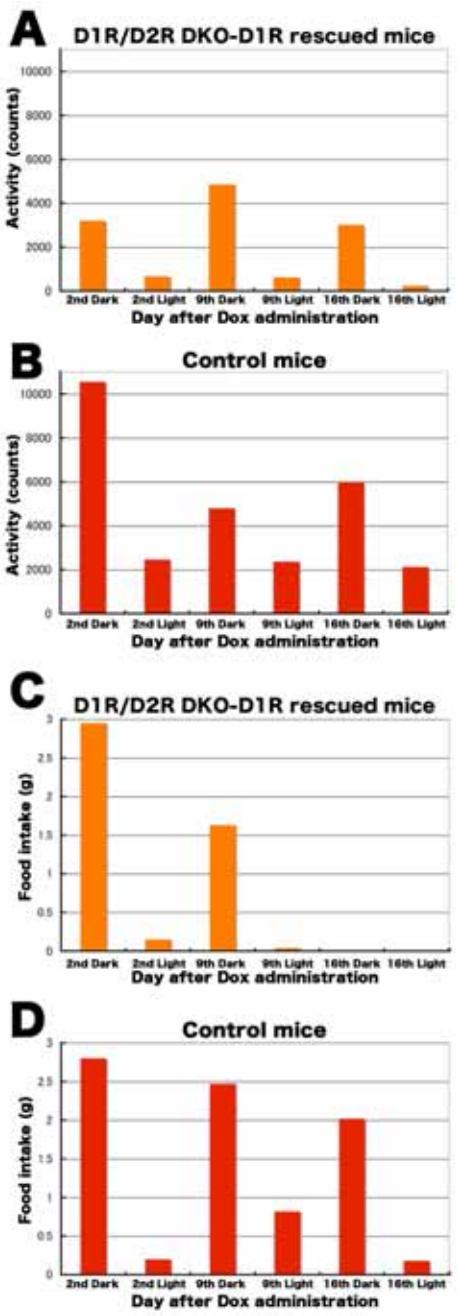

Figure 2. Alteration of locomotion and eating of *D1R/D2R* DKO-*D1R* rescued mice by Dox administration. Locomotive activities of rescued (A) and control (B) mice during light and dark periods are shown as counts of movement using an active sensor. Eating activities of rescued (C) and control (D) mice are shown as amounts of food taken during light and dark periods.

II. Analysis of the function of NMDARs

The NMDARs are widely expressed in the nervous system, fundamental to excitatory neurotransmission, and play a number of important roles. There are many reports on the involvement of the NMDARs in learning and memory. According to one hypothesis schizophrenia may involve a defect in NMDAR function. NMDARs consist of NR1 subunit and at least one subunit of NR2A-NR2D. We generated KO mice lacking each of NMDAR subunits and multiple KO mice lacking two subunits simultaneously, and found the *NR2A* homozygous, *NR2B* heterozygous mutant ($NR2A^{-/-}$,$NR2B^{+/-}$) mice exhibited behavioral alteration similar to that observed in patients with schizophrenia. We are developing an experimental devise to assess behavioral alteration of the $NR2A^{-/-}$,$NR2B^{+/-}$ mice and study the molecular mechanism.

III. *ras* family and their roles in the brain

The *ras* proto-oncogene plays a critical role in cell growth control as a central component of mitogenic signal transduction pathways. In mammals there are H-, N-, K-*ras* identified as the *ras* family. H-, N-, K-*ras* have an overlapped spatial expression pattern as well as an overlapped function. To investigate the distinct function of the individual Ras protein in the brain we generated *ras* DKO mice expressing a single Ras and triple KO mice lacking all H-, N-, and K-Ras and analyzed developmental aspects of these mutant mice.

Publication list:

Original papers

Hara, T., Saito, Y., Hirai, T., Nakamura, K., Nakao, K., Katsuki, M., and Chida, K. (2005). Deficiency of protein kinase C alpha in mice results in impairment of epidermal hyperplasia and enhancement of tumor formation in two-stage skin carcinogenesis. Cancer Res. *65*, 7356-7362.

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DIVISION OF MOLECULAR GENETICS

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The main interest of the group is in understanding the biology of the dynamic genome, namely, genome organization and reorganization and its impact on gene expression and regulation. We are also characterizing various aspects of genetic and epigenetic gene regulations, particularly the flower pigmentation of morning glories. In addition, we are undertaking reverse genetic approaches in order to elucidate the nature of dynamic genome in rice, a model plant for cereals.

I. Spontaneous mutants in morning glories

Considerable attention has recently been paid to the morning glory genus *Ipomoea* because of the experimental versatility of its floral biology including the genetics of floral variation, flavonoid biosynthesis, and transposon-induced mutations. The genus *Ipomoea* includes about 600 species distributed on a worldwide scale that exhibit various flower morphologies and pigmentation patterns. Among them, three morning glories, *Ipomoea nil* (the Japanese morning glory), *Ipomoea purpurea* (the common morning glory), and *Ipomoea tricolor,* were domesticated well as floricultural plants, and many mutants displaying various flower pigmentation patterns were isolated. The wild-type *I. nil* displays blue flowers (Figure 1A) that contain the peonidin (3' methoxyl cyanidin) derivative named Heavenly Blue Anthocyanin or HBA.

I. nil was introduced into Japan from China in the 8th century as a medicinal herb, the seeds of which were used as a laxative, and the plant became a traditional floricultural plant in Japan around the 17th century. The plant has an extensive history of genetic studies, and a number of its spontaneous mutants related to the color and shape of the flowers have been isolated. Genetic studies on the color of *I. nil* have shown that blue flower coloration was mainly controlled by two genetic loci, *Magenta* and *Purple*. Recessive *magenta* and *purple* mutants bloom magenta and purple flowers, respectively, and double mutants carrying both *magenta* and *purple* alleles display red flowers (Figure 1C). The *Magenta* gene encodes flavonoid 3'-hydorxylase, which hydroxylates the 3' position of the B-ring of anthocyanidin precursors. The *Purple* gene encodes a vacuolar Na^+/H^+ antiporter called InNHX1 that increases the vacuolar pH during flower opening, causing a shift towards the bluer coloration. While most of the plant *NHX* genes characterized are generally expressed in leaves, stems and roots and induced by NaCl treatment, the *InNHX1* gene is predominantly expressed in the flower limbs at around 12 hour before flower-opening and is very scarcely expressed in leaves, stems and roots, and no induction occurs in response to NaCl treatment. We also found that the InNHX1 proteins could catalyze both Na^+ and K^+ transport into vacuoles.

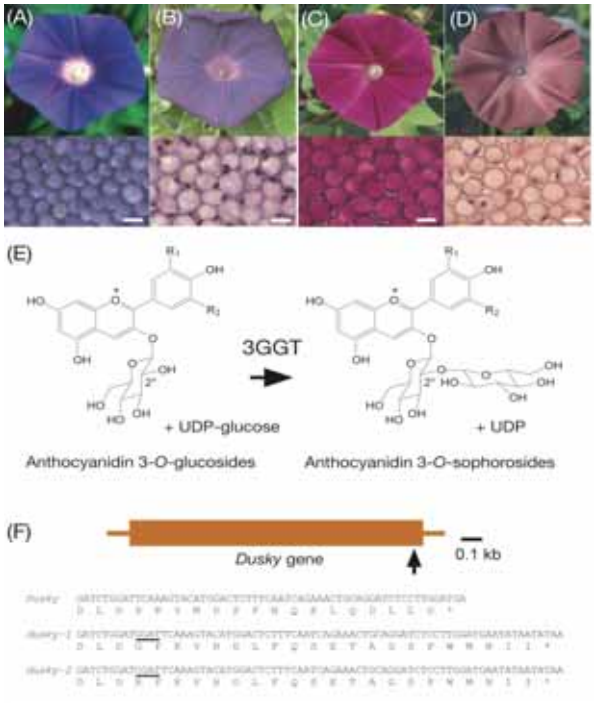

Figure1. Flower phenotypes and the *Dusky* gene for the 3GGT enzyme in *I. nil*. **A-D.** Flower phenotypes (above) and microscopic photographs of adaxial epidermal cells of flower petals (below). (**A**) The wild-type line with the *Magenta*, *Purple*, and *Dusky* alleles. (**B**) The line with the *Magenta*, *Purple*, and *dusky-1* allele. (**C**) The line with the *magenta*, *purple*, and *Dusky* alleles. (**D**) The line with the *magenta*, *purple*, and *dusky-1* allele. Scale bars in microscopic photographs indicate 30 μm. **E.** Reaction mediated by the *Dusky* gene products, 3GGT. **F.** The 3GGT gene and the dusky mutations. The large vertical arrow indicates the site of 4-bp insertions (above), and the 4-bp insertions underlined in the *dusky* mutants (below) result in frameshift mutations.

Among the various colors of *I. nil* flowers, the favorite hue for Japanese floriculturists has been reddish-brown or purplish-grey petals (Figure 1B and D) since the early 19th century, and the flower coloration is mainly caused by recessive *dusky* mutations. We noticed that the petals in all *dusky* mutants often contained intensely pigmented globules, which appeared to affect flower hue. We found that the *Dusky* gene encodes UDP-glucose:anthocyanidin 3-*O*-glucoside-2''-*O*-glucosyltransferase (3GGT), which catalyzes the the conversion of anthocyanidin 3-*O*-glucosides into anthocyanidin 3-*O*-sophorosides (Figure 1E) and that all of the *dusky* mutants tested carry the 4-bp insertion mutations GGAT or CGAT at an identical position near the 3' end of the gene, which resulted in frameshift mutations (Figure 1F). The expected 3GGT enzymatic activities were found in the crude extracts of *Escherichia coli*, in which the *3GGT* cDNA was expressed, and the introduced *3GGT* cDNA could efficiently produce 3GGT that could convert cyanidin 3-*O*-glucoside into cyanidin 3-*O*-sophoroside in transgenic petunia plants.

The transcriptional regulators for anthocyanin biosynthesis include members of proteins containing an R2R3-MYB domain, a bHLH (basic helix-loop-helix) domain, and conserved WD40 repeats. We also found that the recessive *c-1* and *ca* alleles conferring white flowers are frameshift mutations caused by a 2-bp deletion and 7-bp insertions in the genes for the R2R3-MYB and WD40 repeats transcriptional regulators, respectively.

II. Modification of endogenous natural genes by homologous recombination in rice

Rice (*Oryza sativa* L.), with the sequenced genome of 389-Mb, is an important staple food for more than half of the world's population and a model plant for other cereal species. We have developed a large-scale *Agrobacterium*-mediated transformation procedure with a strong positive-negative selection and succeeded in efficient and reproducible targeting of the *Waxy* gene by homologous recombination without concomitant occurrence of ectopic events, which must be an important first step for developing a precise modification system of the genomic sequences in rice. While the *Waxy* gene is a unique gene in the rice genome, 3 copies of the *Adh* gene are present, and both *Adh1* and *Adh2* genes reside on chromosome 11 in the same orientation with an interval of 30 kb and flank highly repetitive *Copia*- and *Gypsy*-like retroelements (Figure 2). The *Adh* genes play a key function in response to an anaerobic condition, and only a single *adh1* mutant has been isolated in rice. By improving our transformation procedure further, we are attempting to modify the *Adh1* and *Adh2* genes, the coding sequences of which are similar to each other. We obtained 9 independent transformed calli having the *Adh2* gene modified and subsequently isolated 8 fertile transgenic plants without concomitant occurrence of undesirable ectopic events. Although we were able to obtain transformed calli with modified *Adh1*, the frequency of homologous recombination at *Adh1* appeared to be about one magnitude lower than that at *Adh2*, indicating that *Adh2* contains a more active hot spot(s) for efficient homologous recombination than *Adh1*.

III. Characterization of mutable *virescent* allele in rice

Leaves of seedlings in the *virescent* mutant of rice are initially pale yellow green due to partial deficient in chlorophyll and gradually become green with the growth of the mutant. We have been characterizing a spontaneous mutable *virescent* allele, *pale yellow leaf-variegated (pyl-v)*, conferring pale yellow leaves with dark green sectors in its seedlings (Figure 3A). The *pyl-v* mutant was isolated among progeny of a hybrid between *indica* and *japonica* rice plants. The leaf variegation is regarded as a recurrent somatic mutation from the recessive pale yellow allele to the dark green revertant allele. From the *pyl-v* line, we also obtained a stable *pyl-stb* (*pyl-stable*) line that exhibits pale-yellow leaves without variegation (Figure 3B), which appeared to carry no active autonomous element acting on the nonautonomous DNA element inserted into the *Pyl* gene. The availability of the genomic sequences of both *japonica* and *indica* subspecies facilitates map-based cloning of the *pyl-v* allele. We identified an active nonautonomous DNA transposon of about 0.6 kb, named *nDart1* (*nonautonomous DNA-based active rice transposon one*), in the untranslated exon 1 of the *Pyl* gene on chromosome 3 (Figure 3D), and excision of the new DNA transposon from the *pyl* gene appears to be responsible for conferring the leaf variegation. We also

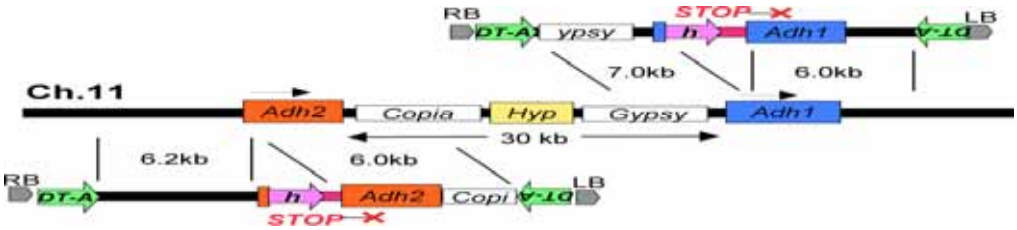

Figure 2. Strategy for gene targeting of the *Adh1* and *Adh2* genes in rice. The symbols *h* and *DT-A* on the T-DNA regions of the vectors used indicate the positive and negative selection markers, respectively. The hypothetical gene flanked by the retroelements on chromosome 11 is indicated by *Hyp*.

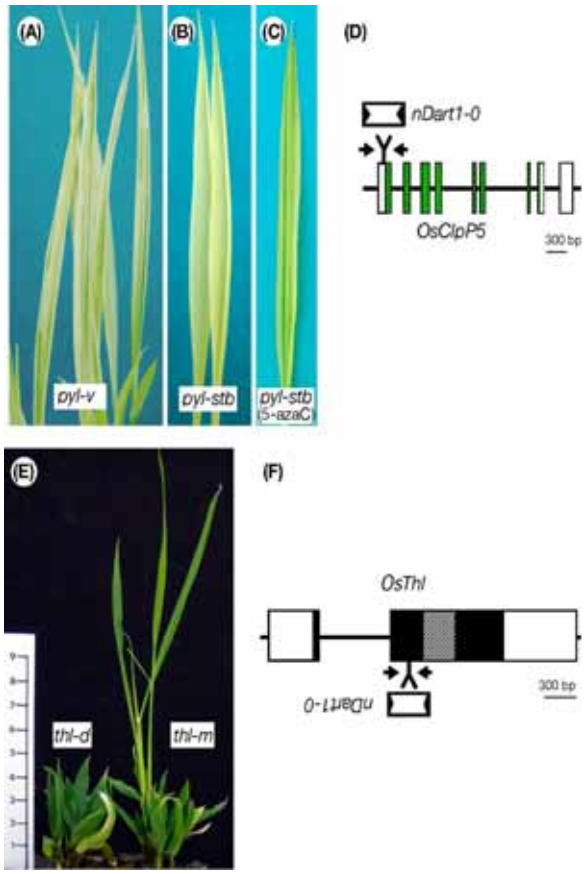

Figure 3. **A-C.** Leaf phenotypes of the mutable *pyl-v* allele, the stable *pyl-stb* allele, and the stable *pyl-stb* allele treated with 5-azaC. Dark-green sectors in a pale-yellow leaf can be seen in *pyl-v* and *pyl-stb* treated with 5-azaC. **D.** Structure of the *pyl-v* allele carrying the *nDart1* insertion. The white and green boxes represent the *Pyl* exons and coding region, respectively. The upper box and small horizontal arrows indicate the *nDart1* element and the positions of the primers to detect the *nDart1* insertion. **E.** A stable *thl-d* mutant and a mutable *thl-m* plant producing a normal revertant tiller. **F.** Structure of the *thl-m* allele carrying the *nDart1* insertion. The open, black and shadowed boxes represent the *Thl* exons, cording region, and a putative esterase/lipase domain.

demonstrated that the transposition of *nDart* could be induced by crossing with a line containing an active autonomous element, *aDart*, and stabilized by segregating out of *aDart* under natural growth conditions. Not only *pyl-stb* but also the *japonica* cultivar Nipponbare carries no *aDart*, although they contain epigenetically silenced *Dart* elements that can be activated by the treatment of 5-azaC (Figure 3C). We also identified a novel mutable dwarf allele, *thl-m* (*thambelina-mutable*), which conferred a tiny and gibberellin-insensitive dwarf phenotype (around 4 cm in height, shorter than a thumb) with occasional appearance on a normal fertile tiller (Figure 3E) and was caused by an *nDart1* insertion (Figure 3F). No somaclonal variation should occur in mutant lines induced by our newly characterizing endogenous element, because no tissue culture is involved in its activation. In this respect, it is important to emphasize here that tissue culture is necessary in all of the currently available rice reverse genetic approaches including transposon tagging systems employing exogenous or endogenous transposons. We are currently attempting to develop a novel transposon tagging system in rice.

Publication List:

Original papers

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Saito, N., Toki, K., Morita, Y., Hoshino, A., Iida, S., Shigihara, A., and Honda, T. (2005). Acylated peonidin glycosides from *duskish* mutant flowers of *Ipomoea nil*. Phytochemistry *66*, 1852-1860.

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DIVISION OF GENOME DYNAMICS

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The genomes of higher organisms contain significant amounts of repetitive sequences which, in general, are unstable. At present, neither the physiological function(s) of repeated sequences nor the mechanism controlling instability is fully understood. To clarify these aspects, we are pursuing several themes using *Saccharomyces cerevisiae* and in 2005 the following four subjects have advanced our knowledge of the dynamics of the genome: (1) identification of non-coding functional elements possibly involved in amplification and stability of the rRNA repeated genes in yeast, (2) a finding of recombination regulation by transcription-induced cohesin dissociation in rDNA, (3) a finding of a new role of condensin in maintaining a long repeated structure of rDNA, (4) development of *in vivo* artificial gene amplification systems.

I. Identifying gene-independent non-coding functional elements in the yeast ribosomal DNA by phylogenetic footprinting

Sequences involved in the regulation of genetic and genomic processes are primarily located in non-coding regions. Identifying such cis-acting sequences from sequence data is difficult because their patterns are not readily apparent, and, to date, identification has concentrated on regions associated with gene-coding functions. We used phylogenetic footprinting to look for gene-independent non-coding elements in the ribosomal RNA gene (rDNA) repeats from several *Saccharomyces* species. Similarity plots of ribosomal intergenic spacer alignments from six closely related *Saccharomyces* species allowed the identification of previously characterized functional elements, such as the origin-of-replication (ARS) and replication-fork barrier (RFB) sites, demonstrating that this method is a powerful predictor of non-coding functional elements. Seventeen previously uncharacterized elements, showing high level of conservation, were also discovered. The conservation of these elements suggests that they are functional, and we demonstrate the functionality of two classes of these elements, a putative bidirectional non-coding promoter (later we named E-pro) and a series of conserved peaks with matches to the origin-of-replication core consensus. Our results paint a comprehensive picture of the functionality of the *Saccharomyces* ribosomal intergenic region and demonstrate that functional elements not involved in gene-coding function can be identified by using comparative genomics based on sequence conservation.

II. Recombination regulation by transcription-induced cohesin dissociation in rDNA repeats in yeast

In most eukaryotic organisms, the rDNAs are clustered in long tandem repeats on one or a few chromosomes. Although the total number of these chromosomal rDNA repeats appears to be maintained at a level appropriate for each organism, genes with such a repeated structure are in general thought to be unstable because of a high frequency of recombinational events. Thus, it might be expected that organisms have developed systems to regulate recombination within rDNA repeats.

In the yeast *S. cerevisiae*, approximately 150 copies of rDNA are maintained on chromosome XII. Recombinational events within the rDNA repeats in normal growing yeast cells appear to be mostly mediated by a *FOB1*-dependent system. *FOB1* is the gene required for fork blocking activity at RFB site, recombination in the rDNA region, and expansion/contraction of rDNA repeats. The latter two activities are likely to be triggered by double-strand breaks at the RFB site and repair of the breaks via gene conversion. On the other hand, the *SIR2* gene plays an important role in decreasing the frequency of recombination in yeast rDNA. Sir2p is a protein required for transcriptional silencing at three yeast chromosomal regions, silent mating type loci, telomeres, and rDNA. It is generally believed that Sir2p, perhaps through its NAD^+-dependent histone deacetylase activity, plays an essential role in forming a higher order of repressive chromatin structure – heterochromatin - which prevents general access of the PolII machinery and some other macromolecules, thus causing silencing as well as decreasing recombination in the chromosomal rDNA repeats. Therefore, mutations in gene *SIR2* increase recombination within rDNA repeats as assayed by marker loss or extrachromosomal rDNA circle formation.

We examined the mechanism involved in the increased frequency of recombination in rDNA repeats that is observed in mutants defective in *SIR2* functions. We measured the frequency of *FOB1*-dependent arrest of replication forks, consequent DNA double-strand breaks, and formation of DNA molecules with Holliday junction structures, and found no significant difference between *sir2Δ* and *SIR2* strains. Formal genetic experiments measuring mitotic recombination rates within individual rRNA genes also showed no significant difference between these two strains. Instead, we found a significant decrease in the association of the cohesin subunit Mcd1p (Scc1p) to the rDNA in *sir2Δ* relative to *SIR2* strains. From these and other experiments, we conclude that *SIR2* prevents unequal sister-chromatid recombination, probably by forming special cohesin structures, without significant effects on recombinational events within

individual rRNA genes.

We found that bidirectional transcription actually occurs from an assuming non-coding bidirectional promoter (E-pro) within the rDNA and is absolutely required for amplification of rDNA. E-pro transcription stimulates the dissociation of cohesin, a DNA binding protein complex that suppresses sister-chromatid-based changes in rDNA copy number. This transcription is regulated by the silencing gene, *SIR2*, and by copy number. These results suggested a model of amplification regulation where transcription of E-pro stimulates unequal recombination by disrupting cohesin association in the rDNA, thus allowing for a change in copy number (Figure 1). Transcription-induced cohesin dissociation may be a general mechanism of recombination regulation.

III. Condensin loaded onto the rDNA in a *FOB1*-dependent fashion to avoid contraction of a long repeat

An average of 200 copies of the ribosomal RNA genes (rDNA) are clustered in a long tandem array in *S. cerevisiae*. *FOB1* is known to be required for expansion/contraction of the repeats by stimulating recombination, thereby contributing in the maintenance of average copy number. In Δ*fob1* cells, the repeats are still maintained without any fluctuation of copy number, suggesting another unknown system acts to prevent repeats contraction. We attempted to understand the putative second *unknown* system by which the long rDNA array is maintained by isolating mutants carrying unstable, shortened rDNA tandem array in a *Δfob1* background. We

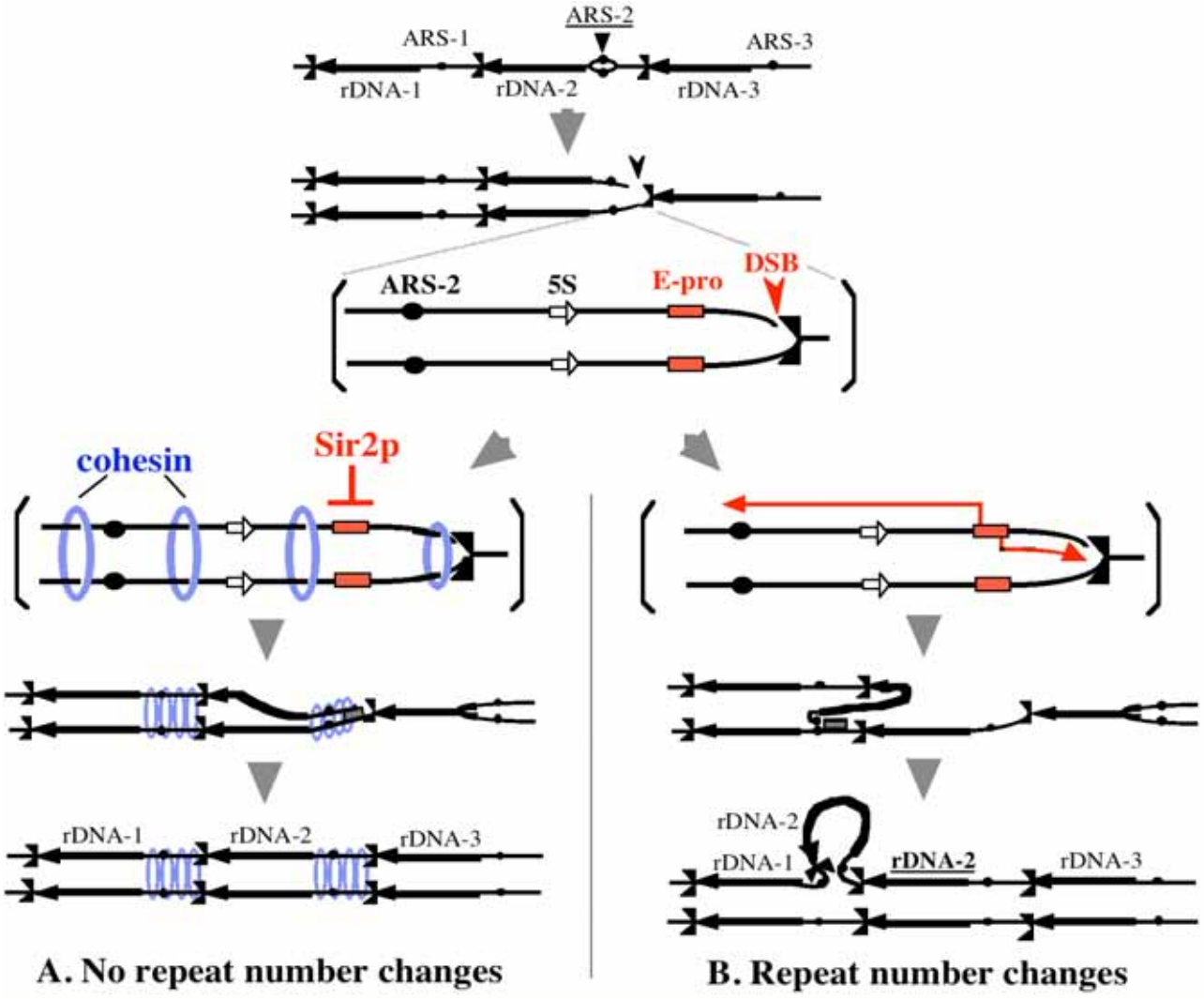

Figure 1. Transcription-induced cohesin dissociation model of rDNA amplification. (A) In normal situation, such as wild-type rDNA copy number, SIR2 repress E-pro activity, allowing cohesin to associates throughout the IGS (intergenic spacer; NTS1). DSBs (double stranded breaks), formed by replication forks pausing at the RFB site, are repaired by equal sister-chromatid recombination, with no change in the rDNA copy number. (B) When *SIR2* repression is removed, such as sir2 mutation or low copy number, E-pro becomes active and transcription displaces cohesin. Unequal sister chromatids can then be used as templates for DSB repair, resulting in changes in the rDNA copy number. Lines represent single chromatids (double stranded DNA). The IGS in which the replication fork is paused is expanded in the bracket.

found that condensin mutants showed severe contraction of rDNA tandem array in a *Δfob1* cells, but not in *FOB1*$^{+}$ cells, thereby suggesting that condensin acts to maintain long repeats with *FOB1* in a functionally complemented fashion. We also found that condensin association with the rDNA was localized to the replication fork barrier (RFB) site in a *FOB1*-dependent fashion. Surprisingly, condensin association with the RFB site was established during S phase and maintained until anaphase. These results indicate that *FOB1* acts in a novel role to prevent repeats contraction by regulating condensin association, and suggest a link between replication termination, and chromosome condensation and segregation.

IV. A novel gene amplification system in yeast based on double rolling-circle replication

Gene amplification is involved in various biological phenomena such as cancer development and drug resistance. However, the mechanism is largely unknown because of the complexity of the amplification process. We developed a gene amplification system in *S. cerevisiae* that is based on double rolling-circle replication (DRCR), utilizing break-induced replication (BIR). This system produced three types of amplification products. Type-1 products contain 5–7 inverted copies of the amplification marker, *leu2d*. Type-2 products contain 13 to ~100 copies of *leu2d* (up to ~730 kb increase) with novel arrangement present as randomly oriented sequences flanked by inverted *leu2d* copies. Type-3 products are acentric multi-copy mini-chromosomes carrying *leu2d*. Structures of type-2 and -3 products resemble those of homogeneously staining region (HSR) and double minutes (DMs) of higher eukaryotes, respectively. Interestingly, products analogous to these were generated at low frequency without deliberate DNA cleavage. These features strongly suggest that the processes described here may contribute to natural gene amplification in higher eukaryotes.

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Original papers

Ganley, A.R., Hayashi, K., Horiuchi, T., and Kobayashi, T. (2005). Identifying gene-independent noncoding functional elements in the yeast ribosomal DNA by phylogenetic footprinting. Proc. Natl. Acad. Sci. USA *102*, 11787-11792.

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DIVISION OF EVOLUTIONARY BIOLOGY

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All living organisms evolved from a common ancestor that lived more than 3.5 billion years ago, and the accumulation of mutations in their genomes has resulted in the present biodiversity. Traces of the evolutionary process are found in the genomes of extant organisms. By comparing the gene sequences and gene networks of different organisms, we can infer (1) the phylogenetic relationships of extant organisms and (2) the genetic changes that caused the evolution of morphology and development. The inferred phylogenetic relationships provide important insights into problems in various fields of evolutionary biology. Our group focuses on biogeography, the evolution of morphological traits, and systematics in a wide range of taxa. Concerning the evolution of morphology and development, we hope to explore the genetic changes that led to the evolution of the plant body plan. We have selected several land plants and some green algae as models to compare the functions of genes involved in the development of both reproductive and vegetative organs in land plants.

I. Origin of the plant cell

The first green alga cell evolved via symbiosis between an ancestral non-photosynthetic eukaryote and a cyanobacterium. Cyanobacteria now exist as chloroplasts in the host cell. The factors and mechanisms of chloroplast movement are being investigated to reveal the molecular mechanisms used to "domesticate" cyanobacteria as organelles. Analyses of (1) cytosolic calcium ion concentration and cytoskeleton organization during chloroplast movement in the moss *Physcomitrella patens* and (2) the functional divergence of photoreceptors and motor proteins involved in chloroplast movement between the moss and angiosperms are in progress by a team directed by Y. Sato.

II. Evolution from unicellular to multicellular organisms

The first evolutionary step from unicellular to multicellular organisms is to form two different cells from a single cell via asymmetric cell division. The first cell division of a protoplast isolated from the protonemata of the moss *Physcomitrella patens* is asymmetric regarding its shape and nature, and gives rise to an apical meristematic cell and a differentiated non-meristematic cell. A systematic overexpression screening for genes involved in asymmetric cell division of protoplasts in *P. patens* is in progress by a team directed by T. Fujita. After eliminating genes that are not directly involved in asymmetric cell divisions, such as photosynthesis genes, we used 3000 clones as materials for the overexpression screening. Individual cDNAs were subcloned under a constitutive promoter and introduced into the protoplasts of *P. patens* for transient expression. We observed and categorized phenotypes of the regenerating protoplasts. Thus far we identified 58 cDNAs, whose overexpression caused the defects in asymmetric cell divisions in two repeated experiments. Overexpression of the genes in protoplasts with GFP-tubulin or GFP-talin, expression analyses of each gene-cytrin fusion protein under its native promoter, loss of function experiments using RNAi are now in progress to characterize what processes these genes are involved in. Functional analyses of these genes should help us to understand the molecular mechanisms of how plants generate distinct cell lineages to build their multicellular bodies.

III. Evolution from cells to tissues

The most prominent difference between plant and animal cells is that plant cells have a cell wall and do not move during development. Therefore, the plane of cell division and the direction of cell elongation, which are regulated by cortical microtubules, determine the morphology of differentiated tissues and organs.

3-1 Microtubule-dependent microtubule nucleation

Almost all microtubule arrays are organized by one or more microtubule organizing centers, such as centrosomes, that regulate nucleation spatially and temporally. It has long been puzzling how, despite the absence of conspicuous organizing centers, higher plant cells form well-organized cortical microtubule arrays, which are essential for cell morphogenesis. A recent report suggests that microtubule nucleation sites for the array are capable of associating with and dissociating from the cortex. We showed that nucleation requires extant cortical microtubules, onto which cytosolic

γ-tubulin is recruited. Microtubule-independent nucleation is rarely observed in living tobacco BY-2 cells and nucleation is minimal in the absence of original microtubules in a cell-free system. In both living cells and the cell-free system, microtubules are nucleated as branches on the extant cortical microtubules. The branch points contain γ-tubulin, which is abundant in the cytoplasm, and microtubule nucleation in the cell free system is prevented by inhibiting γ-tubulin function with specific antibodies. When isolated plasma membrane with microtubules is exposed to purified neuro-tubulin, no microtubules are nucleated, but when the membrane is exposed to a cytosolic extract, γ-tubulin binds microtubules on the membrane, and after a subsequent incubation in neuro-tubulin, microtubules are nucleated on the pre-existing microtubules. We propose that a cytoplasmic γ-tubulin complex shuttles between cytoplasm and the side of a cortical microtubule and has nucleation activity only when bound to the microtubule (Murata *et al*. 2005). T. Murata mainly directed this study.

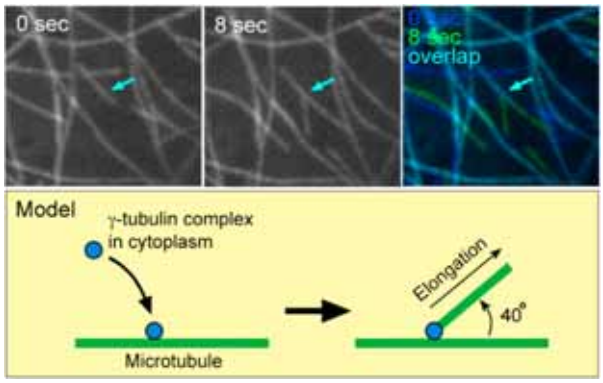

Figure 1. Microtubules in plant cortical arrays nucleate on existing microtubules as branches. Top panels show microtubule nucleation visualized by GFP-alpha-tubulin in living leaf epidermal cells of *Arabidopsis thaliana*. An arrow in each panel indicates a microtubule nucleation site. Images at 0 (left) and 8 (center) seconds, and merged image (right) showing changes of microtubules in 8 seconds are shown. Bottom panel shows proposed model of microtubule nucleation on existing microtubules.

IV. Evolution of molecular mechanisms in the plant development

4-1 Stem cell initiation and maintenance

Postembryonic growth of land plants occurs from the meristem, a localized region that gives rise to all adult structures. Meristems control the continuous development of plant organs by balancing the maintenance and proliferation of stem cells, and directing their differentiation. Meristem initiation and maintenance is a fundamental question in plant development research. Three lines, exhibiting reporter gene (*uid*A) expression preferentially in the apical cells, were isolated from previously established gene- and enhancer-trap lines, and identified as encoding kinesin- and ubiquitin-like proteins, and an unknown protein. Functional analyses of these genes are currently under investigation by a team directed by Y. Hiwatashi. The disruption of the kinesin-like gene did not show any phenotypic differences from the wild type. This is likely caused by the functional redundancy of closely related genes, and the analyses of double disruptions are in progress. Disruption of the gene encoding ubiquitin-like protein suggests that the gene is involved in cell division and elongation through microtubule organization with the proteasome complex.

4-2 Function of gametophytic MADS-box genes

Land plants are believed to have evolved from a gametophyte-dominant ancestor without a multicellular sporophyte; most genes expressed in the sporophyte probably originated from those used in the gametophyte during the evolution of land plants. To analyze the evolution and diversification of MADS-box genes in land plants, eight MADS-box genes predominantly expressed in pollen, male gametophyte, are analyzed by a team directed by N. Aono.

4-3 Origin of floral homeotic MADS-box genes

The MADS-box genes of land plants are extensively diverged to form a superfamily and are important in various aspects of development including the specification of floral organs as homeotic selector genes. The closest relatives of land plants are the freshwater green algae charophyceans. To study the origin and evolution of land plant MADS-box genes, we characterized these genes in three charophycean green algae: the stonewort *Chara globularis* (*CgMADS1*), the coleochaete *Coleochaete scutata* (*CsMADS1*), and the desmid *Closterium peracerosum-strigosum-littorale* complex (*CpMADS1*). Phylogenetic analyses suggested that MADS-box genes diverged extensively in the land plant lineage after the separation of charophyceans from land plants. The stonewort *CgMADS1* mRNA was specifically detected in the oogonium and antheridium together with the egg and spermatozoid during their differentiation. The expression of *CpMADS1* increased when vegetative cells began to differentiate into gametangial cells, and decreased upon fertilization. These expression patterns suggest that the precursors of land plant MADS-box genes originally functioned in haploid reproductive cell differentiation, and that the haploid MADS-box genes were recruited into a diploid generation during the evolution of land plants (Tanabe *et al*. 2005).

4-4 Ancestral function of the floral regulator *FLO/LFY*

After fertilization, the zygote undergoes dynamic changes in the chromosomal and cytoplasmic organizations and begins the cell cycles that eventually lead to formation of multicellular embryo. Specific transcription factors that initiate this cascade of events in land plants have not been identified. We have identified two *FLO*/*LFY* genes, *PpLFY1* and *PpLFY2*, that regulate

the first cell division after formation of the zygote in the moss *Physcomitrella patens*. The deduced amino acid sequences of the two *PpLFY* genes are 94.8% identical to each other and show similar expression patterns. While fertilization occurred in the *PpLFY* disruptants, the development of double disruptant zygotes was arrested at the single-cell stage. When the double disruptants, as female parent, were crossed with the wild type, as male parent, normal sporophytes were formed, supporting the notion that the *PpLFY* genes function after fertilization to regulate the first mitotic cell division in zygotes. The rare sporophytes that formed on the *PpLFY* double disruptants showed mostly normal organogenesis, but had abnormalities in the pattern of cell division, supporting a role of *PpLFY* genes in regulating cell division. The *FLO/LFY* genes in angiosperms are conserved master regulators of floral identity without any obvious effects on cell division. In contrast, our study suggests that *FLO/LFY* genes have functions throughout sporophyte development in the basal land plant lineages (Tanahashi *et al*. 2005).

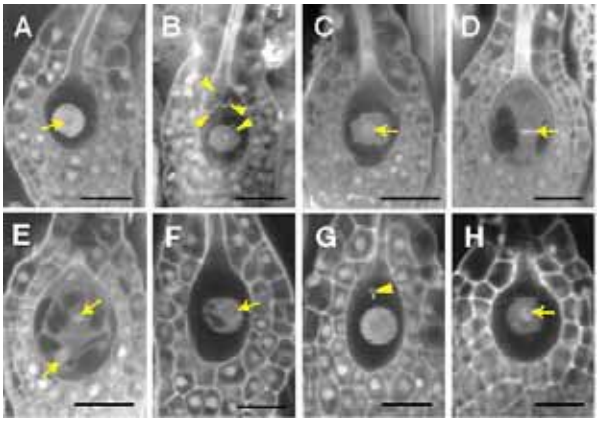

Figure 2. Development of the egg cell, zygote, and embryo. Ventral parts of archegonia in the wild type (A-E) and the *PpLFY* double disruptants (F-H) were observed by confocal laser scanning microscopy. Nuclei and sperm are indicated with arrows and arrowheads, respectively. (A, B, F, G) An egg cell before (A, F) or after (B, G) sperm invasion. (C, H) An unexpanded zygote. (D) An expanded zygote at the first cell division. (E) A two-cell embryo. An expanded zygote and multicellular embryos were hardly observed in the double disruptants. Bars = 25 μm.

4-5 Evolution of the floral regulator FLO/LFY in land plants

The plant-specific transcription factor FLO/LFY controls general aspects of the life cycle in a basal plant, the moss *Physcomitrella patens*. In contrast, FLO/LFY has more specialized functions in angiosperms, where it specifically induces floral fate during the reproductive phase. This raises the question of a concomitant change in the biochemical function of FLO/LFY during the evolution of land plants. We report that the DNA binding domain of FLO/LFY, although largely conserved, has diverged in activity. On the contrary, other, more rapidly evolving portions of the protein have few effects on FLO/LFY activity (Maizel *et al*. 2005).

VI. Molecular mechanisms of speciation

Reproductive isolation is the first step in speciation. To obtain insights into reproductive isolation, several receptors specifically expressed in the pollen tube are being studied to screen for the receptors involved in pollen tube guidance by a team directed by S. Miyazaki.

Polyploidization is a major mode of speciation in plants, although the changes that occur after genome duplication are not well known. Polyploid species are usually larger than diploids, but the mechanisms responsible for the size difference are unknown. To investigate these mechanisms, tetraploid Arabidopsis was established and its gene expression patterns are being compared to those of diploid wild-type plants using microarrays.

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Original papers

Hayashida, A., Takechi, K., Sugiyama, M., Kubo, M., Itoh, R.D., Takio, S., Fujita, T., Hiwatashi, Y., Hasebe, M., and Takano, H. (2005). Isolation of mutant lines with decreased number of chloroplasts per cell from tagged mutant library of moss *Physcomitrella patens*. Plant Biol. *54,* 300-306.

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Shigyo, M., Shindo, S., Hasebe, M., and Ito, M. (2005). Phylogenetic analysis of AP2 domain-containing genes. Gene, in press.

Tanabe*, Y., Hasebe*, M., Sekimoto, H., Nishiyama, T., Kitani, M., Henschel, K., Münster, T., Theißen, G., Nozaki, H., and Ito, M. (2005). Characterization of MADS-box genes in charophycean green alga and its implication for the evolution of MADS-box genes. Proc. Natl. Acad. Sci. USA *102*, 2436-2441. (*both authors contributed equally: Selected as this week in PNAS).

Tanahashi, T., Sumikawa, N., Kato, M., and Hasebe, M. (2005). Diversification of gene function: homologs of the floral regulator *FLO/LFY* control the first zygotic cell division in the moss *Physcomitrella patens*. Development *132*, 1727-1736.

DIVISION OF SPECIATION MECHANISM

(ADJUNCT)

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During the long evolutionary history of vertebrates, they acquired extensive diversity in such areas as morphology, ecology and behavior. It is believed that many organisms inhabiting the earth at present are derived from an ancestral species and became diversified in the evolutionary process with speciation. Therefore, speciation is one of the important factors of diversification. How then does speciation occur? Although various theoretical models have been proposed with respect to speciation, its mechanism has been difficult to clarify so far, especially on a molecular level. The aim of our group's research is to propose the processes and mechanism of the speciation of vertebrates using a molecular approach. To accomplish the aim of our research, we choose the East African cichlid fishes as a model animal for the study of speciation (Figure 1).

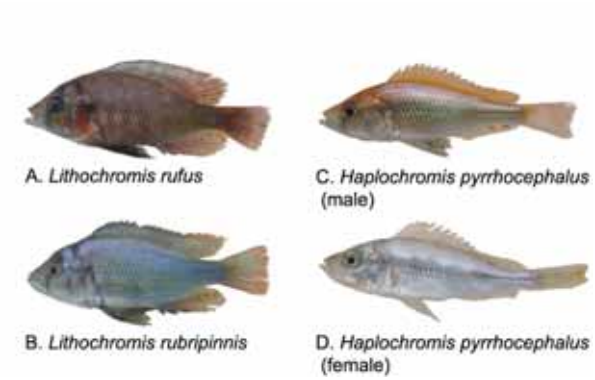

Figure 1. Cichlid fishes in Lake Victoria. These were caught in the field by the expedition of our group.

I. Intention of our research focusing on cichlids in Lake Victoria

Although cichlid fishes are broadly distributed in tropical region throughout the world, our group focuses on the species that are endemic to three great lakes and their drainages in East Africa. There are three lakes, Lakes Tanganyika, Malawi and Victoria, and, in total, 1,000 or more cichlid fishes live in the lakes. It was reported that Lake Tanganyika was established 12 million years ago, that Lake Malawi was established 2 million years ago, and that Lake Victoria was established 12,000 years ago. Thus, it is thought that the cichlids evolved in each lake after the lakes formed. Endemic cichlids of Lake Victoria diversified to 700 or more species from a small number of ancestors during its short history. This fact means that explosive adaptive radiation of the cichlid species occurred in this lake. To date, our group has accomplished certain results in molecular phylogenetic studies of the East African cichlids and other vertebrate animals; for example, elucidation of the evolutionary history of baleen whales. From the results of our phylogenetic analysis of Lake Victoria cichlids using insertions of retroposons (SINEs: short interspersed elements) as markers for elucidation of their evolutionary history, most of selectively-neutral polymorphic alleles (presence/absence of retroposons at orthologous site in the genome) are retained both within and among species of this lake. Namely, polymorphisms among Lake Victoria cichlids are trans-specific. Such homogeneous genome within/among the species of Victorian cichlids provide us with the following criterion for elucidation of the mechanism of speciation. If we can characterize a certain allele that is uniquely fixed at a certain locus in natural populations of a certain species, we can assume that this gene may possibly be related to positive selection (speciation).

II. Field research in Lake Victoria

Lacustrine environment of Lake Victoria highly diverged according to the difference of area such as turbidity, depth and bottom. Depending on such variable habitats, cichlids also show phenotypic diversity adapting to respective habitats. To obtain ecological data and natural fish samples of Victorian cichlids adapting to various habitat, our group and Dr. N. Okada's laboratory (Tokyo Inst. Tech.) conducted field investigation around Mwanza Gulf in the southern shore of Lake Victoria since 2004, August (Figure 2). In 2005, this investigation was conducted twice. The first period started in August, 2004, and finished at the beginning of February 2005. In this first research period, our group sent K. Takahashi to collect approximately 4,000 individuals of Victorian cichlids from various ecological habitats. The second research was conducted over the course of three months (from the beginning of June 2005 to the end of August 2005). In this second research period, our group collected a further 1,400 specimens. At present, our group has almost 130 species of Victorian cichlids in our laboratory.

III. Analysis of candidate genes for elucidation of speciation and diversification

Varied body coloration of cichlids is one of the examples for their phenotypic diversity. Cichlids are known to depend mostly on visual system when they choose their mating partner, and such color variations are considered to affect the female's choice. Therefore, it could be considered that the body colors of males play an important role for recognition by the visual system of females during the course of reproduction. In addition, the visual system of cichlids must have been affected by environmental differences in their habitat such as the turbidity and depth of lake water. In a collaborative work with the Dr. N. Okada's laboratory in Tokyo Institute of Technology, our group proposed that RH1 gene, which is one of the groups of opsin genes, evolved in parallel with

the depth of their habitat among cichlid species in Lakes Tanganyika and Malawi. Based on the actual research in the field, our group focuses on the evolution of opsin genes for visual system in several Victorian cichlid species. *Lithchormis rufus* and *L. rubripinnis* (Figure 1A and B) inhabit shallow water near by the shoreline only in Mwanza Gulf (Figure 2, panel B), and we found geographical clines of nuptial coloration on their male. We are carrying out the analysis of the opsin gene family of these species to detect the genetic variations of chromatic vision as a result of adaptation for each male color among the populations. We also collected *Haplochromis pyrrhocephalus*, which is broadly distributed in the lake (Figure 1C and D), from several localities and depths. The light environment for this species is considerably different among the populations. To elucidate the adaptation of vision system for various light conditions, we are analyzing the six types of the opsin gene family. A more extensive analysis on molecular evolution of opsin genes in Victorian cichlids is in progress in our division.

Publication List:

Original papers

Kijimoto, T., Watanabe, M., Fujimura, K., Nakazawa, M., Murakami, Y., Kuratani, S., Kohara, Y., Gojobori, T., and Okada, N. (2005). cimp1, a novel astacin family metalloproteinase gene from East African cichlids, is differentially expressed between species during growth. Mol. Biol. Evol. *22*, 1649-1660.

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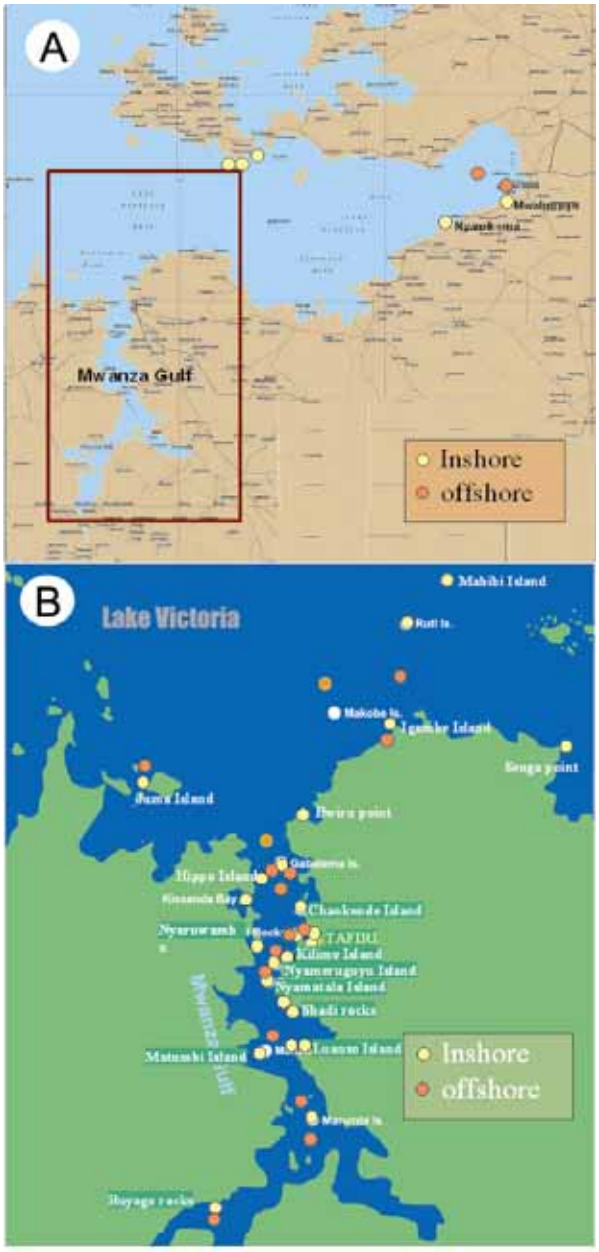

Figure 2. Localities of collection of cichlids in Lake Victoria. Panel A: Sampling localities in southern shore of Lake Victoria. The area surrounded by a brown rectangle in this panel corresponds to the region shown as a magnified map of panel B. Panel B: Sampling localities in Mwanza gulf. Sampling was conducted by angling and trawling and by using gill nets. Sampling was conducted in collaboration with Tanzania Fisheries Research Institute (TAFIRI).

LABORATORY OF MORPHODIVERSITY

Associate Professor: *KODAMA, Ryuji*

The aim of this laboratory is to observe the variety of the morphogenetic processes in the course of the ontogenesis of multicellular ogranisms and to analyze the mechanisms of such processes mainly by morphological methods. Accumulation of such analyses on the embryogenetic processes of related species is expected to give an insight into the evolution of morphogenetic processes. This laboratory uses the wings of lepidopteran insects for the material of morphological studies.

Wings of the lepidopteran insects (butterflies and moths) develop from the wing imaginal disc, which is a hollow sac made of simple epithelium. Due to its simple construction, this tissue is a good material to study cellular interactions in the course of morphogenesis.

The outline shape of the adult wing is often different from that of the pupal wing. This difference is brought about by the programmed cell death of the marginal area of the pupal wing. The marginal dying area is called "the degeneration region" and the internal area, which develops into the adult wing, is called "the differentiation region".

The cell deaths in the degeneration region proceeds very rapidly and completes in a half to one day period in *Pieris rapae* and several other species examined. It was shown that the dying cells in the degeneration region have characteristics common with the apoptotic cell death in mammalian cells, such as fragmented and condensed nuclei containing short DNA fragments detected by TUNEL staining. The cells in the degeneration region are actively engulfed by the macrophages in the cavity beneath the wing epithelium. The macrophages seem to be concentrated beneath the degeneration region by the strong adhesion between basal surfaces of the dorsal and ventral epithelium in the differentiation region, which occurs at the time of prominent cell death and excludes macrophages out of the differentiation region. Thus realized concentration of macrophages seems to accelerate the shrinkage of the degeneration region.

A collaborative work with the laboratory of Dr. K. Watanabe (Hiroshima University) is concerned mostly with the development of trachea and tracheole pattern in swallow tail butterflies. Trachea and tracheoles are both important in delivering air into the wing and their patterns coincide with that of the boundary of degeneration and differentiation zones at the distal end of the wing. According to the observations, the pattern formation of wing epithelium is often dependent on tracheal and tracheole patterns. Basic research on the development of tracheal pattern formation is being done by the scanning electron microscopy and the bright field light microscopy of the fixed or fresh specimens to describe the exact pathway and the time course of the formation of elaborate pattern of trachea and tracheoles and to establish the cytological and developmental relationship between the formation of tracheal pattern and epithelial cell pattern, such as scale cell pattern (Figure 1).

This laboratory also conducts morphological observation of several animal tissues by scanning and transmission electron microscopy and immno-electron microscopic analyses. The training of specimen preparation and instrument operation for such observations is also given. These activities include the Division of Sex Differentiation and the Laboratory of Neurophysiology of our institute.

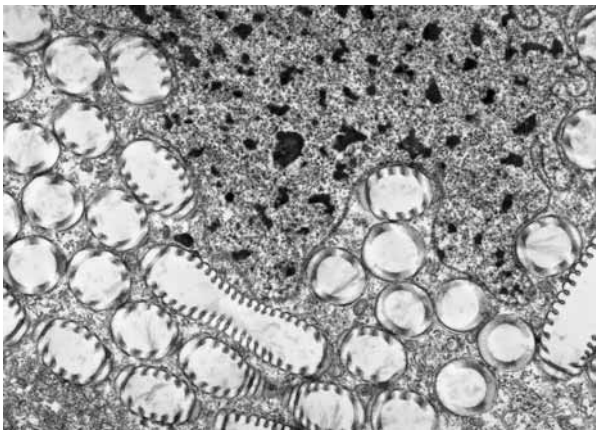

Figure 1. Transmission electron micrograph of the tracheole cell. Many cross-sections of the tracheoles can be observed within the cytoplasm of the tracheole cell. As the cell migrates, the tracheole is laid behind.

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Original paper

Watanabe, E., Hiyama, T.Y., Shimizu, H., Kodama, R., Hayashi, N., Miyata, S., Yanagawa, Y., Obata, K., and Noda, M. (2005). Sodium-level-sensitive sodium channel Nax is expressed in glial laminate processes in the sensory circumventricular organs. Am. J. Physiol., in press.

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Synthetic chemicals found in the environment have the capacity to disrupt the development and function of the endocrine system in both wildlife and humans. This has drawn public concern since many of these chemicals may bind to estrogen receptors (ER) and evoke estrogenic effects. Early evidence that exposure to estrogenic chemicals during development could pose a threat to human health came from studies of a synthetic hormone, diethylstilbestrol (DES), which was used to prevent premature birth and spontaneous abortion. Laboratory experiments showed that exposure of animals to sex hormones during critical windows of perinatal life caused irreversible alterations to the endocrine and reproductive systems of both sexes. In the immune and nervous systems, bone, muscle, and the liver were also affected. Although many of these chemicals can bind to ERs in wildlife and humans, the molecular basis for the action of environmental estrogens remains poorly understood. Thus, understanding the molecular mechanisms through which environmental estrogens and sex hormones act during critical developmental windows is essential.

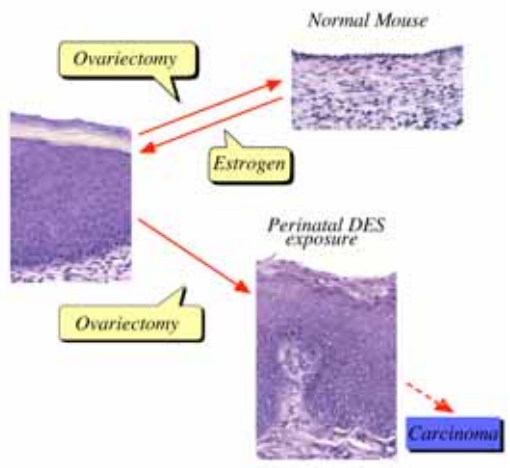

Figure 1. Scheme of estrogen-dependent and -independent vaginal epithelial cell proliferation in mice induced by perinatal estrogen exposure.

I. Perinatal estrogen exposure induces persistent changes in reproductive tracts

Perinatal exposure to sex hormones such as DES induces lesions in the reproductive tracts of female mice. In the early seventies, a close correlation between the occurrence of vaginal clear cell adenocarcinoma in young women and early intrauterine exposure to DES was demonstrated. The possible relevance of the mouse findings to the development of this human cancer has been emphasized. The neonatal mouse model has been especially useful in studying the long-term effects of early sex hormone exposure on the female reproductive tract. Female reproductive tracts in mice exposed prenatally to estrogen show altered expression of Hoxa genes and Wnt genes and knockout mice lacking Hoxa-10 or Wnt7a show uterine hypoplasia. Neonatal treatment of female mice with estrogens induces various abnormalities of the reproductive tract including ovary-independent cervicovaginal keratinization, adenosis, uterine hypoplasia, epithelial metaplasia, oviductal tumors, polyovular follicles and polyfollicular ovaries. The growth response of neonatally DES-exposed reproductive organs to estrogens is reduced, as are ER levels and epidermal growth factor (EGF) receptor levels, in addition to other hormone receptor levels.

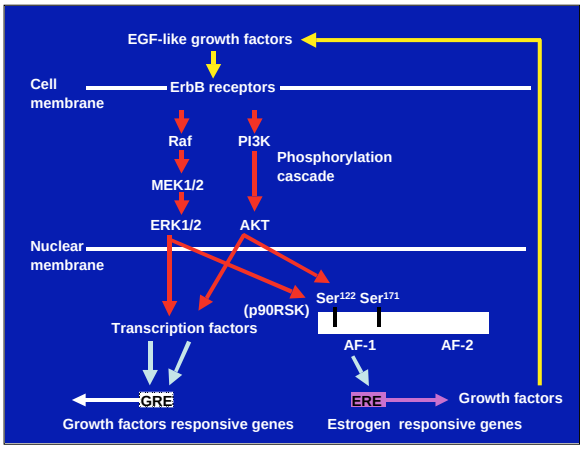

Figure 2. A hypothetical model for the estrogen-independent ER activation pathway in mouse vagina. EGF-like growth factors activate the protein-phosphorylation cascade via erbB receptors. Estrogen receptor is phosphorylated on serine 122 and 171 in AF-1 domain. Furthermore, transcription factors are activated by phosphorylation. These phosphorylations induce the transcriptional activity of ER, and then growth factors are expressed via estrogen-response element (ERE). Growth factors induced by ER activate EGF-receptors.

Growth factor and ER signaling cooperate to play essential roles in cell proliferation, differentiation and tumor progression in mouse reproductive organs, yet the mechanisms underlying the estrogen-dependent and -independent pathways remain unknown. EGFR and erbB2 were activated by estrogen treatment in mouse vaginal epithelium. This activation was also found in vaginae from neonatally DES-exposed mice, along with the expression of EGF, TGF-α, HB-EGF, amphiregulin and neuregulin. Immunohistochemical analysis indicated that erbB2 was primarily expressed in vaginal epithelium.

Serine 118 and 167 located in the AF-1 domain of ERα were phosphorylated in these vaginae. Administration of antagonists for erbB2 (AG825), EGFR (AG1478), and ER (ICI 182,780) blocked proliferation of vaginal epithelium induced by neonatal DES exposure. This suggests that signal transduction via EGFR and erbB2 could be related to the estrogen-induced vaginal changes. Persistent erbB phosphorylation and sustained expression of EGF-like growth factors would lead to ERα activation, resulting in cancerous vaginal lesions later in life in neonatally DES-exposed mice.

We used differential display to identify estrogen-responsive genes related to the proliferation and differentiation of mouse vaginal epithelial cells. A novel c-type lectin that encodes a membrane protein with a c-type lectin domain in the carboxyl-terminal region was identified from this screening. Analysis of mRNA expression showed that this gene is estrogen responsive in the mouse vagina. Furthermore, it is found in epithelial, but not stromal cells, suggesting that this novel c-type lectin may be an important factor in the stratification and/or cornification of the vaginal epithelium of mice. We are continuing efforts to analyze its function during estrogen-induced proliferation and differentiation in the mouse vagina.

Estrogenic compounds such as bisphenol A (BPA) and nonylphenol as well as dioxins and PCBs were found in the human umbilical cord. BPA can easily cross the placenta and enter the fetus in Japanese monkeys and mice. BPA can be found in the fetal brain, testis and uterus when given to pregnant mice and monkeys. Neonatal exposure to a high BPA dose induced ovary-independent vaginal changes, polyovular follciles and infertility lacking corpora lutera. Prenatal exposure to a low BPA dose induced acceleration of vaginal opening in the offspring. This provides further evidence that the developing mammal is sensitive to exposure to estrogenic agents. Neonatal treatment of rats with a high dose of BPA induced infertility in females, but no obvious effects on males.

II. Microarray analysis of estrogen responsive genes

cDNA microarray methods have recently been developed and successfully applied for genome-wide analysis of gene expression stimulated by hormones/or chemicals. Elucidating the expression patterns of estrogen-responsive genes is essential to understanding the mechanisms through which estrogenic chemicals act on mouse reproductive organs. A large number of estrogen-modulated genes were identified in the mouse uterus. Most of these genes were regulated in a dose-dependent manner and their expression was not altered by estrogen treatment in ERα knockout mice. This confirms that expression of these genes is dependent on ERα. Their activation suggests a molecular basis for the marked uterotrophic effect we observed several days following estrogen administration. Intriguingly, characteristic gene expression patterns were observed for each environmental estrogenic chemical and these were distinct from those elicited by estradiol. This suggests that specific mechanisms of action for endocrine disruption exist that could be different from those induced by endogenous estrogens. Physiological estrogens, non-physiological estrogens, and estrogenic dioxins have distinct effects on uterine gene expression. However, nonylphenol and dioxin activate another set of genes in the liver that were distinct from uterine estrogen-responsive genes. These results suggest that only a small number of genes are directly involved in the uterotrophic effects of estrogen treatment, and nonylphenol has very similar effects to estradiol on gene expression in uterine tissue, but not in hepatic tissue. Therefore, potential tissue-specific effects should be considered in order to elucidate the distinct effects of various endocrine disrupting chemicals (EDC) throughout the body.

In order to clarify the molecular mechanisms underlying estrogenic effects, we are studying changes in gene expression patterns induced by perinatal exposure to estrogen and chemicals using differential display and DNA microarray techniques. We have found genes possibly related to the ovary-independent changes by differential display. We also have clustered groups of genes that are responsive to estrogenic stimuli in uterus by using the DNA microarray. Our goals are to understand the molecular background of the critical period during development, the low dose effect of estrogenic chemicals and the molecular metabolism of hormones and hormone-like agents in animals and humans.

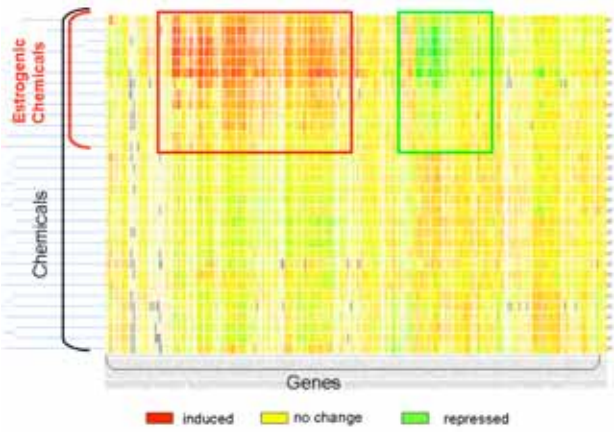

Figure. 3 Scatter plot of average expression levels in control and chemical-treated uterus

III. Effect of estrogen on reptiles, amphibians and fishes

Exogenous estrogen exposure during embryogenesis induces abnormal sex differentiation and abnormal bone formation in the African clawed frog, *Xenopus laevis*, the cyprinodont fish, mummichog (*Fundulus heteroclitus*) and mosquitofish (*Gambusia affinis affinis*). To analyze the estrogen function, we isolated ERα and β cDNA from *F. heteroclitus* and *G. affinis affinis*. Exposure of roach (*Rutilus rutilus* – a common cyprinid fish) to effluents from sewage treatment works containing complex mixtures of EDCs has been shown to alter sexual development and impact negatively on their reproductive capabilities in UK rivers. To unravel the mechanisms of disruption of sexual development in roach exposed to EDCs, we have isolated cDNAs related to sex determination and sex-differentiation such as ERs,

aromatase, StAR, Sox9, vasa, etc., and are examining gene expression during gonadal differentiation with or without EDCs exposure. Furthermore, we have cloned steroid hormone receptors and several oncogenes from the American alligator (*Alligator mississippiensis*), the Nile crocodile (*Crocodilus nicotilus*) and the Florida red-eared slider turtle (*Trachemys scripta*) with the aim of analyzing the ER evolution in reptilian phylogeny. We are also isolating estrogen-responsive genes to understand the molecular physiology of estrogen action with an aim toward understanding temperature-dependent sex determination of alligators at the molecular level.

Other projects related to steroids and physiology are in progress. The Japanese tree frog (*Hyla japonica*) absorbs water through ventral skin. We found that sex steroids and EDCs interfere with water absorption through ventral skin in frogs. Trenbolone, a potent androgenic and anabolic steroid, induced masculinization of anal fin and sperm production in the ovary of mosquitofish. Using the amphibian and fish as model animals we aim to analyze the effects of numerous chemicals released into the environment on endocrine system function in wildlife.

Figure. 4 Evolutionary relationships of estrogen receptor sequences

IV. Male production in Daphnids by juvenile hormones

We found that ten juvenile hormone analogs induce production of males in *Daphnia magna*. Daphnids are susceptible to the male-sex determining effects of juvenoids during oogenesis and the effect of juvenoids is reversible. In order to understand the molecular functional mechanism of juvenoids in induction of male offspring, we are analyzing juvenile hormone binding protein and establishing a microarray system for *D. magna*.

V. Gene zoo

We have established cDNA libraries from various animal species of interest including the Japanese giant salamander (*Andrias japonicus*). Identifying essential genes is indispensable for the basic study and conservation of animal species. We are establishing cDNA library banks of animal species in collaboration with the University of Pretoria, South Africa, the University of Florida, U.S.A., and the Asa Zoo in Hiroshima.

VI. Molecular target search

Abnormalities caused by EDC have been reported but the molecular mechanisms underlying their effects are not well known. Although the ER is one of the most likely candidates responsible for the endocrine disrupting function of many chemicals, ER alone cannot explain the variety of phenomena induced by EDC. Therefore, we are also looking for new target molecules that may be involved in endocrine disruption. We are also studying the ligand-binding mechanisms of nuclear receptors to hormones and other chemicals.

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DIVISION OF PLANT DEVELOPMENTAL GENETICS (ADJUNCT)

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The leaf is the fundamental unit of the shoot system, which is composed of the leaf and stem. The diversity of plant forms is mostly attributable to variation of leaf and floral organs, which are modified leaves. Moreover, leaf shape is sensitive to environmental stimuli. The leaf is therefore the key organ for a full understanding of plant morphogenesis. However, the genetic control of development of these shapes has remained unclear. Recently, studies of leaf morphogenesis reached a turning point after our successful application of the techniques of developmental and molecular genetics using model plants, *Arabidopsis thaliana* (L.) Heynh. (reviewed in Kim *et al.* 2005, Tsukaya 2003).

I. Polarized growth of leaf blades

Focusing on the mechanisms that govern polarized growth of leaves in *Arabidopsis thaliana* we have identified genes for polar-dependent growth of leaf lamina: the *ANGUSTIFOLIA* (*AN*) gene regulates width of leaves and the *ROTUNDIFOLIA3* (*ROT3*) gene regulates length of leaves. Both *AN* and *ROT3* genes control leaf cell shape. In addition to these, we added *ROT4* gene to the list of the genes responsible for the polarity-dependent control of leaf shape. This year, we found that the *ROT3* is involved in biosynthesis of brassinosteroid, a steroid phytohormone (Kim *et al.* 2005). In relation to the function of the *ROT3,* we also found that the different growth responses of the *A. thaliana* leaf blade and the petiole during shade avoidance are regulated by photoreceptors and sugar. Moreover, we identified the *AN3* gene that regulates number of leaf cells (Horiguchi *et al.* 2005) and a homolog of *AN* from *Ipomoea nil* (Cho *et al.* 2005).

1-1 *ROT3* gene function

We found that the *ROT3* gene encodes a cytochrome P450 that catalyzes the conversion of typhasterol to castasterone, an activation step in the biosynthesis pathway of brassinosteroids (BRs) (Kim *et al.* 2005). Differed from already known mutants of genes for biosynthesis of BRs, the loss-of-function mutant of *ROT3* has a specific defect in the length of leaves, suggesting the importance of fine tuning the levels of BRs on the polarized growth of leaves. Interestingly, CYP90D1, the most closely related cytochrome P450 to the ROT3/CYP90C1 enzyme, was suggested to catalyze the other conversion steps of BR biosynthesis (Kim *et al.* 2005). Double mutant for the *ROT3/CYP90C1* and for the *CYP90D1* exhibited extreme dwarf that is observed for the other known mutants of genes for biosynthesis of BRs. Since the loss-of-function mutant of *ROT3* has defect in response of petioles to dark, the *ROT3* might have specific role(s) in the shade-avoidance syndrome. In relation to this topic, the adaptive responses of arabidopsis leaves against gravity and other environmental factors were also analyzed and the interaction between light signal and gravity-response in leaves was suggested (Kim *et al.* 2005, Tsukaya 2005).

1-2 Shade avoidance syndrome

As mentioned above, we also focused on the effects of environmental factors on leaf morphogenesis. In darkness, the expansion of leaf lamina is inhibited, while at the same time petiole elongation is enhanced. This phenomenon is termed the shade-avoidance syndrome. We analyzed the nature of the shade-avoidance syndrome and found that phytochromes and cryptochromes specifically regulate the contrasting growth patterns of the leaf blade and petiole in shade (Kozuka *et al.* 2005). Differed from photomorphogenesis of hypocotyl, cell elongation was stimulated in the petiole in dark conditions without an increase in the ploidy level. By examining the effects of sucrose on the growth of the leaf blade and petiole, we revealed that the growth promotional effects of sucrose are highly dependent on the light conditions.

1-3 *AN3* gene regulates leaf cell numbers

How are cell proliferation and cell enlargement coordinated in leaf morphogenesis? In a determinate organ - a leaf- the number of leaf cells is not necessarily reflected in leaf shape or, in particular, in leaf size. Genetic analyses of leaf development in *A. thaliana* shows that a compensatory system(s) acts in leaf morphogenesis and an increase in cell volume might be triggered by a decrease in cell number (Tsukaya 2003). Thus, leaf size is, at least to some extent, uncoupled from the size and number of cells by the compensatory system(s). Recently, we have revealed that *ANGUSTIFOLIA3* (*AN3*) gene is involved in the maintenance/establishment of activity of cell proliferation in leaf primordia. *AN3* encodes a co-activator, and is found to control cell cycling in leaf primordia by interacting with a transcription activator, *AtGRF5* (Horiguchi *et al.* 2005). Interestingly, the *an3* shows clear "compensation", namely, accelerated cell expansion in relation to a decrease in the number of leaf cells (Figure 1) whereas overexpression of the *AN3* caused simple increase of leaf area in association with an increased number of leaf cells. By using various mutants with altered number and/or size of leaf cells, we are currently analyzing the genetic system of the compensation.

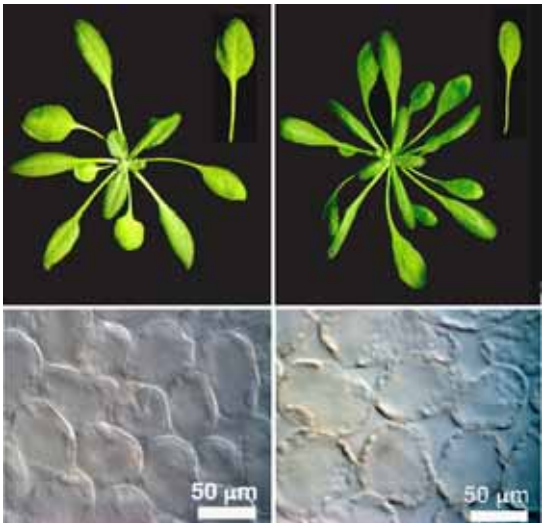

Figure 1. Left, Columbia wild type; right, *an3* mutant. Lower panels show paradermal views of palisade tissue. Note enlarged palisade cells in the *an3* mutant that has narrow and small leaves.

1-4 Evolution of *AN* gene family

How have these genes evolved the function of leaf-shape control? The *AN* is a homolog of animal *CtBP/BARS* gene family which has varied roles in morphogenesis and organelle control. To understand the common role(s) of *AN* subfamily in the plant kingdom, we analyzed a homolog of *AN* from *Ipomoea nil*, *IAN*, and showed that the *IAN* exhibits the same function with the *AN* on the control of leaf shape when introduced to *A. thaliana* (Cho *et al*. 2005).

II. Biodiversity of plant form

2-1 Biodiversity in leaf size

We are also interested in biodiversity in wild plants. This year, *Spiranthes sinensis* (Orchidaceae) in Japan was analyzed (Tsukaya 2005). *S. sinensis* shows a wide range of morphological diversity and a dwarf form has been known on Yakushima Island. Analysis on molecular variation of this species revealed that the dwarf form has not yet gained any specific variations in *ITS* and *trnL-F* loci, suggesting recent evolution of the dwarf form.

2-2 Genetic analyses of plant diversity

The so-called "Evo/Devo" study of leaf morphogenesis is also one of our research projects. In the course of field research of natural evolution of leaf shape/size, we have revealed some aspects of the biodiversity of plants (Ikeda *et al.* 2005, Okada *et al.* 2005, Tsukaya and Okada 2005, Tsukaya *et al.* 2005, Yokoyama *et al.* 2005). *Thismia mullerensis* and *Didymoplexiella cinnabarina* are new plant species discovered in our botanical survey in central Kalimantan, Borneo, Indonesia (Tsukaya and Okada 2005, Tsukaya *et al.* 2005).

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Original papers

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DIVISION OF PHOTOBIOLOGY (ADJUNCT)

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TSUBOI, Hidenori

Plants respond to light as an environmental factor to optimize development and regulate other physiological phenomena. Phytochrome (phy) and blue light receptors, such as cryptochrome (cry) and phototropin (phot), are the main photoreceptors for plant photomorphogenesis. The goal of our research is to elucidate the photoperception and signal transduction pathways of photo-morphogenesis.

I. Chloroplast relocation movement

One of our major subjects is chloroplast photo-relocation movement, which is thought to be one of the simplest model systems to study photomorphogenesis. We use the fern *Adiantum capillus-veneris* and the moss *Physcomitrella patens* as model plants for our cell biological approach since the gametophytes are very sensitive to light and the organization of the cells is very simple. We also use *Arabidopsis* mutants to identify the genes regulating chloroplast photo-relocation movement.

1-1 *Arabidopsis*

Chloroplasts relocate in a cell according to ambient light conditions: accumulation response occurs under low-light condition and avoidance response under high-light condition. In *Arabidopsis thaliana*, the accumulation response is mediated redundantly by two blue light receptors, i.e. phototropins (phot1 and phot2) (Sakai *et al.* 2001) and the avoidance response is mediated only by phot2 (Kagawa *et al.* 2001). We isolated a mutant, *jac1* (*J*-domain protein required for chloroplast *ac*cumulation response 1) which lacks accumulation response under weak light, but shows normal avoidance response under strong light. Positional cloning of *JAC1* revealed that this gene encodes a J-domain protein at its C-terminus similar to clathrin uncoating factor auxilin. In dark-adapted wild type cells, chloroplasts sediment on the cell bottom, but the *jac1* mutant lacks this response. The green fluorescent protein (GFP)-JAC1 fusion protein showed the similar localization pattern to GFP protein at the transient assay with onion epidermal cells, suggesting that JAC1 protein may be a soluble cytosolic protein. The results suggest that JAC1 is one of the essential components of phototropin-mediated chloroplast movement.

1-2 *Mougeotia*

Most plant species from alga to flowering plants utilize blue light for inducing phototropism and chloroplast movement; many ferns, however, as well as some mosses and green alga utilize red as well as blue light for the regulation of these responses, resulting in efficient capture of white light under low light levels. During their evolution, ferns have created a chimeric photoreceptor (phy3 in *Adiantum*) between phytochrome and phototropin enabling them to utilize red light effectively. We have identified two genes resembling *Adiantum* PHY3, NEOCHROME1 and 2 (MsNEO1 and MsNEO2), from the green alga *Mougeotia scalaris*, a plant famous for its phytochrome-dependent chloroplast movement. Like *Adiantum* PHY3, both MsNEO gene products show phytochrome-typical bilin binding and red/far-red reversibility, the difference spectra closely matching the known action spectra of light-induced chloroplast movement in *Mougeotia*. Furthermore both rescue red-light-induced chloroplast movement in *Adiantum* phy3 mutants, indicating functional equivalence. The fern and algal genes seem to have arisen independently in evolution, demonstrating an intriguing example of convergent evolution.

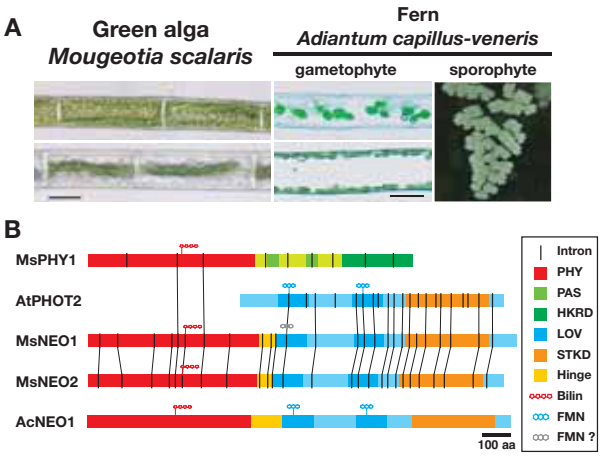

Figure 1. A, Giant chloroplasts of *Mougeotia scalaris* in their face-on (upper panel) and side-on orientation (lower panel) and those of *Adiantum capillus-veneris* protonemal cells in which the chloroplast accumulation to the upper (upper panel) or side wall (lower panel) are induced by polarized red light vibrating parallel or perpendicular to the cell axis irradiated from horizontal direction. Scale bar 20 μm. The right panel shows *A. capillus-veneris* sporophyte. B, The domain structure of fern and algal neochromes and canonical plant phytochrome and phototropin. MsNEO1 LOV1 domain has so far failed to bind FMN in vitro, the other MsNEO LOV domains are unlikely to bind flavins. Introns whose positions are equivalent in each gene are connected with thin lines. PHY: phytochrome photosensory region. HKRD: histidine kinase-related domain. STKD: serine/threonine kinase domain. PHOT: phototropin region.

1-3 *Adiantum*

The avoidance movement response in *Adiantum* phot2 deficient mutants can be restored by transient expression of non-mutant AcPHOT2 cDNA, indicating that

chloroplast avoidance movement in this fern is mediated by the Acphot2 protein as is the case in Arabidopsis. To know whether Acphot1 functions as a photoreceptor of blue light-induced chloroplast relocation movement, we need either triple mutant or at least three double mutants of Acphot1, Acphot2 and Acphy3, because phy3 might also be one of the blue light receptors in *Adiantum*. We are screening double mutants based on *Acphot2* or *Acphy3* mutant lines. As some double mutants have been obtained, the function of Acphot1 might be clarified soon.

II. Photomorphogenesis

2-1 Branching in *Physcomitrella*

Side branch formation in the moss *Physcomitrella patens* has been shown to be light dependent and cryptochrome 1a and 1b (Ppcry1a and Ppcry1b) were assigned as the blue light receptors for this response (Imaizumi *et al*. 2002). Here we analyzed this phenomenon in detail, and revealed the complex nature of the response where multiple photoreceptors are involved. For branch induction, blue light of a fluence rate higher than 6μmol $m^{-2}s^{-1}$ for period longer than 3 h was required. Number of branches is dependent on fluence rate of blue light but further increased when red light was applied together with the blue light, although red light alone had much less effect. By partial irradiation of a cell, both receptive sites for blue and red light were found to be located around the nucleus.

Both red and blue light determine the position of branches in a way affected by the vibration plane of polarized light. The red light effect was nullified by simultaneous far-red light irradiation, indicating phytochrome involvement. The blue light effect was not found in phototropin disruptants. Thus, dichroic phytochrome and phototropin possibly on the plasma membrane regulate branch position. Together, at least four distinct photoreceptor systems- namely cryptochromes and red light receptors around or in the nucleus, and dichroic phytochrome and phototropin around the cell periphery - are involved in the light induction of side branches in the moss *Physcomitrella patens*.

III. Gene targeting and gene silencing

In order to elucidate the role of genes in *Adiantum* and rice, we have tried to establish new methods for gene targeting in these organisms.

3- 1 Miniature transposable element

Transposable elements constitute a large portion of eukaryotic genomes and contribute to their evolution and diversification. We identified active transposable elements, miniaturePing (mPing), Ping and Pong in rice (Kikuchi *et al*. 2003). The mPing element was identified as the first active MITE from any organism. mPing is a short 430 base pair element with 15 base pair terminal inverted repeats that lacks a transposase. mPing elements are activated in calli derived from another culture and excise efficiently from original sites to reinsert into new loci. *Ping* and *Pong* transposable elements were isolated as putative autonomous elements encoding an IS/PIF/Harbinger superfamily of transposases. We are now trying to detect its transposase function.

3- 2 DNA interference in *Adiantum*

Silencing of gene expression by RNA interference (RNAi) is a useful technique for determining the roles of genes of unknown function in a wide range of organisms. In fern we found a simple method for gene-silencing using DNA fragments homologous to the target gene, called DNAi. It has the advantage of being faster and simpler than current RNAi approaches. To make DNAi a more powerful tool to study function-unknown genes in pteridophytes, expressed sequence tags (ESTs) were obtained from a normalized cDNA library of *Adiantum capillus-veneris* constructed from prothallia grown under white light. Clustering of 10,552 sequences in total resulted in 7,132 non-redundant groups. Of these, 1,608 EST groups were found to be similar to sequences of known function and 1,092 EST groups showed similarities to sequences of unknown function.

Publication List:

Original papers

Suetsugu, N., Kagawa, T., and Wada, M. (2005). An auxilin-like J-domain protein, JAC1, regulates phototropin-mediated chloroplast movement in *Arabidopsis thaliana*. Plant Physiology *139*,151-162.

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LABORATORY OF PHOTOENVIRONMENTAL BIOLOGY

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PRESTO Researcher: *ISEKI, Mineo*
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SUZUKI, Takeshi
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ITO, Makiko

Photosynthetic microorganisms, such as cyanobacteria and flagellate algae, respond to light to locate themselves at an appropriate photoenvironment. Our research is aimed at the elucidation of photoreceptive and signal transduction mechanisms of the light responses in microorganisms. This approach has lead us to the discovery and characterization of a remarkably unique light sensor molecule as described below.

I. Photoactivated Adenylyl Cyclase (PAC)

In 2002, we found a novel blue-light receptor with an effector role from *Euglena* (Iseki *et al*., Nature *415*, 1047-1051, 2002): *Euglena gracilis*, a unicellular flagellate, shows blue-light type photomovements. The action spectra indicate the involvement of flavoproteins as the photoreceptors mediating them. The paraflagellar body (PFB), a swelling near the base of the flagellum, has been considered as a photosensing organelle for the photomovements. To identify the photoreceptors in the PFB, we isolated PFBs and purified the flavoproteins therein. The purified flavoprotein (ca. 400 kDa), with noncovalently bound FAD, seemed to be a heterotetramer of α- and β-subunits. Predicted amino acid sequences of each of the subunits were similar to each other and contained two FAD-binding domains (BLUF: sensor of blue light using FAD) each followed by an adenylyl cyclase catalytic domain. The flavoprotein showed an adenylyl cyclase activity, which was elevated by blue-light irradiation. Thus, the flavoprotein (PAC, photoactivated adenylyl cyclase) can directly transduce a light signal into a change in the intracellular cyclic AMP level without any other signal transduction proteins.

1-1 Kinetic properties of PAC photoactivation

Although PAC appeared to be a photoreceptor for the step-up photophobic response (Figure 1), physiological evidence demonstrating that the photoactivation of PAC actually causes the step-up photophobic response is lacking. Recently, we reported the kinetic properties of in vitro activation of PAC by light, comparing them with those of the step-up photophobic response (Yoshikawa *et al*. 2005). We showed that activation of PAC is dependent both on photon fluence rate and the duration of irradiation and that reciprocity held well in the range of 2-50 μmol m^{-2} s^{-1} (total fluence of 1,200 μmol m^{-2}), suggesting that activation of PAC is entirely dependent on total photon fluence. We also examined the effects of intermittent irradiation on PAC activation and showed that intermittent irradiation using pulses of light and dark of equal length (0.1-180 s) caused activation of PAC to almost the same extent irrespective of the cycle periods. This means that elevation of PAC activity occurs only during the light period and that elevated PAC activity falls off within 0.1 s after the termination of irradiation. Such responsiveness is fast enough for PAC to mediate the step-up photophobic response that occurs with several subseconds' latency.

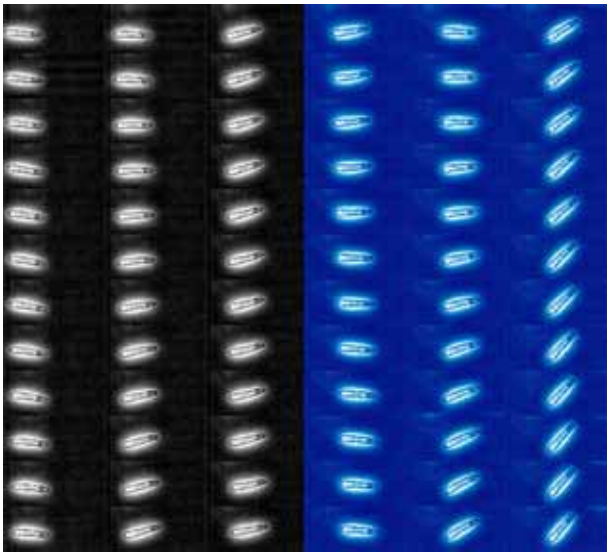

Figure 1. A serial recording of the step-up photophobic response of a *Euglena* cell by a high-speed video camera under infrared observation light (from top of left column to bottom of right column, 20 ms intervals).

We also reported wavelength dependency of PAC activation between 260-650 nm at equal quanta. The curve of wavelength dependency showed prominent peaks at 290 nm, 390 nm and 450 nm, which agrees well both with the absorption spectrum of FAD and the action spectrum for the step-up photophobic response of *Euglena*. All the above properties of PAC activation confirm that PAC actually acts as a photoreceptor for the step-up photophobic response.

1-2 The change in intracellular cAMP levels upon blue light irradiation

Since in vitro activity of PAC was elevated by blue light irradiation, it is easy to think that an increase in intracellular cAMP level evoked by photoactivation of PAC is a major cause of the step-up photophobic response. So far, however, there has been no experimental evidence that blue light irradiation induces an increase in intracellular cAMP level of *Euglena*. We measured intracellular cAMP before and after onset of blue light irradiation, which showed that the intracellular cAMP level remarkably increased within 1 s of the onset of irradiation and the increased cAMP level decreased within 10 s and gradually returned to the initial level even if irradiation continued. The time course of intracellular

cAMP coincided well with the process of the step-up photophobic response. This strongly suggests that the increase in intracellular cAMP evoked by photoactivation of PAC is a key event in the step-up photophobic response.

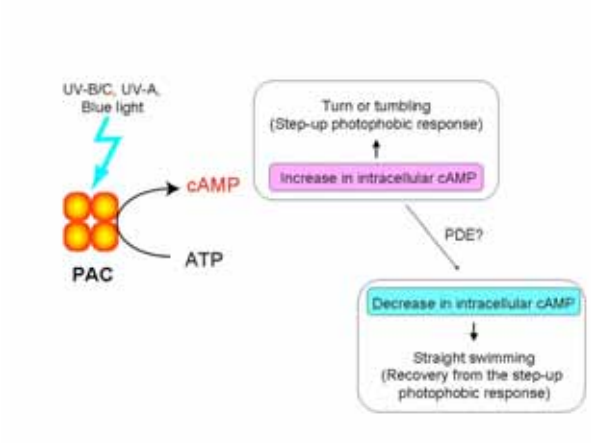

Figure 2. Schematic representation of PAC involvement in the step-up photophobic response of *Euglena*. PAC is activated by UV-B/C, UV-A and blue lights in a photon fluence-dependent manner to produce cAMP, which increases intracellular cAMP. The increase in intracellular cAMP triggers the step-up photophobic response, being followed by a decrease in intracellular cAMP, probably mediated by PDE, which brings about recovery from the step-up photophobic response.

1-3 Photoinduced spectral shift of BLUF domains

The mechanism of photoactivation of PAC is a subject of great interest. However, it is difficult to obtain enough PAC from *Euglena* cells for photochemical and structural analyses because the efficiency of PAC purification is low; only several micrograms of PAC can be obtained from 30 litres of *Euglena* culture. It is also difficult to obtain heterologously expressed PAC while keeping its activity intact because most of the expressed protein goes into the insoluble fraction, so-called inclusion bodies. Recently, we succeeded in obtaining the soluble recombinant flavin binding domain (F2) of PACα by heterologous expression in *E. coli* by fusing the domain to glutathion-S-transferase (GST). The recombinant F2 sample contained both FAD and FMN with trace amounts of riboflavin and showed a spectral red shift upon blue-light irradiation followed by recovery in darkness. Such photoinduced spectral shifts were first reported and well characterized for the BLUF domain of AppA, a regulator of photosynthesis gene expression in *Rhodobacter sphaeroides*. The spectral shift is considered to be caused by alterations in π-π stacking and hydrogen bonding between FAD and the tyrosine residue (Tyr21). Recent reports on the crystal structures of BLUF domains of AppA and a cyanobacterial protein Tll0078 indicate that the glutamine residue (Gln63 in AppA and Gln50 in Tll0078) play a crucial role in rearrangement of the hydrogen bond network to the flavin. We reported that when recombinant F2 proteins were mutated at the tyrosine (Tyr472) or glutamine (Gln514) residues, corresponding to Tyr21 and Gln63 in AppA, no photoinduced spectral shift was observed. Thus, the mechanism of photoactivation of the recombinant F2 in PACα seems essentially the same as that of the prokaryotic BLUF proteins, though the kinetic properties are slightly different from each other: e. g., the half-life was 34-44 s at 25 ˚C for the recombinant PACα F2, whereas that reported for prokaryotic BLUF domains varies from ca. 3.5 s (Tll0078) to ca. 900 s (AppA).

Publication List:

Original papers

Fujita, S., Iseki, M., Yoshikawa, S., Makino, Y., Watanabe, M., Motomura, T., Kawai, H., and Murakami, A. (2005). Identification and characterization of a flagellar fluorescent protein of brown alga *Scytosiphon lomentaria* (Scytosiphonales, Phaeophyceae): a flavoprotein homologous to Old Yellow Enzyme. Eur. J. Phycol. *40*, 159-167.

Ito, S., Murakami, A., Sato, K., Nishina, Y., Shiga, K., Takahashi, T., Higashi, S., Iseki, M., and Watanabe, M. (2005). Photocycle features of heterologously expressed and assembled eukaryotic flavin-binding BLUF domains of photoactivated adenylyl cyclase (PAC), a blue-light receptor in *Euglena gracilis*. Photochem. Photobiol. Sci. *4*, 762-769.

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Okajima, K., Yoshihara, S., Fukushima, Y., Geng, X., Katayama, M., Higashi, S.-I., Watanabe, M., Sato, S., Tabata, S., Shibata, Y., Itoh, S., and Ikeuchi, M. (2005). Biochemical and functional characterization of BLUF-type flavin-binding proteins of two species of cyanobacteria. J. Biochem. *137*, 741-750.

Yoshikawa, S., Suzuki, T., Watanabe, M., and Iseki M. (2005). Kinetic analysis of the activation of photoactivated adenylyl cyclase (PAC), a blue-light receptor for photomovements of *Euglena*. Photochem. Photobiol. Sci. *4*, 727-731.

Review article

Watanabe, M., and Iseki, M. (2005). Discovery and characterization of photoactivated adenylyl cyclase (PAC), a novel blue-light receptor flavoprotein, from *Euglena gracilis*. In Handbook of Photosensory Receptors, W.R. Briggs, and J.L. Spudich, eds. (Weinheim, Wiley-VCH Verlag), pp. 247-260.

LABORATORY OF STRESS RESPONSE

Associate Professor: *MIKAMI, Koji*

As their sessile nature, growth and development of plants are largely influenced by environmental factors; thus, plants must have highly sophisticated systems to sense and respond to these factors. Much effort has been put into the analysis of stress-signal transduction in plants, which led us to the identification of stress-responsive genes and transcription factors responsible for the stress-dependent transcription. However, it is still unknown how plants sense various kinds of environmental stresses separately and how environmental stresses are converted into intracellular signals. Based on our knowledge in mammalian cells, it is possible that, in plants, the transmission of the signals via cell surface receptors or sensors may result in the production of intracellular second messengers, such as inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DG), both of which initiate cascades of intracellular signal transduction in response to environmental stresses. Moreover, since phosphatidylinositols, some of which are precursors of the second messengers or are second messengers themselves, are produced by sequential phosphorylation and dephosphorylation via lipid kinases and lipid phosphatases in the PI pathway (Figure 1), these reactions could be activated by environmental stimuli. Indeed, stress-dependent production of PI 3,4-bisphosphate [$PI(3,4)P_2$] and IP_3 has already been reported in various kinds of plant species. It is, therefore, important to analyze the PI pathway to understand the molecular mechanisms of sensing of and response to extracellular signals in plants.

Since phosphoinositide-specific phospholipase C (PI-PLC) plays an essential role in the PI pathway through generation of IP_3 and DG by the hydrolysis of $PI(3,4)P_2$ (Figure 1), I have investigated the functions of PI-PLCs, PpPLC1 and PpPLC2, isolated from the moss *Physcomitrella patens*, because *Physcomitrella* is now recognized as a model system for plants with easy application of molecular genetic approaches such as gene-targeted mutagenesis via the homologous recombination. However, there is no information about PI pathway-regulating enzymes other than PI-PLCs in *Physcomitrella*, thus it is necessary to analyze the lipid kinases and phosphatases to confirm the similarity of the PI pathways between *Physcomitrella* and higher plants. Accordingly, the structural characterization of *Physcomitrella* lipid kinases and PI-PLCs were performed.

I. PI 4-kinase

PI 4-kinase (PI4K) phosphorylates the D4 position of PI to yield PI 4-phosphate [PI(4)P]. Two major types of PI4Ks, type II and type III, have been identified in animals and yeasts. The type II PI4Ks are membrane-bound 55 kD enzymes, which consists of the conserved catalytic domain and small N-terminal extension. There are two forms, a and b, for type III PI4Ks; the former consists of the PH domain and catalytic domain with a long N-terminal extension and 200 to 230 kD in size, whereas the latter is 110 kD protein composed with LKU, NH and catalytic domains. Plants also have all forms of PI4K.

Until to date, I have obtained only one full-length cDNA encoding a β form of type III PI4K, PpPI4Kβ1, whose structure is very close to those of the β form of type III PI4Ks from other species. Interestingly, although the repetitive sequence domain between LKU and NH domains was reported as a unique domain in *Arabidopsis* AtPI4Kβ1, the same domain was found in PpPI4Kβ1 (Figure 2), indicating that this domain is specific in plants. Thus, PpPI4Kβ1 is useful to investigate the plant-specific function of the type III PI4Kβ.

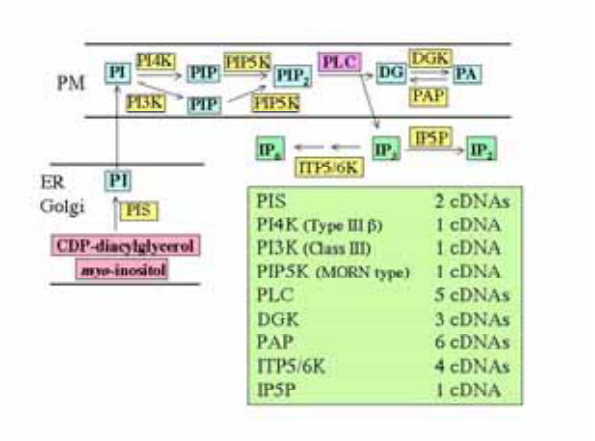

Figure 1. Schematic representation of the PI pathway. Light blue and yellow boxes indicate phosphoinositides and enzymes (PI synthase, lipid kinases or lipid phosphatases), respectively. Current situation of the cloning of *Physcomitrella* full-length cDNAs encoding enzymes involved in the PI pathway was indicated in the large box. PM, plasma membrane.

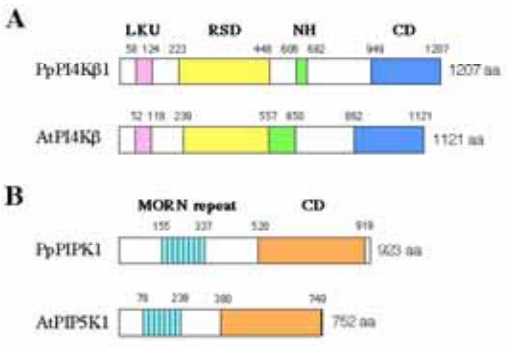

Figure 2. Structural characteristics of plant β form of type III PI4Ks and PIPKs. Protein domains represent in PpPI4Kβ1 (A) and PpPIPK1 (B), in comparison with *Arabidopsis* homologues. Numbers indicate amino acid positions. Accession nos. are as follows; AtPI4Kβ, AJ002685; AtPI5K1, Q56YP2. RSD, repetitive sequence domain; CD, catalytic domain.

II. PI-phosphate kinase

PI-phosphate kinase (PIPK) catalyzes the synthesis of PI(5)P, PI(3,5)P2 and PI(3,4)P2 by phosphorylation of PI and PI-phosphates. In animals, PIP5Ks are classified into three subfamilies, type I to III, according to their substrate specificity. Type I PIPKs are PI 5-kinases that phosphorylate PI and PI(3)P to generate PI(5)P and $PI(3,5)P_2$, while type II enzymes produce $PI(3,4)P_2$ from PI(3)P by their PI 4-kinase activity. Type III enzymes catalyze phosphorylation of the D5 position of PI as PI 5-kinase. The first plant PIPK was *Arabidopsis* AtPIP5K1 (Mikami *et al*., Plant J. *15*, 563-568, 1998) whose catalytic domain is similar to both animal type I and II enzymes, thus AtPIP5K1 was classified as type I/II. Indeed, it has been reported that AtPIP5K1 has both the PI 4-kinase and PI 5-kinase activities. Interestingly, AtPIP5K1 had a long N-terminal extension containing an unidentified repetitive sequence that was named recently a MORN repeat; however, the function of this repeat is still unknown. Taken together, the structure of type I/II PIPKs is plant-specific and, thus, the activation mode of them may also be plant-specific. Plants have the type III PIPKs as is in animals.

I obtained a *Physcomitrella* full-length cDNA encoding PIPK, designated PpPIPK1. As shown in Figure 2, PpPIPK1 consists of a N-terminal extension containing the MORN repeat and the catalytic domain classified as the type I/II enzyme. As the conservation of plant-specific structure between *Physcomitrella* and *Arabidopsis*, PpPIPK1 is useful to analyze the plant-specific mode of activation and functions of plant type I/II PIPKs.

III. PI-PLC

As mentioned above, PLC catalyzes the hydrolysis of $PI(4,5)P_2$ to generate two second messengers in a Ca^{2+}-dependent manner. Although animal PI-PLCs were classified into 5 isozymes, named β, γ, δ, ε and ζ types. All of them contain the X and Y domains to compose their catalytic domain and C2 domain as Ca^{2+}-dependent membrane-interacting module, although isozyme specific-domain structures are composed by the combination with other protein motifs in relation to their activation modes. It is well known that β and γ isozymes are activated by G protein-coupled receptors and receptor tyrosine kinases, respectively, whereas the activation of ε isozymes is regulated by heteromeric G proteins and Ras. In contrast to the variety of isozymes with isozyme-specific activation modes in animal PI-PLCs, plant PI-PLCs reported showed the same structure that resembles closely those of the ζ isozymes, which are sperm-specific enzymes composed by the two EF-hand repeats and X, Y and C2 domains. In plant PI-PLCs, there is one EF-hand repeat called the N domain. Until to date, it is unclear how plant PI-PLCs are activated.

It has been reported that two *Physcomitrella* PI-PLC, PpPLC1 and PpPLC2, are structurally close to plant PLCs reported, however PpPLC2 containing point mutations in the Y domain and an insertion in the N domain is inactive

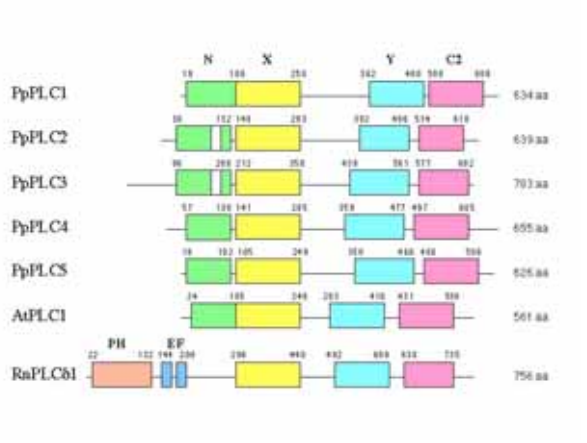

Figure 3. Structural characteristics of five *Physcomitrella* PI-PLCs. Protein domains represent in five *Physcomitrella* PI-PLCs, PpPLC1 to PpPLC5, in comparison with AtPLC1 from *Arabidopsis* and RnPLCδ1 from *Rattus norvegicus*. All PI-PLCs indicated have the X, Y and C2 domains, although the PH (pleckstrin homology) domain, which regulates membrane localization of animal PI-PLCs, is not found in any plant PI-PLCs. Numbers indicate amino acid positions. Accession nos. are as follows; PpPLC1, BAD02919; PpPLC2, BAD02918; AtPLC1, BAA07547; RnPLCδ1, NP_058731.

form of PI-PLC (Mikami *et al*., J. Exp. Bot. *55*,1437-1439, 2004). In addition, I have cloned other 3 full-length cDNAs encoding PpPLC3, PpPLC4 and PpPLC5. As shown in Figure 3, PpPLC4 and PpPLC5 have structures similar to that of PpPLC1, although PpPLC3 has mutations in the Y domain and an insertion in the N domain, which is similar to PpPLC2. Thus, *Physcomitrella* seems to have three PI-PLC isozymes whose structural organization is identical to those of higher plant PI-PLCs, although the inactivated forms such as PpPLC2 and PpPLC3 are found in only *Physcomitrella*. These findings indicate that *Physcomitrella* is useful to investigate the activation mode of plant PI-PLCs and the functional diversity among PI-PLC isoforms.

IV. Conclusion

Structures of lipid kinases and PI-PLCs involved in the PI pathway in *Physcomitrella* are basically similar to those in higher plants, suggesting the conservation of the activation modes and, probably, functions between lower and higher land plants. As gene-targeted disruption by homologous recombination is available in *Physcomitrella*, the functions of enzymes presented above could be analyzed directly. In fact, since I already produced gene-targeted mutants of PpPLC5 and PpPIPK1 in addition to PpPLC1, analyses of these mutants may provide new insight into the activation modes and functions of PIPK and PI-PLC in stress responses and development.

DIVISION OF THEORETICAL BIOLOGY

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NIBB Research Fellows: *TOHYA, Shusaku*
FUJITA, Hironori
Graduate Student: *IMAMURA, Hisako*
Visiting Scientists: *AYABE, Yoshiko*
YAMAGAMI, Ayumi
Secretary: *UMEBAYASHI, Hiromi*

We are studying biological phenomena using mathematical models. This method gives us an integrative understanding of the behavior of complex systems in biology including gene regulatory networks.

Mathematical models are also useful in understanding pattern formation in development. Study of the mechanisms responsible for morphological differences between species is an important research focus of current developmental biology. Theoretical studies would be useful in identifying candidates of cell-cell interaction that are likely to be responsible for the systems in real organisms.

I. Transcriptional autoregulation in cyanobacterial circadian rhythms

Among the wide variety of organisms exhibiting circadian rhythms, cyanobacteria are the simplest organisms and may provide a model to understand the basic mechanism of the sustained rhythmicity physiologically. In the cyanobacterium *Synechococcus elongatus* PCC 7942, clock genes kaiA, kaiB and kaiC have been characterized as the indispensable clock regulators. The kai genes form a gene cluster and kaiB and kaiC are co-transcribed as kaiBC mRNA. KaiC plays a central role and exhibits rhythms in transcription, translation and phosphorylation status under continuous illumination conditions. The other clock proteins KaiA and KaiB modulate KaiC autophosphorylation: KaiA enhances autophosphorylation of KaiC, and KaiB inhibits this action of KaiA.

The transcriptional regulation of kaiBC is not fully understood yet, though experimental evidences suggest the autoregulation by KaiC, which may work as a positive or negative regulator depending on the phosphorylation status. The gene of kaiB, which forms an operon with kaiC, also seems to affect this regulation process, inhibiting KaiA-enhanced phosphorylation of KaiC.

In this study, we investigated and predicted the possible mechanisms of the transcriptional regulation by KaiC and its phosphorylated state to realize circadian oscillation using a mathematical model. Considering the experimental results, phosphorylated and non-phosphorylated KaiC may play different roles in the transcriptional regulation. We developed a dynamical model including the concentrations of phosphorylated, non-phosphorylated KaiC, and kaiBC mRNA. We can choose different transcriptional regulation functions which may switch their values increasingly or decreasingly depending on both phosphorylated and non-phosphorylated KaiC. We examined all the possible regulation patterns without using biological assumption and determined the condition for oscillation by means of the linear stability analysis. We determined that there are only two possible patterns in the transcriptional regulation to realize circadian oscillation (Figures 1 & 2). One of the two patterns does not include the direct negative feedback regulation, but successfully exhibits the oscillatory behavior by positive feedback of the transcriptional regulation. Based on this result, we proposed that positive autofeedback may be a novel mechanism for circadian oscillation.

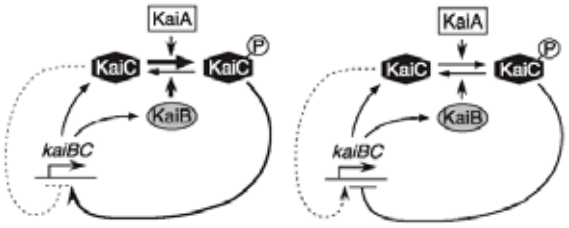


Figure 1. Functional schemes of two models. (Left) The Transcriptional Activation Model, where P-KaiC induces *kaiBC* transcription. NP-KaiC does not affect on transcription, or has a small effect of repressing transcription. (Right) The Transcriptional Repression Model, where P-KaiC represses *kaiBC* transcription.

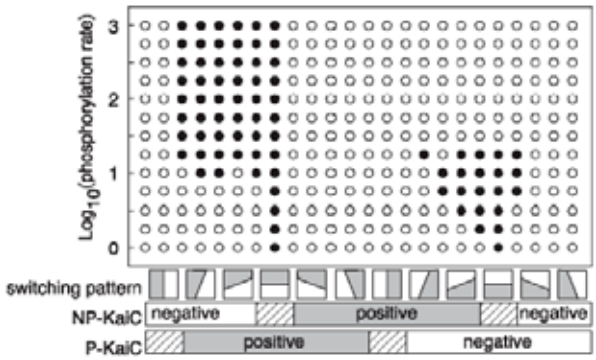


Figure 2. The result of linear stability analysis. The horizontal axis is θ, the angle of the threshold line of transcriptional switching, and the vertical axis is the phosphorylation rate. The θ specifies patterns of the transcriptional regulation, which are shown schematically below the horizontal axis. Along the horizontal axis, the regulation changes continuously; effects of NP-KaiC and P-KaiC are positive or negative. Open circles indicate conditions of the transcription and phosphorylation rates where oscillation never occurs, and filled circles indicate where oscillation can occur.

Secondly we verified if the determined conditions can explain the cyanobacterial circadian mechanism, comparing the behaviors in computer simulation with the experimentally observed phenotypes.

Experiments in circadian systems of *Neurospora*, *Drosophila*, and mammals as well as theoretical studies have long underlined the importance of the negative transcriptional regulation for the generation of oscillations.

cAMP coincided well with the process of the step-up photophobic response. This strongly suggests that the increase in intracellular cAMP evoked by photoactivation of PAC is a key event in the step-up photophobic response.

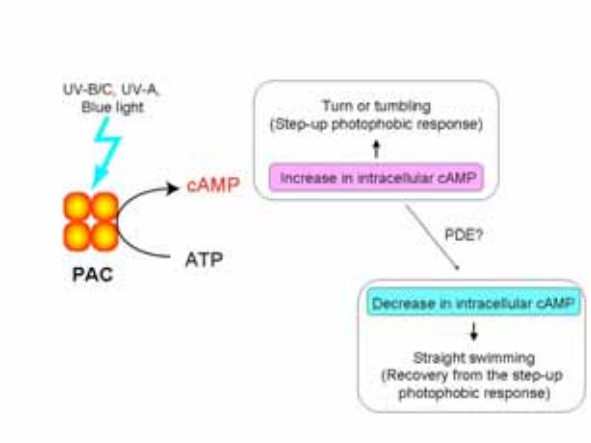


Figure 2. Schematic representation of PAC involvement in the step-up photophobic response of *Euglena*. PAC is activated by UV-B/C, UV-A and blue lights in a photon fluence-dependent manner to produce cAMP, which increases intracellular cAMP. The increase in intracellular cAMP triggers the step-up photophobic response, being followed by a decrease in intracellular cAMP, probably mediated by PDE, which brings about recovery from the step-up photophobic response.

1-3 Photoinduced spectral shift of BLUF domains

The mechanism of photoactivation of PAC is a subject of great interest. However, it is difficult to obtain enough PAC from *Euglena* cells for photochemical and structural analyses because the efficiency of PAC purification is low; only several micrograms of PAC can be obtained from 30 litres of *Euglena* culture. It is also difficult to obtain heterologously expressed PAC while keeping its activity intact because most of the expressed protein goes into the insoluble fraction, so-called inclusion bodies. Recently, we succeeded in obtaining the soluble recombinant flavin binding domain (F2) of PACα by heterologous expression in *E. coli* by fusing the domain to glutathion-S-transferase (GST). The recombinant F2 sample contained both FAD and FMN with trace amounts of riboflavin and showed a spectral red shift upon blue-light irradiation followed by recovery in darkness. Such photoinduced spectral shifts were first reported and well characterized for the BLUF domain of AppA, a regulator of photosynthesis gene expression in *Rhodobacter sphaeroides*. The spectral shift is considered to be caused by alterations in π-π stacking and hydrogen bonding between FAD and the tyrosine residue (Tyr21). Recent reports on the crystal structures of BLUF domains of AppA and a cyanobacterial protein Tll0078 indicate that the glutamine residue (Gln63 in AppA and Gln50 in Tll0078) play a crucial role in rearrangement of the hydrogen bond network to the flavin. We reported that when recombinant F2 proteins were mutated at the tyrosine (Tyr472) or glutamine (Gln514) residues, corresponding to Tyr21 and Gln63 in AppA, no photoinduced spectral shift was observed. Thus, the mechanism of photoactivation of the recombinant F2 in PACα seems essentially the same as that of the prokaryotic BLUF proteins, though the kinetic properties are slightly different from each other: e. g., the half-life was 34-44 s at 25 ˚C for the recombinant PACα F2, whereas that reported for prokaryotic BLUF domains varies from ca. 3.5 s (Tll0078) to ca. 900 s (AppA).

Publication List:

Original papers

Fujita, S., Iseki, M., Yoshikawa, S., Makino, Y., Watanabe, M., Motomura, T., Kawai, H., and Murakami, A. (2005). Identification and characterization of a flagellar fluorescent protein of brown alga *Scytosiphon lomentaria* (Scytosiphonales, Phaeophyceae): a flavoprotein homologous to Old Yellow Enzyme. Eur. J. Phycol. *40*, 159-167.

Ito, S., Murakami, A., Sato, K., Nishina, Y., Shiga, K., Takahashi, T., Higashi, S., Iseki, M., and Watanabe, M. (2005). Photocycle features of heterologously expressed and assembled eukaryotic flavin-binding BLUF domains of photoactivated adenylyl cyclase (PAC), a blue-light receptor in *Euglena gracilis*. Photochem. Photobiol. Sci. *4*, 762-769.

Ohnishi, N., Allakhverdiev, S.I., Takahashi, S., Higashi, S.-I., Watanabe, M., Nishiyama, Y., and Murata, N. (2005). Two-step mechanism of photodamage to photosystem II: step 1 occurs at the oxygen-evolving complex and step 2 occurs at the photochemical reaction center. Biochemistry *44*, 8494-8499.

Okajima, K., Yoshihara, S., Fukushima, Y., Geng, X., Katayama, M., Higashi, S.-I., Watanabe, M., Sato, S., Tabata, S., Shibata, Y., Itoh, S., and Ikeuchi, M. (2005). Biochemical and functional characterization of BLUF-type flavin-binding proteins of two species of cyanobacteria. J. Biochem. *137*, 741-750.

Yoshikawa, S., Suzuki, T., Watanabe, M., and Iseki M. (2005). Kinetic analysis of the activation of photoactivated adenylyl cyclase (PAC), a blue-light receptor for photomovements of *Euglena*. Photochem. Photobiol. Sci. *4*, 727-731.

Review article

Watanabe, M., and Iseki, M. (2005). Discovery and characterization of photoactivated adenylyl cyclase (PAC), a novel blue-light receptor flavoprotein, from *Euglena gracilis*. In Handbook of Photosensory Receptors, W.R. Briggs, and J.L. Spudich, eds. (Weinheim, Wiley-VCH Verlag), pp. 247-260.

The negative feedback regulation of clock genes has been considered responsible for generating oscillation. On the other hand, the contribution of positive feedback in circadian clock is much less well understood. The Transcriptional Activation Model we proposed in this report is distinct from circadian oscillator models that have been proposed previously.

II. Mathematical models for pattern formation of leaf vascular networks

The vascular system of a plant is a network of bundles that connects within major organs. The development or differentiation of vascular systems is one of the most important subjects in botany, and a lot of studies have focused on the problem. In dicot systems, veins in a leaf are usually diverse in their size and they can be classified distinctively by their appearance. The largest vein, located at the center of a leaf, is called the midvein (or primary vein), and is generated first. Veins differentiate progressively in an early developmental stage of the leaf. A vein, which is continuous with the stem vascular bundle, extends from the proximal edge to the distal end of the leaf, and then become a midvein. Secondary veins are generated from branches of the midvein and are thinner than the midvein. By repeated branching and extension, higher order veins are generated sequentially with the growth of the leaf and the reticulate network system is formed.

Although a lot of experimental studies have attempted to identify the mechanism of vascular patterning in leaves, it is still a problem to solve. It has been revealed that the auxin is important for vascular differentiation. Auxin is one of the major plant hormones. In the higher plant, indole acetic acid (IAA) is one of the most important auxin chemicals. Auxin is a diffusible molecule; however, it is believed that auxin is also transported from apical to basal in a stem, from distal to proximal in a leaf. This is called auxin polar transport. In a leaf, auxin is thought to be produced in the apical margins of leaves and transported toward the proximal regions. Many researchers believe that this kind of auxin flow plays an important role in the vascularization of plants.

Three hypotheses have been proposed so far to explain the leaf venation pattern formation. One is the auxin canalization hypothesis proposed by T. Sachs (1981) that is based on the assumption of the positive feedback regulation between auxin flow and flow capacity. Auxin is a diffusible plant hormone of small molecules and has important roles in various developmental events including vascular differentiation in plants. In this hypothesis, auxin pathways are thought to be generated from a uniform field and are extended in a sequential manner. The second is the activator-inhibitor type reaction-diffusion hypothesis proposed by H. Meinhardt (1982) that is based on two interactive factors of weakly diffusible activator and strongly diffusible inhibitor. We proposed the third hypothesis, the substrate-depletion type reaction-diffusion model, which is based on two interactive factors of strongly diffusible auxin resource and its consumer (Figure 3).

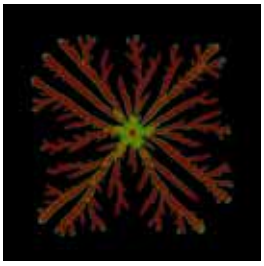


Figure 3. Branching pattern generated by computer simulation based on substrate-depletion type reaction-diffusion model. Veins grow toward the margin with repeating branching. In the final state, regular interval branching patterns are formed.

On the other hand, we tried to integrate the canalization hypothesis and the experimental knowledge. We introduce auxin transport by PIN1 efflux carrier into the auxin canalization hypothesis. We investigated a model based on the assumption of the positive feedback regulation between auxin flow and PIN1 localization (Figure 4).

The substrate-depletion type reaction-diffusion model can regenerate growth and branching patterns. On the other hand, the canalization model can generate closed circuits of leaf veins under a condition. Both mechanisms seem to be responsible for the leaf vein formation.

Figure 4. Leaf vein pattern generated by computer simulation based on canalization model. Veins grow toward the margin with repeating branching. The final patterns are similar to ones observed in maple leaves.

Publication List:

Original papers

Feugier, F.G., Mochizuki, A., and Iwasa, Y. (2005). Self-organization of the vascular system in plant leaves: Inter-dependent dynamics of auxin flux and carrier proteins. J. theor. Biol. *236*, 366-375.

Mochizuki, A. (2005). An analytical study of the number of steady states in gene regulatory networks. J. theor. Biol. *236*, 291-310.

Mochizuki, A., Yahara, K., Kobayashi, I., and Iwasa, Y. (2005). Genetic addiction: selfish gene's strategy for symbiosis in the genome. Genetics, in press.

Takigawa-Imamura, H., and Mochizuki, A. (2005).

Transcriptional autoregulation by phosphorylated and non-phosphorylated KaiC in cyanobacterial circadian rhythms. J. theor. Biol. in press.

LABORATORY OF GENOME INFORMATICS

Research Associate: *UCHIYAMA, Ikuo*

The accumulation of biological data has recently been accelerated by various high-throughput so-called “omics” technologies such as genomics, transcriptomics, proteomics and so on. The field of genome informatics is aimed at utilizing this data, or finding some principles behind the data, for understanding complex living systems by integrating the data with current biological knowledge using various computational techniques. In this laboratory we focus on developing computational methods or tools for comparative genome analysis, which is a useful approach for finding functional or evolutionary clues to interpreting genomic information of various species. The current focus of our research topics is on comparative analysis of microbial genomes, the number of which is now beyond a hundred, as a basic model system for understanding the variety of life through the comparative analysis of numerous genomic sequences simultaneously.

I. Construction of microbial genome database for comparative analysis

The number of completed microbial genome sequences is growing rapidly, and nearly three hundred genome sequences of various levels of relatedness have already been made available. The role of comparative genomics becomes much more important in order to utilize this large number of sequences not only for elucidating commonality in all of life, but also for understanding the evolutionary diversity within various groups, as well as for understanding the evolutionary processes or mechanisms producing such diversity.

We have been developing and maintaining a database system for comparative analysis of microbial genomes named MBGD (http://mbgd.genome.ad.jp/) (Figure 1). The key components of MBGD include i) an efficient algorithm that can classify genes into orthologous groups using precomputed all-against-all similarity search results (see below), ii) a user interface that is designed for users to explore the resulting classification in detail, and iii) an incremental updating process for similarities between genes and other data, which enables the system to provide the latest data rapidly. By this approach, MBGD is now the world’s largest database of its kind. Moreover, by specifying a set of organisms, users can obtain the appropriate classification results that they require using the latest data available.

The constructed classification table can be used for comparative analyses from various points of view, such as phylogenetic pattern analysis, gene order comparison and detailed gene structure comparison (Figure 1).

II. Hierarchical clustering algorithm for constructing orthologous groups of multiple genomes

Although ortholog identification is a crucial first step in comparative genomics, a scheme for large-scale ortholog grouping is yet to be established. The conventional approach to the identification of orthologs between two genomes is the so-called bidirectional best-hit (BBH) criterion, where two genes, *a* and *b*, in the genomes *A* and *B*, respectively, are considered to be orthologs if *a* is the best hit of *b* in genome *A* and vice versa. The Clusters of Orthologous Groups (COGs) Database, a widely used curated database for ortholog grouping, was constructed using this approach, although the overall construction process has included additional complex procedures such

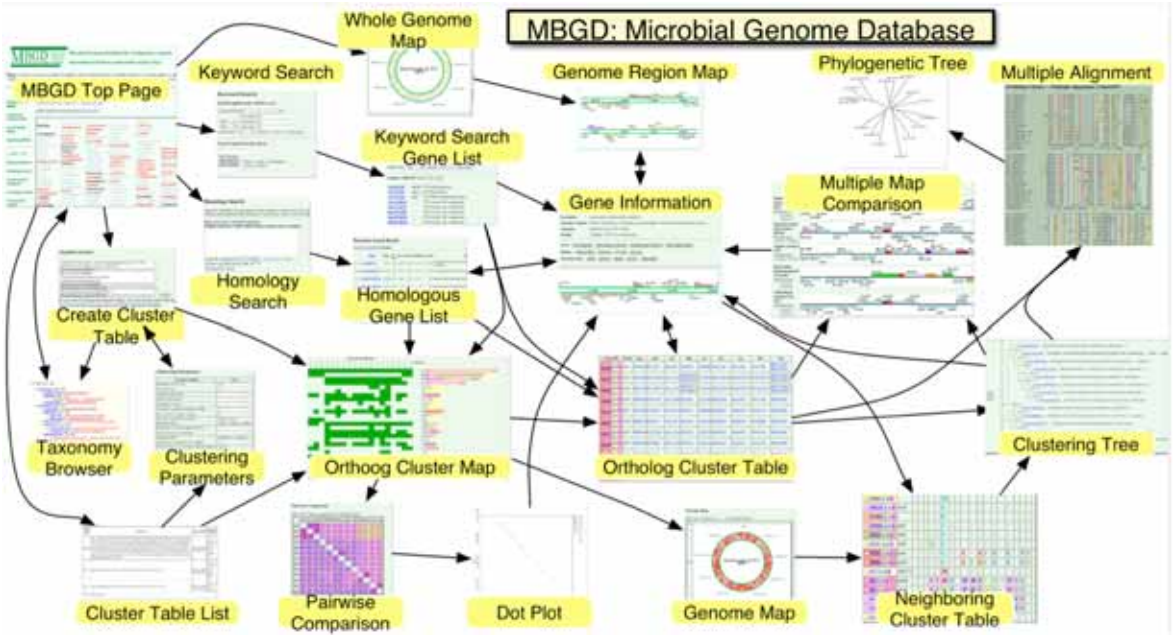


Figure 1. A flow diagram of the MBGD user interface

as the addition of species-specific paralogs, the splitting of proteins into multiple domains if required, and other case-by-case manual modifications

As a part of the core technologies of the MBGD system, we have developed a rapid automated method of ortholog grouping which is effective enough to allow the comparison of hundreds of genomes simultaneously. The method takes as input all-against-all similarity data and classifies genes based on the traditional hierarchical clustering algorithm UPGMA. In the course of clustering, the method detects domain fusion or fission events, and splits clusters into domains if required. The subsequent procedure splits the resulting trees such that intra-species paralogous genes are divided into different groups so as to create plausible orthologous groups. As a result, the procedure can split genes into the domains minimally required for ortholog grouping.

The procedure, named DomClust, was tested using the COG database as a reference. When comparing several clustering algorithms combined with the conventional BBH criterion, we found that our method generally showed better agreement with the COG classification. By comparing the clustering results generated from datasets of different releases, we also found that our method showed relatively good stability in comparison to the BBH-based methods.

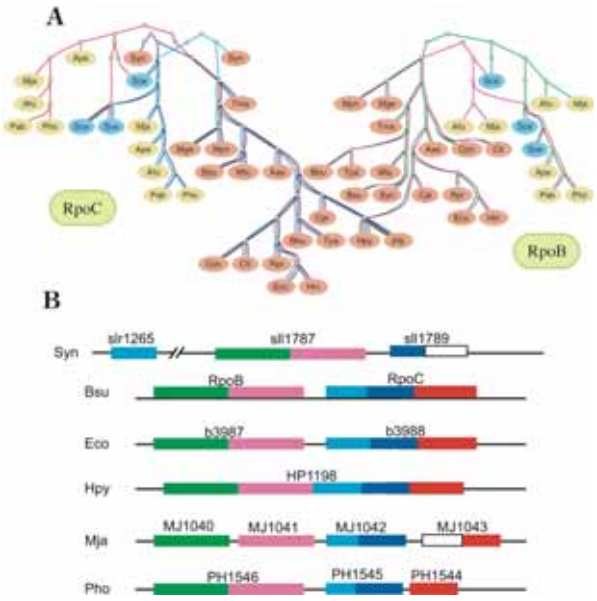


Figure 2. Orthologous groups of RNA polymerase beta (RpoB) and beta' (RpoC) subunits, as an example of DomClust classification. A) Hierarchical clustering trees constructed by the DomClust program. Each domain is drawn in a different color. An abbreviated species name (taken from the COG database) is shown on each leaf, which is colored according to the kingdom: salmon, bacteria; khaki, archaea; sky-blue, eukaryotes. B) Schematic illustration of the gene structures of RpoB and RpoC in selected genomes.

III. Identification of the common core structure of phylogenetically related genomes

It is known that horizontal transfer as well as vertical transfer has played important roles in prokaryotic evolution. Because of this complexity, further investigation is required in order to obtain a clearer picture of the bacterial genome evolution. Extensive comparison of multiple genomes that are closely or moderately related to each other should give many clues to understanding evolutionary processes. Such data is now rapidly accumulating in our MBGD database.

We are trying to identify a common "core structure" of phylogenetically related genomes, which is defined as a set of sufficiently long consecutive genomic segments in which gene orders are conserved among multiple genomes so that they are likely to have been inherited from the common ancestor mainly through vertical transfer. For this purpose, we have developed a graph-based algorithm for aligning conserved regions of multiple genomes by ordering orthologous groups so as to retain the conserved gene orders as much as possible.

The method was applied to the comparison of *Bacillus*-related species whose genome sequences have been determined including alkaliphilic *B. halodurans*, halotolerant *Oceanobacillus iheyensis* and thermophilic *Geobacillus kaustophilus*, in addition to well-known laboratory strain *B. subtilis* and pathogenic *B. anthracis* and *B. cereus*. These organisms – except for *B. anthracis* and *B. cereus* – are moderately diverged each other and belong to distinct major clusters in the 16S rRNA phylogenetic tree. Overall genomic structures are primarily well conserved between them, which can be confirmed by dotplot analyses where large collinear regions along the diagonal lines can easily be seen.

Using orthologous groups of *Bacillus*-related species with *Staphylococcus aureus* as an outgroup generated by the DomClust program, we constructed genome alignments by the above algorithm. From this alignment, we were able to identify the common core structure of *Bacillus* genomes comprising about 1500 genes. It appears that most of the important genes are included in the resulting core gene set. Indeed, the set contains most of 271 *B. subtilis* essential genes that were primarily determined by a systematic inactivation experiment. Further investigation of the core gene set revealed characteristic distributions of function categories in the core and non-core gene sets.

Publication List:

Original paper

Ishikawa, K., Watanabe, M., Kuroita, T., Uchiyama, I., Bujnicki, J.M., Kawakami, B., Tanokura, M., and Kobayashi, I. (2005). Discovery of a novel restriction endonuclease by genome comparison and application of a wheat-germ-based cell-free translation assay: PabI (5'-GTA/C) from the hyperthermophilic archaeon *Pyrococcus abyssi*. Nucleic Acids Res. *33*, e112.

STRATEGIC PLANNING DEPARTMENT

Chair:	*NAGAHAMA, Yoshitaka*
Vice-Chairs:	*UENO, Naoto*
	MOROHASHI, Ken-ichirou
Professor:	*WADA, Masamitsu*
Associate Professor:	*KODAMA, Ryuji*
Technical Assistants:	*MAKIHARA, Nobuko*
	MUKOHDA, Yasuyo
	OTA, Misaki
	SUGIYAMA, Tomomi
	TANAKA, Megumi
	TSUGE, Toyoko

The Strategic Planning Department was founded in April 2005 to perform and/or to support efficient management for cooperation with other organizations, distribution of public information, planning conferences, symposia and other activities and administration of the National Institute for Basic Biology.

The main activities of the Department in 2005

1) Supporting international conferences (Table 1)
2) Management of education-related programs
   - 20th Bioscience Training Course
   - Internship Program 2005
3) Editing of publications
   - Bulletin of NIBB 2005 (in Japanese)
   - Annual Report of NIBB 2005 (in English)
4) Production of posters and leaflets for international conferences and advertisements for entrance examination of graduate school(Table 2, Figure1)
5) Updating and maintenance of NIBB home page
6) Publication of NIBB News
   (Intra-institutional newsletter in Japanese)
7) Maintenance of archives
8) Dealing with visitors
9) Other various performances related to NIBB activities

Table 1. International conferences managed by Strategic Planning Department

Meetings	Date	Title	Organizer
51st NIBB Conference	November 5-8, 2005	New Aspects of Gene Amplification Mechanisms and Biological Function	T. Kobayashi
52nd NIBB Conference	January 20-23, 2006	Reproductive Strategies	M. Hoshi
3rd Okazaki Biology Conference	March 12-17, 2006	Biology of Extinction 2	T. Yahara C. Roberts
2nd NIBB-EMBL Symposium	March 22-24, 2006	Frontiers in Bioimaging	N. Ueno J. Ellenberg

Table 2. Products of Strategic Planning Department in the occasion of events listed bellow

Events	Homepage	Booklet	Panel	PC Presentation	Movie	Photographic Chronicle
Education						
Bioscience Training Course	✔	✔				✔
Internship Program	✔					✔
Graduate University Explanatory Meeting	✔		✔		✔	✔
Meetings						
50th NIBB Conference			✔			✔
51st NIBB Conference	✔	✔	✔			✔
52nd NIBB Conference	✔					✔
3rd Okazaki Biology Conference	✔					✔
2nd NIBB-EMBL Symposium	✔					✔
Exhibition Booth						
NIFS[1] Open House			✔			✔
MBSJ[2] Meeting			✔	✔	✔	✔

[1] National Institute for Fusion Science, [2] Molecular Biology Society of Japan

Figure 1. Some examples of posters and panels designed by Strategic Planning Department

RESEARCH SUPPORT FACILITIES

Head: *NISHIMURA, Mikio*

Large Spectrograph Laboratory
Professor (Adjunct): *WATANABE, Masakatsu*
Technical Staffs: *HIGASHI, Sho-ichi*
NAKAMURA, Takanori
Technical Assistant: *ICHIKAWA, Chiaki*

Tissue and Cell Culture Laboratory
Research Associate: *HAMADA, Yoshio*
Technical Assistant: *TAKESHITA, Miyako*

Computer Laboratory
Research Associate: *UCHIYAMA, Ikuo*
Technical Staffs: *MIWA, Tomoki*
NISHIDE, Hiroyo
Technical Assistants: *MAKIHARA, Nobuko*
YAMAMOTO, Kumi

Plant Culture, Farm, Plant Cell Laboratory
Technical Staff: *NANBA, Chieko*
Technical Assistant: *SUZUKI, Keiko*

1. The Large Spectrograph Laboratory

This laboratory provides, for cooperative use, the largest spectrograph in the world, the Okazaki Large Spectrograph (OLS), dedicated to action spectroscopical studies of various light-controlled biological processes. The spectrograph runs on a 30kW Xenon arc lamp and has a compound grating composed of 36 smaller individual gratings. It projects a spectrum of a wavelength range from 250nm (ultraviolet) to 1,000nm (infrared) onto its focal curve of 10m in length. The fluence rate (intensity) of the monochromatic light at each wavelength is more than twice as much as that of the corresponding monochromatic component of tropical sunlight at noon (Watanabe *et al.*, Photochem. Photobiol. *36*, 491-498, 1982).

Figure 1. The Large Spectrograph

The NIBB Cooperative Research Program for the Use of the OLS supports about 20 projects every year conducted by visiting scientists including foreign sicientists as well as those in the Institute.

Action spectroscopical studies for various regulatory and damaging actions of light on living organisms, biological molecules, and artificial organic molecules have been conducted (Watanabe, *In* "CRC Handbook of Organic Photochemistry and Photobiology, 2nd ed.". pp. 115-1~115-16, 2004).

An advanced irradiation system composed of CW lasers (364nm, 390-410nm, 440-460nm, 532nm, 655nm, 752nm) and uniform-fluence-rate irradiation optics interconnected by optical fibers was constructed in 2003. An advanced observation system for cellular and intracellular photobiological responses utilizing a two-photon microscope (FV300-Ix71-TP with a MaiTai laser) and a microbial photomovement analyzer (WinTrack2000/Ecotox) was also introduced.

2. Tissue and Cell Culture Laboratory

Various types of equipment for tissue and cell culture are provided. This laboratory is equipped with safety rooms which satisfy the P2/P3 physical containment level. This facility is routinely used for DNA recombination experiments.

3. Computer Laboratory

The Computer Laboratory provides computation resources and the means for electronic communication. Currently, the main system consists of three servers and two terminal workstations: biological information analysis server (SGI Origin 2000), database server (Sun Enterprise 450), file server (Sun Enterprise 220R), data visualization terminal and molecular simulation terminal (both are SGI Octanes). Some personal computers and color/monochrome printers are also available. On this system we provide various biological databases and data retrieval/analysis programs, and support large-scale data analysis and database construction for the institute members. At the end of this year, a new computer system with enhanced performance will be introduced as a replacement for the current system. The new system will be available for use at the beginning of next year.

The Computer Laboratory also provides network communication services within the institute. Most of the computers in each laboratory, as well as all of the above service machines, are connected to each other by a local area network (LAN) which is linked to the high performance multimedia backbone network of Okazaki National Research Institute (ORION). Many local services including sequence analysis service, file sharing service and printer service are provided through this LAN. We also maintain a public World Wide Web server that contains the NIBB home pages (http://www.nibb.ac.jp/).

4. Plant Culture Laboratory

This laboratory contains a large number of culture boxes and a limited number of rooms with environmental control for plant culture. In some of these facilities and rooms, experiments can be carried out at the P1 physical

containment level under extraordinary environments such as strong light intensity, low or high temperatures, etc.

5. Experimental Farm

This laboratory consists of two 20 m^2 glass-houses with precise temperature and humidity control, three green houses (each 6 m^2) at the P1 physical containment level, a small farm, and two greenhouses (45 and 88 m^2) with automatic sprinklers. The laboratory also includes a building with storage and work space.

6. Plant Cell Laboratory

This laboratory is equipped with autotrophic and heterotrophic culture devices and equipment for experimental cultures of plant and microbial cells. Facilities for preparation of plant cell cultures, including an aseptic room with clean benches, are also provided.

Publication List:

Original Papers

Hasegawa, E. (2005). Comparison of the spectral sensitivity of juvenile red sea bream investigated by the physiological technique and by the behavioral technique. Fisheries Science *71*, 79-85.

Ohnishi, N., Allakhverdiev, I.S., Takahashi, S., Higashi, S., Watanabe, M., Nishiyama, Y., and Murata, N. (2005). The two mechanism of photodamage to photosystem II: Step one occurs at the oxygen-evolving complex and step two occurs at the photochemical reaction center. Biochem. *44*, 8494-8499.

Okajima, K., Yoshihara, S., Fukushima, Y., Geng, X., Katayama, M., Higashi, S., Watanabe, M., Sato, S., Tabata, S., Shibata, Y., Itho, S., and Ikeuchi, M. (2005). Biochemical and functional characterization of BLUF-type flavin-binding proteins of two species of cyanobacteria. Japanese Biochemical Society *137*, 741-750.

Nagai, Y., Nakamura, D., Ueno, H., Matsumoto, N., and Ohishi, F. (2005). Photodegradation mechanisms in poly (2,6-butylenenaphthalate-co-tetramethyleneglycol) (PBN-PTMG). II: Wavelength sensitivity of the photodegradation. Polymer Degradation and Stability *88*, 256-260.

RESEARCH CENTER FOR INTEGRATIVE AND COMPUTATIONAL BIOLOGY

Head: *TAKADA, Shinji*
Associate Professor: *MOCHIZUKI, Atsushi*
Secretary: *UMEBAYASHI, Hiromi*

The aims of the research center for integrative and computational biology are (1) investigating fundamental principles of various biological phenomena based on the method of integration of computational science and biology; (2) establishing new methodologies for integrative biology; and (3) providing the new technology and knowledge to researchers. Our final goal is to establish a new bioscience that includes methods originally utilized in different fields: informatics, mathematics, and biology.

I. Research activity

The research center for integrative and computational biology was founded in 2001 in a time of rapid progress in modern biology. The success in world wide genome projects has provided a huge amount of new information on genes, leading to expectations of advances in the development of new medicines for intractable diseases, the exploitation of new cultivated plants resistant to noxious insects, and so on. In addition, it is the object of present-day biology to research higher-order phenomena that are constructed from complex interactions between many genes. To grapple with these questions, it is essential to decipher huge amounts of gene information, and to derive the essence for the biological behavior of cells or organisms.

Mathematical and computational sciences have strong capacities for dealing with these questions. Computational methods make it possible to process ever-increasing amounts of data. Hypothetical experiments (including the evolution of past organisms) based on mathematical or computational models make it possible to consider conditions which are impossible in real experiments. We are continuing to research higher-order phenomena in biology by using mathematical or computational methods, and to develop new methodology for studying such complex phenomena.

Figure 1. A laboratory room for computational studies

For example, integrative methods are especially important in understanding pattern formation in development. Morphological difference between species is an important research focus of the current developmental biology. What is the mechanism responsible for the difference of morphogenesis between species? Theoretical studies are useful in identifying candidates of cell or gene interaction that are likely to be responsible for the systems in real organisms. This method gives us an integrative understanding for the behavior of complex systems in biology, including gene regulatory networks.

II. Collaboration activity

Interaction between researchers is essential to make the best use of mathematical and computational methods for experimental biology. The theoretical methods provide testable predictions which the experimental biologists are able to investigate before returning the results to the theoreticians for the next round of predictions. By repeating these predictions and tests, the integrative methods are continually developed. The research center for integrative and computational biology has continued to enhance interaction between theoretical biologists and experimental biologists.

The center provides equipment such as cluster machines and Unix-based machines for computation and experiments. Members can also use computational equipment at the Research Center for Computational Science in Okazaki. The center also provides experimental equipment for collaborations with experimental biologists.

Figure 2. A cluster machine

In 2005, we held a symposium on March 8-9 entitled "Mathematical and Computational Approaches to Biology." Dozens of researchers studying mathematical, computational, or experimental biology participated. The center is dedicated to encouraging young researchers who can use these methods for continued research into complex biological phenomena.

CENTER FOR TRANSGENIC ANIMALS AND PLANTS

Head:	*TAKADA, Shinji*
Associate Professors:	*WATANABE, Eiji*
	SASAOKA, Toshikuni
	TANAKA, Minoru
Technical Staffs:	*HAYASHI, Kohji*
	ICHIKAWA, Youko
	ICHIKAWA, Hiromi
	TAKAGI, Yukari
Supporting Staffs:	*KOBAYASHI-NISHIMURA, Keiko*
	YASUDA, Mie
	YAMAGUCHI, Kyoko
	NOGUCHI, Yuji
	YOSHIDA, Etsuko
	KAWAMURA, Motofumi

The worldwide genome project has been almost completed and research on basic biology has arrived at a post-genome era in which researchers focus on investigating the function of individual genes. To promote functional analysis of a gene of interest it is essential to utilize genetically altered model organisms, which are generated by the genetic engineering technology, the creation of gene deletion, gene replacement or point mutation.

The NIBB Center for Transgenic Animals and Plants (CTAP) was established in April 1998 to support research using transgenic and gene targeting techniques at NIBB. The CTAP is managed by the head (professor, a concurrent post) and an associate professor.

Figure 1. A new center facility for transgenic animals in the Yamate area.

Technical staff and supporting staff develop and promote research-supporting activities. In 2003 two associate professors joined the CTAP. A new CTAP building for transgenic animals in the Yamate area opened at the end of 2003.

The expected activities of the CTAP are as follows:

1. Provision of information, materials, techniques and animal housing space to researchers.
2. The use of various kinds of instruments to analyze mutant, transgenic, and gene-targeted animals and plants.
3. Development of novel techniques related to transgenic and gene targeting technology.
4. Cryopreservation and storage of transgenic strains.

I. Research supporting activity (mouse)

In 2001 the NIBB mouse facility (built under specific pathogen free (SPF) conditions) opened in the Myodaiji area and the production, breeding, analysis, cryopreservation and storage of genetically manipulated mouse strains has been conducted there since then. The new CTAP building in the Yamate area strengthened research activities using genetically altered organisms. The building has five floors and a total floor space of 2,500m^2, in which we can generate, breed, store and analyze transgenic, gene targeting and mutant mice under SPF conditions. This building is also equipped with breeding areas for transgenic small fish, birds and insects on the first floor. The mouse housing area of this building is constructed based on a barrier system in which the clean area and the semi-clean area are clearly separated and designed for an efficient and one-directional flow of mice, personnel, articles of animal housing and equipment from the clean area side to the semi-clean area side.

Figure 2. Autoclaves of a large scale for sterilization

In 2005, 4,122 mice were brought into the CTAP in the Yamate area, and 20,521 mice (including pups bred in the facility) were taken out from the CTAP from April 01, 2005 to October 31, 2005.

A number of strains of genetically altered mice outside the CTAP were brought into the mouse housing area by microbiological cleaning using the *in vitro* fertilization-embryo transfer techniques, and stored by cryopreservation.

A new mouse facility in the Myodaiji area opened in the beginning of 2005. The facility provides research-supporting activities to researchers in the Myodaiji area. In 2005, 958 mice were brought into the CTAP in the Myodaiji area, and 2,321 mice (including pups bred in the facility) were taken out from the CTAP from April 01, 2005 to October 31, 2005.

Figure 3. Equipments to generate mutant mice under condition of specific pathogen-free

II. Research supporting activity (small fish, birds, and insects)

The first floor of the CTAP building in the Yamate area provides space and facilities to maintain small fish, chick embryos and insects. In a laboratory room for chick embryos, a large incubation chamber is equipped and set at 42 degrees (suitable for chick embryogenesis). For researchers who need fish, 480 tanks (1 liter) and 450 tanks (3 liters) are available for medaka and zebrafish, respectively. Water circulates and can be maintained to suit conditions desired for fish breeding in the aquarium systems. Currently, three mutant lines and two transgenic lines of medaka are maintained in our facility. In addition to the rooms mentioned above, another room is available for insects. All the rooms are qualified to meet the criteria for P1A transgenic animals, allowing researchers to generate and maintain transgenic animals.

In 2005 (as of the end of November from the beginning of January), 944 medaka (452 fertilized eggs, 440 embryos and 52 adults) were brought to the facility and 46,842 medaka (45,923 fertilized eggs, 700 embryos, and 219 adults, including animals bred in the facility) were taken out from the CTAP. In a laboratory for chick embryos 44,531 fertilized chicken eggs were brought in and 452 animals (190 fertilized eggs and 262 embryos) were taken out from the CTAP. These animals were used for research activities in neurobiology and developmental biology

Figure 4. Breeding equipments for transgenic chickens

III. Academic activity

The associate professors of this center - E. Watanabe, T. Sasaoka, and M. Tanaka - are the principal investigators of the Laboratory of Neurophysiology, the Laboratory of Neurochemistry and the Laboratory of Molecular Genetics for Reproduction, respectively. The Laboratory of Neurophysiology is studying the brain sensing system for the water and sodium homeostasis in the body fluid by using gene-targeting mice, the Laboratory of Neurochemistry is studying a physiological role of dopaminergic system using genetically altered mice, and the Laboratory of Molecular Genetics for Reproduction is studying molecular mechanism of reproductive organ development and sex differentiation using mutagenized or transgenic medaka. For details, please refer to the academic activity of each laboratory.

THE CENTER FOR ANALYTICAL INSTRUMENTS

(managed by NIBB)

Head of Facility: *KOBAYASHI, Satoru*
Technical Staffs: *MORI, Tomoko*
MAKINO, Yumiko
TAKAMI, Shigemi
Technical Assistants: *MORIBE, Hatsumi*
KUROYANAGI Asuka
Secretary: *ICHIKAWA Mariko*

The Center is responsible for amino acid sequence analysis, amino acid analysis, and chemical syntheses of peptides, as well as supporting researchers at NIBB and NIPS. Luminescent Image Analyzers (Fujifilm LAS 3000 mini) were newly installed in 2005. The Center's instruments can be used by researchers outside the institute upon proposal.

Figure 1. Protein sequencers

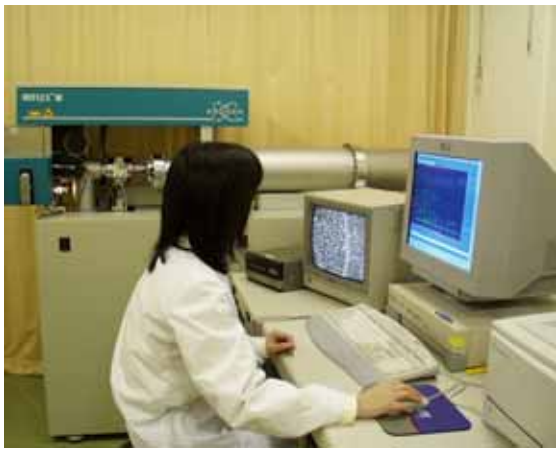


Figure 2. MALDI/TOF-MS

Representative Instruments

Protein Sequencers (ABI Procise 494HT, 492cLC)
Amino Acid Analyzer (Hitachi L8500A)
Peptide Synthesizers (ABI 433A)
Plasmid Isolation Systems (Kurabo PI-100∑, PI-50, PI-200)
Automatic Nucleic Acid Isolation System (Kurabo NA-2000)
Genetic Anlyzer (ABI PRISM 3100, 310)
Thermal Cyclers (Perkin Elmer PJ-9600, Takara TP-300, Biometra TGRADIENT)
Integrated Thermal Cyclers (ABI CATALYST Turbo 800)
Particle Delivery System (Bio-Rad BiolisticPDS-1000/He)
Gas Chromatograph (Shimadzu GC-14APF-SC)
Glycoprotein Analysis System (Takara Glyco-Tag)
High Performance Liquid Chromatographs (Shimadzu LC-10AD, LC-6AD, Waters 600E, Alliance UV system)
Integrated Micropurification System (Pharmacia SMART)
Flow Cytometer (Coulter EPICS XL)
Biomolecular Interaction Analysis Systems (Pharmacia BIACORE 2000, Affinity Sensors IAsys)
Laboratory Automation System (Beckman Coulter Biomek 2000)
ESR Spectrometer (Bruker ER-200D)
GC/Mass Spectrometer (JEOL DX-300)
MALDI/TOF-MS (Bruker Daltonics REFLEX III)
Inductively Coupled Plasma Atomic Emission Spectrometer (Seiko SPS1200A)
Spectrofluorometers (Hitachi 850, F-4500, Simadzu RF-5000)
Spectrophotometers (Hitachi 330, 557, U-2001,Varian Cary 5G, Perkin Elmer Lambda-Bio)
Microplate Luminometer (Berthold MicroLumat LB 96P)
Microplate Readers (Corona MTP-120, MTP-100F)
FT-IR Spectrophotometer (Horiba FT-730)
Laser Raman Spectrophotometer (JASCO R-800)
Bio Imaging Analyzers (Fujifilm BAS 2000)
Luminescent Image Analyzer (Fujifilm LAS 3000 mini)
Fluorescence Bio Imaging Analyzer (Takara FMBIO)
Electrophoresis Imaging System (BIOIMAGE)
Microscopes (Carl Zeiss Axiophot, Axiovert)
Environmental Scanning Electron Microscope (PHILIPS XL30 ESEM)
Confocal Laser Scanning Microscope (Leica TCS SP2)
Color Laser 3D Profile Microscope (KEYENCE VK-8500)
High-Resolution Quick Microscope (KEYENCE VH-5000)

TECHNICAL DIVISION

Head: FURUKAWA, Kazuhiko

Common Facility Group

Chief: *MIWA, Tomoki*

Research Support Facilities

Unit Chief: *HIGASHI, Sho-Ichi*
Subunit Chief: *NANBA, Chieko*
Technical Staff: *NISHIDE, Hiroyo*
NAKAMURA, Takanori
Technical Assistants: *SUZUKI, Keiko*
MAKIHARA, Nobuko
ICHIKAWA, Chiaki
TAKESHITA, Miyako
NISHIMURA, Noriko
YAMAMOTO, Kumi

Center for Analytical Instruments

Unit Chief: *MORI, Tomoko*
Subunit Chief: *MAKINO, Yumiko*
Technical Staff: *TAKAMI, Shigemi*
Technical Assistants: *MORIBE, Hatsumi*
DODO, Yukiko
KUROYANAGI, Asuka

Transgenic Animal Facility

Subunit Chief: *HAYASHI, Kohji*
Technical Assistants: *ICHIKAWA, Hiromi*
ICHIKAWA, Yoko
TAKAGI, Yukari

Disposal of Waste Matter Facility

Unit Chief: *MATSUDA, Yoshimi*

Radioisotope Facility

Unit Chief: *MATSUDA, Yoshimi*
Subunit Chief: *SAWADA, Kaoru*
Technical Staff: *IINUMA, Hideko*
Technical Assistant: *ITO, Takayo*

Research Support Group

Chief: *KOBAYASHI, Hiroko*

Cell Biology

Unit Chief: *KONDO, Maki*
Subunit Chief: *KABEYA, Yukiko*

Developmental Biology

Technical Staff: *TAKAGI, Chiyo*
UTSUMI, Hideko
OKA, Sanae
NODA, Chiyo

Neurobiology

Unit Chief: *OHSAWA, Sonoko*
Subunit Chief: *TAKEUCHI, Yasushi*

Evolutionary Biology and Biodiversity

Unit Chief: *TANAKA, Sachiko*
Subunit Chief: *YAMAGUCHI, Katsushi*
Technical Staff: *MOROOKA, Naoki*
SUMIKAWA, Naomi

Environmental Biology

Subunit Chief: *MIZUTANI, Takeshi*

Reception

Supporting Staff: *SAKAGAMI, Mari*
TSUZUKI, Shihoko
KATAOKA, Yukari
NAKANE, Kaori
HIRONAKA, Tomie
MUKOHDA, Yasuyo
UNO, Satoko
SUGIYAMA, Tomomi

The Technical Division is a supporting organization for researchers and research organizations within the NIBB. The Division develops and promotes the institute's research activities and maintains the research functions of the institute.

The Division is organized into two groups: the Common Facility Group, which supports and maintains the institute's common research facilities, and the Research Support Group, which assists the research activities as described in the reports of individual research divisions.

Technical staff members participate, through the Division, in mutual enlightenment and education to increase their capabilities in technical areas. Generally, technical staff members are attached to specific divisions so that they may contribute their special biological and biophysical techniques to various research activities.

The Division hosts an annual meeting for technical engineers who work in various fields of biology at universities and research institutes throughout Japan. At this meeting, the participants present their own activities and discuss technical problems. The proceedings are published after each meeting.

CENTER FOR RADIOISOTOPE FACILITIES

Head:	*NODA, Masaharu*
	(Professor, concurrent post)
Associate Professor:	*OGAWA, Kazuo*
	(Radiation Protection Supervisor)
Technical staff:	*MATSUDA, Yoshimi*
	(Radiation Protection Supervisor)
	SAWADA, Kaoru
	IINUMA, Hideko
Supporting staff:	*ITO, Takayo*
	KATAGIRI, Izumi
	KAMIYA, Kiyomi
	KANEUJI, Kimie

The technical and supporting staffs of the Center for Radioisotope Facilities (CRF) maintain three controlled areas according to Japanese law. The CRF is responsible for monitoring the purchase of radioisotopes from JRA (Japan Radioisotope Association) and the transfer of radioisotope wastes to JRA.

Matsuda, Iinuma, Ito, and Kaneuji maintain CFBI (Common Facilities Building I)-branch and LGER (Laboratory of Gene Expression and Regulation)-branch at the Myodaiji-Area, and Ogawa, Sawada, Katagiri, and Kamiya work at the Yamate-Area.

The numbers of registrants and users from January 2005 to November 2005 are presented in Table 1.

Users counted by the monitoring system going in and out of the controlled areas numbered 4,485 persons during this period. The percentages and numbers for each area are shown in Figure 1 and Table 2. The annual changes of registrants and the number of totals per year are shown in Figure 2.

The balance of radioisotopes received at the CRF is shown in Table 3. No ^{125}I was used at Myodaiji-Area.

The Yamate-Area has passed the periodical inspection by the Nuclear Safety Technology Center. The same inspection will be carried out at the Myodaiji-Area 2005 on December 16th, 2005.

The air supply system of CFBI-branch was repaired in order to decrease the amount of dust from the air supply duct ports (Figure 3) and increase the comfort of those working at CFBI-branch's controlled rooms.

Table 1. Numbers of registrants and users at Myodaiji-Area and Yamate-Area in the 2005

	Myodaiji-Area	Yamate-Area
registrants	137	113
users	66	50

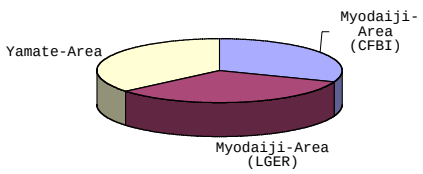


Figure 1. Percentage of users going in and out of each controlled area in 2005

Table 2. The numbers of users and visitors who entered each controlled area in 2005

	Myodaiji-Area		Yamate-Area	total
	CFBI-branch	LGER-branch		
users	1167	1412	1444	4023
visitors	215	74	173	462
total	1382	1486	1617	4485

Figure 2. Annual changes of registrants and number of totals per fiscal year

Table 3. Balance of radioisotopes received and used (MBq) at each controlled area

	Myodaiji-Area		Yamate-Area	total
	CFBI-branch	LGER-branch		
^{125}I Received	0.000	0.000	2220.000	2220.000
^{125}I Used	0.000	0.000	2590.000	2590.000
^{35}S Received	18500.000	265000.000	64750.000	348250.000
^{35}S Used	9990.000	132500.000	39868.701	182358.701
^{32}P Received	185000.000	841750.000	1082250.000	2109000.000
^{32}P Used	139860.000	711500.000	958672.000	1810032.000
^{14}C Received	0.000	11100.000	9520.000	20620.000
^{14}C Used	46213.000	416.000	9520.000	56149.000
^{3}H Received	64750.000	0.000	0.000	64750.000
^{3}H Used	74796.610	0.000	177600.000	252396.610

Figure 3. Photograph showing a controlled area during clearing the air supply duct ports

50th NIBB conference
Structure and Dynamics of Complex Biological Networks

Organizing Chair : Atsushi Mochizuki
February 8 (Tue) -10 (Thu), 2005

Thanks to progress in molecular genetics technologies the past several years have seen rapid increases in the amount of biological information available to us; the next order of business, therefore, is to find the essential principles of higher-order phenomena in biology hidden within enormous amounts of information. Non-experimental methods are now attracting attention as new methods for integrating information or for understanding whole systems of biology. Many researchers from physics, mathematics and computer science are starting to grapple with problems in biology.

Enhanced interaction between researchers working in different disciplines is essential for progress in theoretical methods in biology. For this purpose we held an international conference on February 2005 in Okazaki, Japan. For this conference we called researchers studying various biological phenomena, network systems, spatio-temporal patterns, ecology and evolution using different theoretical methodologies, with a special focus on the network structures in different fields of biology, including gene regulation, metabolic pathway and ecology. One of the notable themes was the relation between the structural and dynamic behaviors of these complex systems.

The conference featured 39 research presentations including 8 short talks and 12 poster presentations. Ninety one participants – including 12 reseachers from foreign countries - enjoyed discussions and the interaction between different fields of theoretical biology. We have received many mails from participants thanking us for holding this conference. We hope that this conference leads to the start of a new theoretical bioscience in the future.

Scientific topics:

Network systems in biology
Mathematical property of network
Application and understanding for biological network

Regulations in cells, tissues and organisms
Dynamical regulation in metabolism or cellular systems
Morphogenesis in development

Ecology and evolution
Dynamics in ecological network system

Speakers

AKUTSU, Tatsuya	(Kyoto University, Japan)
ALBERT, Réka	(Pennsylvania State University, USA)
ALMAAS, Eivind	(University of Notre Dame, USA)
ALVAREZ-BUYLLA, Elena Roces	(Universidad Nacional Autonoma de Mexico, Mexico)
ARITA, Masanori	(University of Tokyo, Japan)
CHING, Wai Ki	(University of Hong Kong, China)
CRACIUN, Gheorghe	(Ohio State University, USA)
IWASA, Yoh	(Kyushu University, Japan)
KANEKO, Kunihiko	(University of Tokyo, Japan)
KING, Ross	(University of Wales, UK)
KONDO, Shigeru	(Riken, Kobe / Nagoya University, Japan)
MARTINEZ, Neo	(Pacific Ecoinformatics and Computational Ecology Laboratory, USA)
MOCHIZUKI, Atsushi	(National Institute for Basic Biology, Japan)
MURATOV, Cyrill	(New Jersey Institute of Technology, USA)
NAKAI, Kenta	(University of Tokyo, Japan)
PAULSSON, Johan	(University of Cambridge, UK)
REINITZ, John	(Stony Brook University, USA)
TOKITA, Kei	(Osaka University, Japan)
VERT, Jean-Philippe	(Ecole des Mines de Paris, France)

51st NIBB conference
New Aspects of Gene Amplification-Mechanisms and Biological Function

Organizing Chair : Takehiko Kobayashi
November 5 (Sat) - 8 (Tue), 2005

Gene amplification not only results in genome alteration, it also plays wide-ranging roles in many biological functions. In multicellular organisms, it is observed in many stages of the life-cycle: development, differentiation, senescence, and tumorgensis. In unicellular organisms, it is one of the main strategies for adaptation to surroundings. Moreover, it is well-known that gene amplification has played critical roles in evolution.

Despite its involvement in these important biological functions, gene amplification has not been a central focus for discussion. One of the reasons is that the mechanisms responsible have remained elusive. In this conference, researchers studying DNA recombination, DNA replication, chromatin structure and evolution discussed the molecular mechanisms and biological functions of gene amplification (including maintenance mechanism of amplified genes).

The conference featured over 50 participants (including 11 researchers from foreign countries) and included 22 oral and 11 poster presentations. Tremendous amounts of information were exchanged during many exciting discussions.

Now, with the enormous increases in genomic information and the rapid progression of molecular biology, we are in a good position to look at new aspects of gene amplification. This timely conference, therefore, was an excellent opportunity to develop amplification study.

Scientific topics:

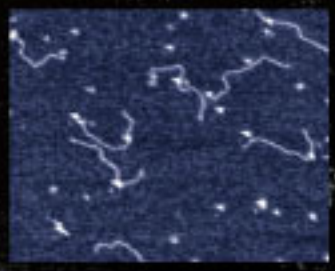
Molecular Mechanism of Gene Amplification

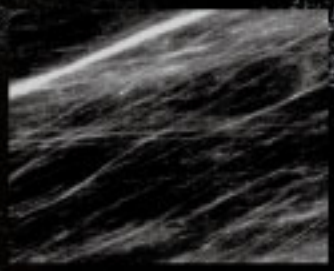
Adaptation and Gene Amplification

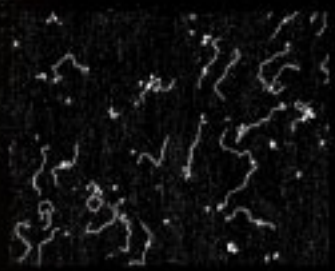
Evolution and Gene Amplification

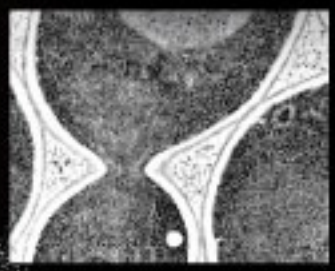
Speakers

ARCANGIOLI, Benoit	(Pasteur Institute, France)
BENSIMON, Aaron	(Pasteur Institute, France)
DEBATISSE, Michelle	(Curie Institute, France)
GANLEY, Austen	(National Institute for Basic Biology, Japan)
HERNANDEZ, Pablo	(CSIC, Spain)
HISHIDA, Takashi	(Osaka University, Japan)
HORIUCHI, Takashi	(National Institute for Basic Biology, Japan)
IWASAKI, Hiroshi	(Yokohama City University, Japan)
JOHZUKA, Katsuki	(National Institute for Basic Biology, Japan)
KIKUCHI, Akihiko	(Nagoya University, Japan)
KOBAYASHI, Takehiko	(National Institute for Basic Biology, Japan)
LOBACHEV, Kirill	(Georgia Institute of Technology, USA)
MAKI, Hisaji	(Nara Institute of Science and Technology, Japan)
ROTH, John	(University of California-Davis, USA)
SASAKI, Hiroki	(National Cancer Center Research Institute, Japan)
SCHVARTZMAN, Jorge	(CSIC, Spain)
SHIBATA, Takehiko	(RIKEN Institute, Japan)
SHIMIZU, Noriaki	(Hiroshima University, Japan)
SHORE, David	(University of Geneva, Switzerland)
SOGO, José	(ETH, Switzerland)
STRUNNIKOV, Alexander	(NIH, USA)
TOWER, John	(University of Southern California, USA)









http://www.nibb.ac.jp/

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Aichi 444-8585, Japan