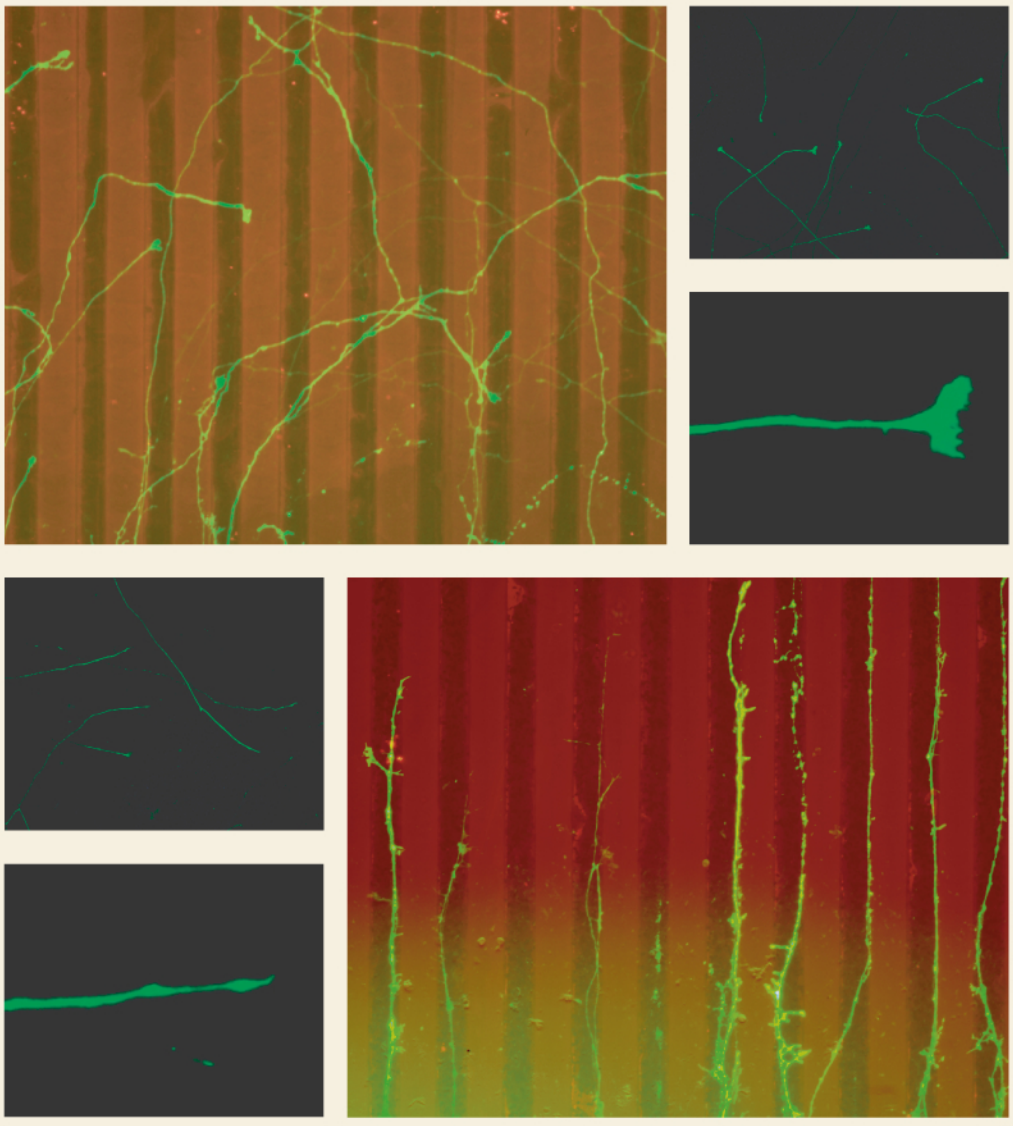


National Intitute for Basic Biology

2006 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 four Inter-University Research Institute Corporations. At that 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 2006 were as follows. Assoc. Prof. Koji Mikami moved to Hokkaido University as an associate professor on August 31 and Assoc. Prof. Takehiko Kobayashi moved to the National Institute of Genetics as a professor on November 30. Dr. Kazuhiro Kikuchi moved to Boyce Thompson Institute for Plant Research as a research associate and Dr. Takeshi Noda moved to Osaka University as an associate professor on January 31. Dr. Goro Horiguchi moved to the University of Tokyo as an associate professor on October 31.

The following researchers joined our institute over the past year. Dr. Shigenori Nonaka from UCSF as an associate professor on January 16, Dr. Osamu Sadakane, a JSPS post-doctoral fellow, as a research associate on August 1, and Dr. Takahiro Yamaguchi, a JSPS post-doctoral fellow, as a research associate on October 1. Dr. Atsushi Miyawaki became an adjunct professor and Dr. Kazuhiro Sakamaki became an adjunct associate professor on April 1.

The collaboration program between NIBB and the European Molecular Biology Laboratory (EMBL) under the sponsorship of NINS, which began in 2005, is a continuing success. The program aims to promote joint research activities between the two organizations and to prompt mutual visits by researchers and graduate students, thereby encouraging an exchange of information and intimate communication, both of which are indispensable for the prosperity of joint research. Activities seen this year are as follows. Two symposia were held at NIBB: "Frontiers in Bioimaging" in March and "Biology of Protein Conjugation: Structure and Function" in December. A meeting on mouse biology was held at Moterotondo EMBL outstation in April. Four seminars were held at NIBB by visiting researchers of EMBL, while NIBB researchers stayed at EMBL to prepare for the introduction of the SPIM microscope to NIBB.

Finally, I would like to congratulate Prof. Mikio Nishimura for winning the Chunichi Cultural Prize in May, Prof. Norihiro Okada for winning the Fujihara Prize in June, and Prof. Yoshinori Ohsumi for winning the Japan Academy Prize in July. It was our great honor that NIBBís former Director General, Emer. Prof. Hideo Mohri, was decorated with the Order of the Sacred Treasure, Gold and Silver Star in November.

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) ñ renamed the Okazaki Institute for Integrative Bioscience on April 1st, 2004 ñ 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.

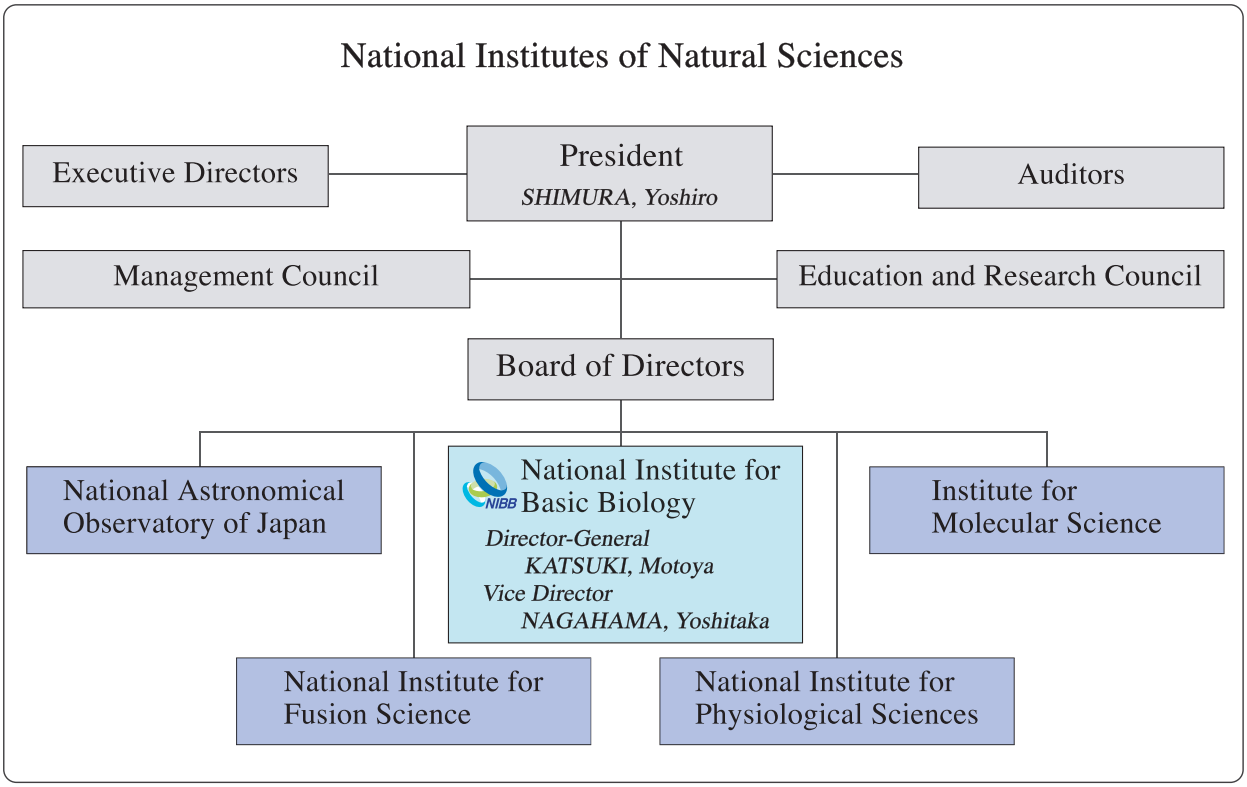
Research Support Facilities

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 Cell Structure
  - Laboratory of Cell Sociology
- **Developmental Biology**
  - Division of Reproductive Biology
  - Division for Sex Differentiation
  - Division of 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 Molecular Environmental Endocrinology †††
  - Division of Plant Developmental Genetics (adjunct)
  - Division of Photobiology (adjunct)
  - Laboratory of Photoenvironmental Biology
  - Laboratory of Stress Response[1)]
- **Theoretical Biology**
  - Division of Theoretical Biology
  - Laboratory of Genome Informatics
- **Imaging Science**
  - Division of Developmental Dynamics (adjunct)
  - Laboratory of Bioimaging

Research Support

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

Research Facilities run jointly with other Institute(s) in Okazaki

- **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 ***
- **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

*〜***)These divisions also belong to NIBB as shown with †〜†††), respectively. 1) Until August 31, 2006

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

Chairperson

YAMAMORI, Tetsuo	Professor, National Institute for Basic Biology

Vice-Chair

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, Ken-ichirou	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 masterís 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 Joint Seminar of the School of Life Science in October 2006.

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 52nd NIBB Conference was ìR eproductive Strategiesî and for the 53rd was "Dynamic Organelles in Plants" (see pages 84, 85). 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 (see pages 82, 83).

The NIBB plays a key role in the academic exchange programs between the European Molecular Biology Laboratory (EMBL) and National Institutes of Natural Sciences (NINS). Efforts were made 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 4th Okazaki Biology Conference: "Terra Microbiology 2" in September 2006.

At the excursion of the 2nd NIBB-EMBL symposium on "Frontiers of Bioimaging".

DIVISION OF CELL MECHANISM

Professor:	*NISHIMURA, Mikio*
Associate Professor:	*HAYASHI, Makoto*
Research Associates:	*MANO, Shoji*
	YAMADA, Kenji
Technical Staff:	*KONDO, Maki*
Postdoctoral Fellows:	*ARAI, Yuko*
	KAMIGAKI, Akane
	OIKAWA, Kazusato
Graduate Students:	*OGASAWARA, Kimi*
	GOTO, Shino
Visiting Scientists:	*LU, Zhongpen*
	CHRISTELLER, John
	SINGH, Tanuja
Technical Assistants:	*NAKAMORI, Chihiro*
	YOSHINORI, Yumi
	SUZUKI, Iku
	FUKAZAWA, Mitsue
	KATO, Kyoko
	NISHINA, Momoko
	SATO, Yori
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 the 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 ìOrganelle Differentiation as the Strategy for Environmental Adaptation in Plantsî 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 the 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 peroxisomes 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* and soybean. Peptide MS fingerprinting analyses allowed us to identify novel proteins existing 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 with a new insight into plant peroxisomal functions.

Bioinfomatic analysis of *Arabidopsis* genome predicted the presence of 15 kinds of genes for peroxisomal biogenesis factors, called *PEX* genes. We comprehensively investigated whether or not 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. In contrast, PEX7, a receptor for PTS2, is involved only in photorespiration by regulating import of PTS2-containing proteins.

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 (Figure 1A).

Two of these *apm* mutants, *apm2* and *apm4* mutants, showed GFP fluorescence in the cytosol as well as in peroxisomes (Figure 1B, 1C), indicating the decrease of efficiency of PTS1-dependent protein transport to peroxisomes. Interestingly, both mutants are defective in PTS2-dependent protein transport as well. Both mutants exhibit dwafism (Figure 1D), and this phenotype was

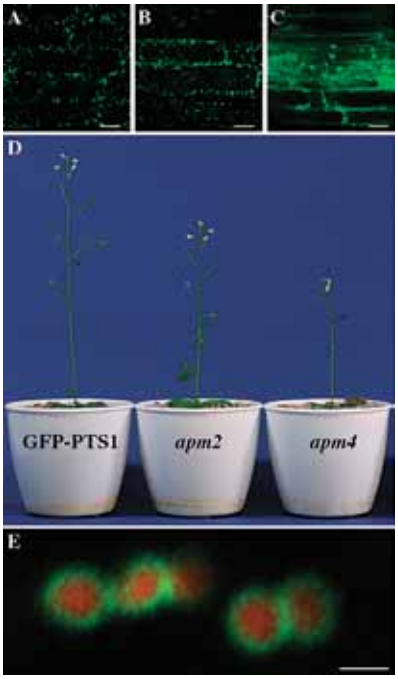


Figure 1. Contribution of APM2 and APM4 proteins for protein transport to peroxisomes. GFP fluorescence in cells of *apm2* (B) and *apm4* (C) mutants is observed in the cytosol as well as in peroxisomes, whereas the parent plant, GFP-PTS1, shows the fluorescence in only peroxisomes (A). These results show the decrease of efficiency of protein transport in both mutants. Both mutants exhibit dwarfism compared to the GFP-PTS1 (D). *APM2* and *APM4* genes encode Perxin 13 (PEX13) and PEX12, respectively, which are involved in protein transport on peroxiomal membranes (E, green and red signals represent PEX12-GFP and RFP-PTS1, respectively). Bars indicate 20 μm for (A) to (C), and 1 μm for (E).

related to the frequency of appearance of GFP fluorescence in the cytosol, apparently because protein transport was more severely decreased in *apm4* than in *apm2* mutant. *APM2* and *APM4* were found to encode proteins homologous to PEX13 and PEX12, respectively. It was revealed that APM2/PEX13 and APM4/PEX12 are localized on peroxisomal membranes (Figure 1E), and that APM2/PEX13 interacts with PEX7. In addition, we found that PEX5 binds to, and does not move on peroxisomal membranes in both mutants. These results show that APM2/PEX13 and APM4/PEX12 are components of the protein-translocation machinery on peroxisomal membranes. Analyses of other *APM* genes will help to identify components responsible 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. We have isolated a couple of *Arabidopsis* mutants that have a defect in ER body formation. *Arabidopsis nai1* mutant has no ER bodies in whole plants and does not accumulate PYK10 (Figure 2). *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.

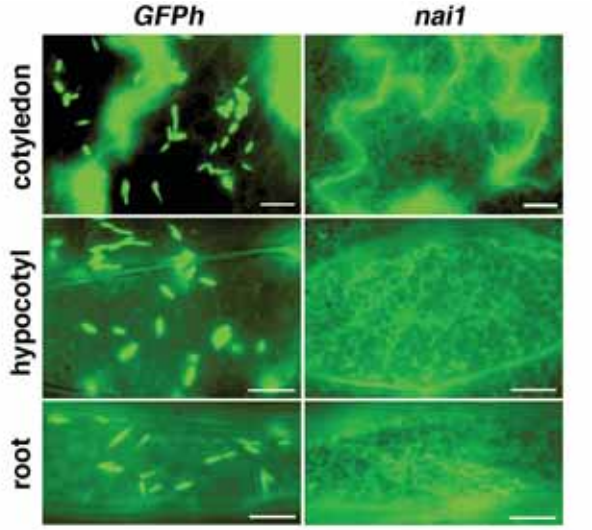


Figure 2. Fluorescent images of transgenic *Arabidopsis* (*GFPh*) and *nai1* mutant that express ER-localized GFP. Five-day-old seedlings were inspected with a fluorescence microscope. The GFPh seedlings had many ER bodies (rod -shape structures) in cotyledons, hypocotyls and roots. On the contrary, the *nai1* seedlings had no ER bodies in these organs. Bars= 10 μm.

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 seed-coat type. Seed type VPE is responsible for the maturation of seed storage proteins. On the other hand, the function of vegetative and seed-coat type VPEs was obscure. Recently, we revealed a novel function of VPE in various programmed cell death (PCD) in plants. The 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-deficient

tobacco plants. Fumonisin B1 (FB1), a fungal toxin, induced cell death in *Arabidopsis*. The features of FB1-induced cell death were completely abolished in the VPE-null *Arabidopsis* mutant. *Arabidopsis* δVPE expresses specifically and transiently in two cell layers of the seed coat that causes PCD accompanying cell shrinkage. In a *δvpe* mutant, shrinkage of these cell layers was delayed. 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, such as heat shock and hormone responses, in *Arabidopsis*. The evolutional and functional characterization is now under experiment.

VII. The Plant Organelles Database ñ Databases of plant organelles visualized with fluorescent probes, and protocols for functional analysis

Figure 3. The plant organelles database (http://podb.nibb.ac.jp/Orgenellome).

The plant organelles database is a specialized database project dedicated to plant organelle research and is maintained by the Scientific Research of Priority Areas on ìO rganelle Differentiation as the Strategy for Environmental Adaptation in Plantsî. This database consists of three individual databases: the organellome database, the functional analysis database and external links about transcriptomics and proteomics. The organellome database provides information of various plant organelles visualized with fluorescent probes. The functional analysis database contains useful protocols for analyses of plant organelles and integrated functions. This database is 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.

Publication List:

Original papers

Li, L., Shimada, T., Takahashi, H., Ueda, H., Fukao, Y., Kondo, M., Nishimura, M., and Hara-Nishimura, I. (2006). MAIGO2 is involved in exit of seed storage proteins from the endoplasmic reticulum in *Arabidopsis thaliana*. Plant Cell *18*, 3535-3547.

Mano, S., Nakamori, C., Nito, K., Kondo, M., and Nishimura, M. (2006). The Arabidopsis *pex12* and *pex13* mutants are defective in both PTS1- and PTS2-dependent protein transport to peroxisomes. Plant J. *47*, 604-618.

Shimada, T., Koumoto, Y., Li, L., Yamazaki, M., Kondo, M., Nishimura, M., and Hara-Nishimura, I. (2006). AtVPS29, a putative component of a retromer complex, is required for the efficient sorting of seed storage proteins. Plant Cell Physiol. *47*, 1187-1194.

Ueda, H., Nishiyama, C., Shimada, T., Koumoto, Y., Hayashi, Y., Kondo, M., Takahashi, T., Ohtomo, I., Nishimura, M., and Hara-Nishimura, I. (2006). AtVAM3 is required for normal specification of idioblasts, myrosin cells. Plant Cell Physiol. *47*, 164-175.

Review articles

Hatsugai, N., Kuroyanagi, M., Nishimura, M., and Hara-Nishimura, I. (2006). A cellular suicide strategy of plants: vacuole-mediated cell death. Apoptosis *11*, 905-911.

Hayashi, M., and Nishimura, M. (2006). Arabidopsis thaliana-A model organism to study plant peroxisomes. Biophys. Biochim. Acta *1763*, 1382-1391.

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:	*SEKITO, Takayuki*
	YOSHIMOTO, Kohki
	HANADA, Takao
	FUJIKI, Yuki
	OHNEDA, Mamoru
	OITA, Eiko
	ONODERA, Jun
	OKU, Masahide
	HARASHIMA, Toshiaki
	YAMAMOTO, Hayashi
	OKAMOTO, Koji
Graduate Students:	*KAGEYAMA, Takuya*
	OOOKA, Kyoko
Visiting Scientists:	*BABA, Misuzu*
	KAWAMATA, Tomoko
Technical Assistants:	*TSUKESHIBA, Kumi*
	ICHIKAWA, Rie
	KONDO, Chika
	NIIMI, Kaori
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. It is then 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. Structural studies of Atg proteins

To understand the molecular basis of the functions of Atg proteins, structural information is crucial. For four years we have collaborated closely with Prof. F. Inagaki's lab at Hokkaido University. Our final goal is to elucidate the 3-D structures of every Atg protein, two conjugates and protein complexes involved in autophagy. We have succeeded in obtaining the crystal structures of LC3 (mammalian homolog of Atg8), human Atg4B, *Arabidopsis* Atg12 and the human Atg4B-LC3 complex, and yeast Ape1, Atg3, complex of Atg5-N-terminal Atg16 fragment, These results revealed the unique structure of Atg4 enzyme, a closed active site without binding of LC3. It was found that Atg5, the target of Atg12 conjugation, contains two ubiquitin-like domains and a helical region connecting them and Atg3, an E2-like enzyme for Atg8, has a distinct domain from other typical E2 enzymes. This structural data gives us many critical ideas to elucidate the mechanism of Atg protein functions. Atg proteins other than the two conjugation systems are not easy to crystallize, so we are now trying to determine the essential domains for their functions.

III. Functions of two ubiquitin-like conjugates in autophagosome formation

Autophagosome formation involves two ubiquitin-like proteins, Atg8 and Atg12. We showed that Atg12 forms a conjugate with another Atg protein, Atg5, whereas Atg8 is conjugated to a lipid, phosphatidylethanolamine (PE). Although the functions of these conjugates have remained long-standing questions, we have recently made good progress on these issues using *in vitro* reconstitution systems consisting of purified protein components and PE-containing liposomes.

First, we found that Atg8 mediates the tethering and fusion of membranes (liposomes), which are evoked by lipidation of the protein and reversibly modulated by Atg4 that catalyzes the deconjugation of Atg8-PE. Moreover, mutational analyses suggested that these activities of Atg8 observed *in vitro* are required for autophagosome formation *in vivo*; membrane tethering and fusion represent bona fide functions of Atg8. These results provide key insights into the unique membrane dynamics of autophagy, which cannot be explained based on the mechanisms that have been elucidated in conventional vesicular trafficking systems.

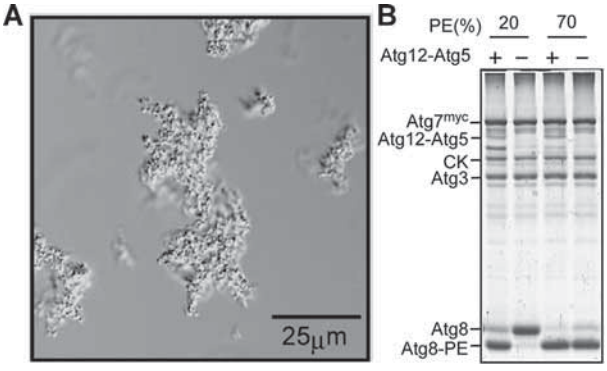


Figure 1. Functions of two ubiquitin-like protein conjugates. (A) The production of Atg8-PE *in vitro* leads to the clustering of liposomes, suggesting that Atg8-PE conjugates have an ability to tether together membranes to which they are anchored. The reaction mixture after incubation was observed under a light microscope. (B) The stimulation of the Atg8-PE conjugation reaction by the Atg12-Atg5 conjugate. If purified Atg12-Atg5 was added, Atg8 was effectively conjugated to PE even when liposomes containing a low concentration of PE (20%) were used.

Previous *in vivo* observations have suggested that two ubiquitin-like conjugation systems have an interrelationship: the Atg12-Atg5 conjugate is required for effective formation of Atg8-PE. We also examined this possibility *in vitro* and found that the Atg12-Atg5 conjugate directly facilitates Atg8-PE formation, in which Atg3, an E2-like enzyme, is catalytically activated for Atg8 transfer to PE by the Atg12-Atg5 conjugate. Further studies will shed light on understanding how these ubiquitin-like systems cooperate in autophagosome formation in the cell.

IV. Assortment of phosphatidylinositol 3-kinase complexes ñ Atg14 directs association of complex I to the pre-autophagosomal structure in *S. cerevisiae* ñ

Phosphatidylinositol 3-kinase (PI3-K) is a lipid kinase which phospholyrates phosphatidylinositol to produce phosphatidylinositol 3-phosphate (PI3-P). The produced PI3-P often serves as a ìm arkî to concentrate downstream molecules to specific sites of the cell. This process is essential for autophagy and the transport of vacuolar hydrolases into the vacuole. We previously identified two distinct PI3-K complexes, complexes I and II, in yeast (Kihara et al., 2001). Complex I functions in autophagy, while complex II functions in the transport of vacuolar hydrolases. How the two complexes can function in distinct biological processes despite producing the same primary product, PI3-P, is unknown. Each complex contains a unique subunit, Atg14 for complex I and Vps38 for complex II, in addition to three common subunits shared by both complexes. We focused on the specific subunits and analyzed them in detail. Atg14 localized to the vacuolar membrane and the PAS, a possible site of autophagosome formation, whereas Vps38 localized to the vacuolar membrane and endosomes. We then monitored the localization of the other subunits and found that complex I localized to the vacuolar membrane and the PAS. The PAS-localization of complex I was entirely dependent on Atg14. In contrast, complex II localized to the vacuolar membrane and endosomes. Localization of complex II to the endosome was dependent on Vps38. From these results and other biochemical data, we proposed a model that the distinct functions of PI3-K complexes are acquired by the specific association of each complex to a distinct compartment that is mediated by the specific components (Figure 1). Next, we further dissected the key molecule, Atg14, by truncation analysis. Atg14 has three putative coiled-coil domains within the N-terminal half. Surprisingly, the region covering the first two coiled-coil domains was shown to be sufficient for the function of Atg14 in autophagy, if not fully active, which indicates that the primary role of Atg14 is protein-protein interaction. Through the protein-protein interaction at the coiled-coil domains, Atg14 was able to form functional PI3-K complex I and localize to the PAS.

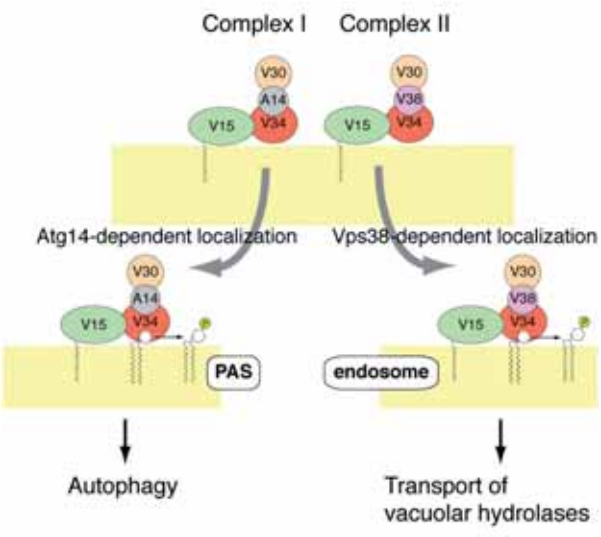


Figure 2. Function of PI3-K complexes is specified by distinct localization. PI3-K complex I localizes to the PAS in Atg14-dependent manner and functions in autophagy. In contrast, Vps38 mediates the localization of complex II to endosomes to function in sorting of vacuolar hydrolases. V30, Vps30; A14, Atg14; V34, Vps34; V15, Vps15; V38, Vps38.

V. Hierarchy of Atg proteins in pre-autophagosomal structure organization

Autophagy is a bulk degradation process that is conserved in eukaryotic cells and functions in the turnover of cytoplasmic materials and organelles. When eukaryotic cells face nutrient starvation, the autophagosome, a double-membraned organelle, is generated from the pre-autophagosomal structure (PAS). In the yeast *Saccharomyces cerevisiae*, at least 17 *ATG* (autophagy-related) genes are essential for autophagosome formation. Most of the Atg proteins are

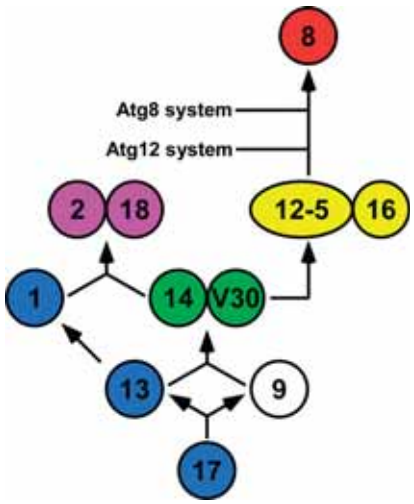


Figure 3. Hierarchy diagram of Atg proteins required for PAS organization. Tails of arrows are placed at Atg proteins that affect the PAS localization of other Atg proteins located at the heads of the arrows. Numbers: Atg proteins; V30: Vps30p/Atg6p.

localized to the PAS, leading to autophagosome production. However, the mechanism of PAS organization remains to be elucidated. Here, we performed a systematic and quantitative analysis by fluorescence microscopy to develop a hierarchy map of Atg proteins involved in PAS organization. This analysis suggests that Atg17p is the most basic protein in PAS organization: when it is specifically targeted to the plasma membrane, other Atg proteins are recruited to that location, suggesting that Atg17p acts as a scaffold protein to organize Atg proteins to the PAS.

VI. Arabidopsis Atg6/Vps30 is essential for pollen germination

Many *ATG* genes are conserved in plants and reverse genetic studies have demonstrated that Arabidopsis *atg* mutants were hypersensitive to nutrient starvation and exhibited accelerated senescence even under favorable growth conditions. In addition, we proposed that autophagy is involved in hypersensitive response cell death, an immune response of plants. In contrast to the previously characterized *AtATG* genes, all of which are fertile, we found that deletion of *AtATG6/VPS30* resulted in male sterility. Detailed microscopic observations have revealed that *AtATG6/VPS30* is essential for pollen germination (Figure 4). *AtATG6/VPS30* was able to restore vacuolar protein sorting as well as autophagy in yeast *atg6/vps30* mutant. Results have suggested that AtAtg6/Vps30 not only functions in autophagy but also has an autophagy-independent role, possibly in vesicle trafficking, which is responsible for pollen germination.

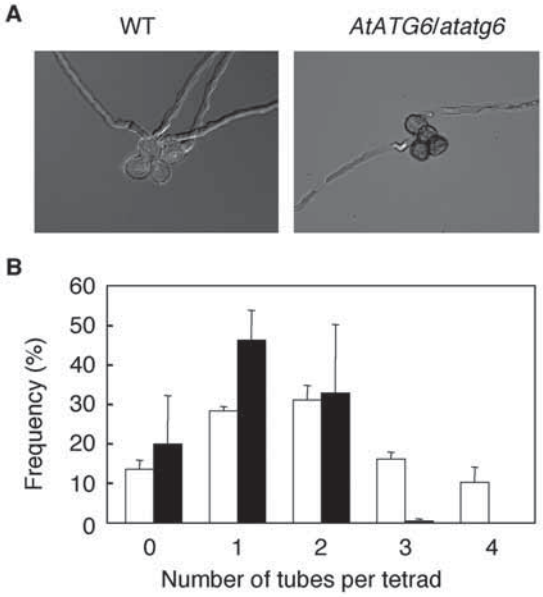


Figure 4. *AtATG6* is essential for pollen germination. (A), *In vitro* pollen germination of *AtATG6/atatg6* heterozygotes in the *qrt* background (right) and wild type (*qrt*, left). (B), Frequency (%) of wild type (*AtATG6/AtATG6/qrt/qrt*, open bars) and heterozygous mutant (*AtATG6/atatg6/qrt/qrt*, closed bars, $n > 500$) tetrads with zero to four pollen tubes. The values shown are the means of three different experiments (±SD).

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Original papers

Amar, N., Lustig, G., Ichimura, Y., Ohsumi, Y., and Elazar, Z. (2006). Two newly identified sites in the ubiquitin-like protein Atg8 are essential for autophagy. EMBO Rep. *7*, 635-642.

Inoue, Y., Suzuki, T., Hattori, M., Yoshimoto, K., Ohsumi, Y., and Moriyasu, Y. (2006). *AtATG* genes, homologs of yeast autophagy genes, are involved in constitutive autophagy in *Arabidopsis* root tip cells. Plant Cell Physiol. *47*, 1641-1652.

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Laboratory OF Cell Structure

Associate Professor: *OGAWA, Kazuo*

Dynein is a molecular motor that carries cargo in the direction of the centriole or basal body from the cell periphery along the microtubules in cells. The heavy chain is the dynein motor subunit, composed of three domains: stem, head, and stalk. The targeting of dynein to specific cargo may be related to the NH_2-terminal third constituting the ìstemî domain, where amino acid sequences are different between the two dynein families i.e., cytoplasmic and axonemal dynein. The COOH-terminal two-thirds, which constitute the ìheadî domain, exhibit sequence conservation even in distantly related species. The ìstalkî possesses an extended flexible structure which binds to microtubules in an ATP-dependent manner. Thus, dynein binds to microtubules at the "stalk" domain and to cargo at the "stem" domain.

The mechanism by which cytoplasmic and axonemal dyneins target the cargo has been gradually made clear in terms of the molecules concerned. Cytoplasmic dynein is linked with a dynactin complex containing at least 10 proteins. Via the dynactin complex it targets a receptor of cargo membranes. In flagellar and ciliary axonemes, outer and inner dynein arms are projected from the A-subfiber of peripheral doublet microtubules. These bind to the B-subfiber of neighboring doublet microtubules in an ATP-dependent manner. In this case, the A-subfiber corresponds to their cargo. Axonemal dynein barely detaches from the cargo, A-subfiber, while the cargo of cytoplasmic dynein is thought to be detached when it arrives at the cell center to recruit another motor. Thus, the targeting mechanism is different between the two dynein families.

When the Triton-model sperm are exposed to a high salt solution containing 0.5 M KCl or NaCl, the outer dynein arm detaches from the A-subfiber. Model sperm without an outer dynein arm swim with half the beat frequency of control sperm. Since the extracted sperm are able to recover normal beat frequency by both mixing with the extract and lowering the ionic concentration of the mixture, a high salt extract might contain certain proteins necessary for correct positioning of the outer dynein arm as well as certain scaffold proteins needed to mediate the binding of the outer dynein arm onto the A-subfiber. During the course of characterizing such proteins in high salt extract of the axonemes in sea urchin sperm, we found a novel protein with a molecular mass of 58 kDa which was designated ap58. Immuno-electron microscopy using antibodies raised against recombinant ap58 revealed gold-particles at ~25 nm repeats along the length of the axoneme coinciding with the repeat of the outer dynein arm (longitudinal sections of Figure 1). Thus, ap58 in situ binds to the outer dynein arm.

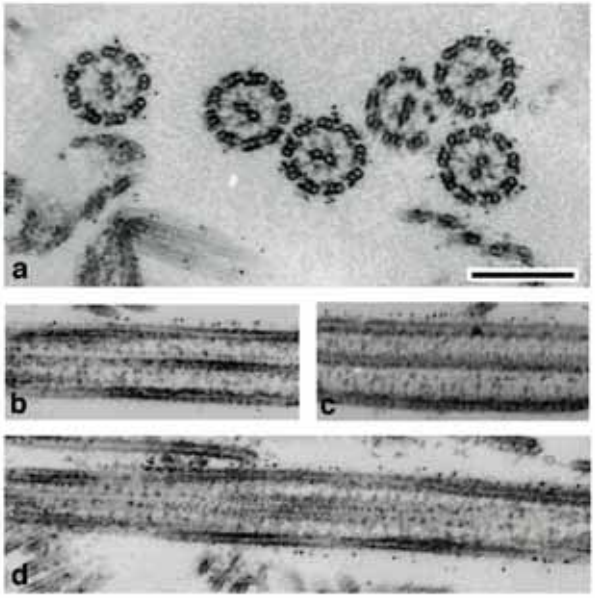

Figure 1. Immuno-gold localization of ap58 in the axonemes of sea urchin sperm. Cross section (a) and longitudinal images (b-d) are shown. The scale bar in (a) represents 200 μm for all images (Ogawa and Inaba).

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Original papers

Ushimaru, Y., Konno, A., Kaizu, M., Ogawa, K., Sato, N., and Inaba, K. (2006). Association of a 66 kDa homolog of *Chlamydomonas* DC2, a subunit of the outer arm docking complex, with outer arm dynein of sperm flagella in the Ascidian *Ciona intestinalis*. Zool. Sci. *23*, 679-687.

Hozumi, A., Satouh, Y., Makino, Y., Toda, T., Ide, H., Ogawa, K., King, S.M., and Inaba, K. (2006). Molecular characterization of *Ciona* sperm outer arm dynein reveals multiple components related to outer arm docking complex protein 2. Cell Motil. Cytoskel. *63*, 591-603.

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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 it has been postulated that *Notch* plays a role in cell fate decision by mediating cell-cell interactions, we are trying to find out the 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 an interface with the maternal deciduas. The polar trophectoderm gives rise to cells of the chorion, and the ectoplacental cone. They produce the labyrinthine and spongiotrophoblast layers, respectively. While maternal red blood cells begin to perfuse into trophoblast cell layers and reach the labyrinthine layer by E9.5, the invasion of embryonic allantoic mesenchyme into the labyrinthine layer and the differentiation of fetal red blood and endothelial cells which line the 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 likely uninvolved 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 compromisedin the 4N chimeric placenta. These results indicate that *Notch2* promotes vasculogenesis. Thus, *Notch2* is not 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.

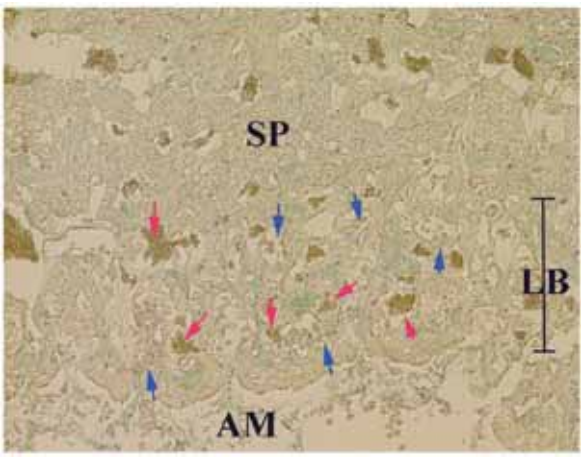

Figure 1. Expression of *Notch2* gene in developing mouse placenta. A cryo-section of *Notch2*$^{+/LocZ}$ placenta at E10.0 was incubated in X-gal staining solution. Trophoblast in maternal vascular bed side expresses the gene, but not in allantoic mesenchyme (AM) side in labyrinth layer (LB). The expression 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.

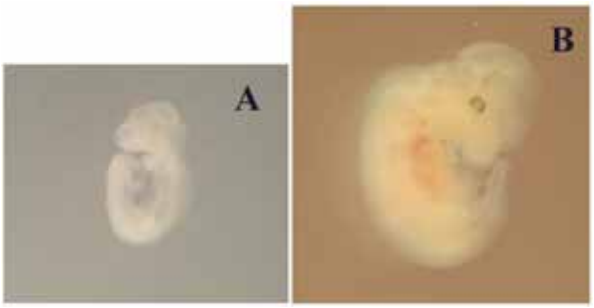

Figure 2. Survival of *Notch2* mutant embryo in a chimera with tetraploid wild type embryo. The mutant obtained in the intercrosses by the heterozygotic mice shows developmental retardation at E10.5 (A) and die before E11.5. The mutant is able to survive in a chimera with wild type tetraploid embryo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

DIVISION OF REPRODUCTIVE BIOLOGY

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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 medaka (*Oryzias latipes*) to investigate the molecular mechanisms of sex determination and Nile tilapia (*Oreochromis niloticus*) to investigate gonadal sex differentiation.

Medaka possess 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. The involvement of *DMY* in the process of sex-determination was first confirmed by the advent of two naturally occurring sex-reversed mutants, in which *DMY* was either truncated or expressed at reduced levels. More recently, we performed over-expression experiments using the *DMY* genomic region or *DMY* cDNA, which can induce testis development in genetic females (XX) (Figure 1). A 117-kb genomic DNA fragment carrying *DMY* was able to induce testis differentiation and subsequent male development in XX medaka. In addition, over-expression of *DMY* cDNA under the control of the CMV promoter also caused XX sex reversal. These results demonstrate that *DMY* is sufficient for male development in medaka, and suggest that the functional difference between the X and Y chromosomes in medaka is a single gene. These data indicate that *DMY* is an additional sex-determining gene in vertebrates.

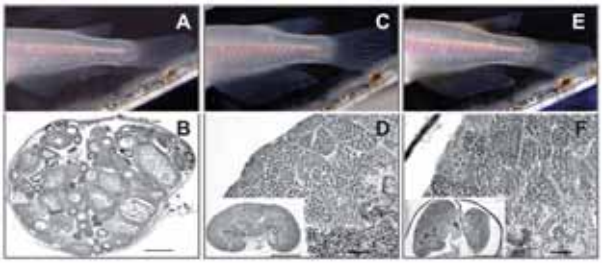

Figure 1. Phenotypic analyses of *DMY* transgenic adult medaka. Transgenic medaka include: a white (XX) female (A) with an ovary (B), a white (XX) male (C) with testes (D), and an orange-red (XY) male (E) with testes (F).

We also used two fundamentally different ways of sex reversal, *DMY* knock down and estradiol-17β (E2) treatment, to determine the possible function of DMY during early gonadal sex differentiation in XY medaka. Our findings revealed that the mitotic and meiotic activities of the germ cells in *DMY* knock-down XY larvae (the day of hatching) were identical to that of the normal XX larvae, suggesting the microenvironment of these XY gonads to be similar to that of the normal XX gonad, where *DMY* is naturally absent. Conversely, E2 treatment failed to initiate mitosis in the XY gonad, possibly due to an active *DMY*, even though it could initiate meiosis. The present study is the first to prove that the germ cells in the XY gonad can resume the mitotic activity, if *DMY* was knocked-down.

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 gonads 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 then investigated the plausible role of Foxl2 in ovarian differentiation through transcriptional regulation of aromatase gene (*Cyp19a1*), using mono-sex tilapia fry. *Foxl2* expression, like that of *Cyp19a1*, is sexually dimorphic in gonads prior to the occurrence of morphological sex differentiation, co-localizing with *Cyp19a1* and *Ad4BP/SF-1* in the stromal cells and interstitial cells in gonads of normal XX and sex-reversed XY fish. Under *in vitro* conditions, Foxl2 binds to the sequence, ACAAATA in the promoter region of the *Cyp19a1* gene directly through its forkhead domain (FH),

and activates the transcription of *Cyp19a1* with its C-terminus. Foxl2 can also interact through the FH with the ligand binding domain of Ad4BP/SF-1 to form a heterodimer and enhance the Ad4BP/SF-1 mediated *Cyp19a1* transcription. Disruption of endogenous Foxl2 in XX tilapia by over-expression of its dominant negative mutant induces varying degrees of testicular development with occasional sex reversal from ovary to testis. Such fish display reduced expression of *Cyp19a1* as well as a drop in the serum levels of E2 and 11-ketotestosterone (11-KT) (Figure 2). Although the XY fish with wild type tilapia *Foxl2* over-expression never exhibited a complete sex reversal, there were significant structural changes, such as tissue degeneration, somatic cell proliferation and induction of aromatase, with increased serum levels of E2 and 11-KT. These results suggest that Foxl2 plays a decisive role in the ovarian differentiation of tilapia by regulating aromatase expression.

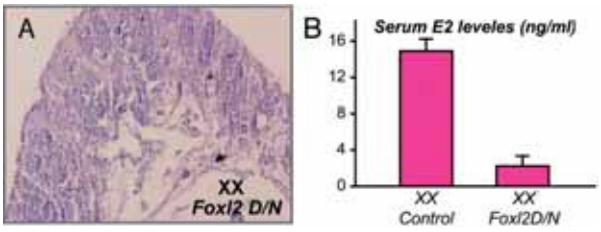

Figure 2. Transgenic XX tilapia with over-expression of Foxl2 dominant negative mutant (D/N) showing a complete sex change from ovary to testis (A). Serum E2 levels in XX tilapia with over-expression of Foxl2 D/N were 7 times lower than that of the XX control fish (B).

In XY tilapia 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 sex change 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. As sex change in both directions can be socially manipulated, *T. okinawae* provides an excellent animal model to investigate the molecular mechanisms of sex change.

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 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 3). 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.

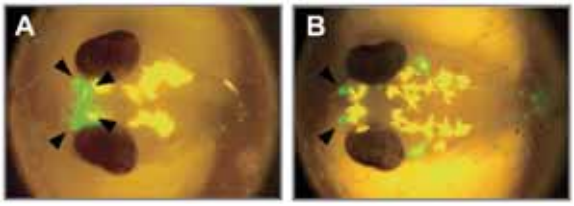

Figure 3. Knockdown of *kal1* in the transgenic medaka that express GFP in gnrh1 (A) and gnrh3 (B) neurons resulted in the deficient migration of GnRH neurons (arrowheads).

IV. Endocrine regulation of oocyte maturation

A period of oocyte growth is followed by a process called oocyte maturation (the resumption of meiosis) which occurs prior to ovulation and 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 in 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: gonad-stimulating substance (GSS), 1-methyladenine (maturation-inducing hormone, MIH), and maturation-promoting factor (MPF) in starfish, and gonadotropin (LH), 17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-DP) (MIH), and MPF in fish.

We recently purified GSS 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 (M. Mita, M. Yoshikuni *et al.*, unpublished).

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 demonstrated that the 26S proteasome initiates cyclin B degradation through the first cut of its NH_2 terminus at lysine 57.

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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, mammals carrying XY and XX sex chromosomes develop the testis and ovary, respectively. This gonad 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 gonad 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 gonad 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 gonad differentiation. However, it remains to be elucidated how the genes are expressed by upstream regulators. Studies considering this aspect of sex differentiation are quite important in order 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: the signal for male (testicular) differentiation and the signal for female (ovarian) differentiation. It is possible to assume that the signals are transcriptional activities driven by the transcription factors expressed in the sexually differentiating gonads or other types of growth factors. This divisionís research has focused primarily on the transcriptional control of the genes implicated in gonad sex differentiation.

I. Structure and Function of *Ad4BP/SF-1* gene locus

Ad4BP/SF-1 is expressed in the testicular Leydig and Sertoli cells, ovarian theca and granulosa cells, adrenocortical cells, pituitary gonadotropes, and ventromedial hypothalamic nucleus, and the expressions are tightly controlled during the tissue development processes. It is well accepted that the spatial and temporal control of gene expression is essential for the establishment of cell fates, and gene transcription is thought to be controlled through appropriate interaction between enhancers and the basal promoter. In the case of *Ad4BP/SF-1*, the tissue-specific enhancers should be localized somewhere in the gene locus and functionally correlated with the basal promoter localized upstream of the first exon. Evidently, functional interaction between the enhancers and the basal promoter is one of the fundamental elements required for tissue specific expression. Therefore, it was expected that the primary structure of the enhancer would provide information to understand the mechanisms underlying tissue development and gonad sex differentiation. Based on this concept, we have conducted transgenic mouse assay (TG mouse assay) with fragments prepared from the *Ad4BP/SF-1* gene locus and bacterial *lacZ* gene as the reporter. As indicated in Figure 1, we analyzed whether 20-30 kb long fragments prepared from a mouse BAC clone contain enhancer sequences or not. Two fragments, cGcnf5 and cIA3, gave lacZ signal in the fetal adrenal, ventromedial hypothalamus, and pituitary. Positive fragments were further examined through the TG mouse assays using deletion mutants. Consequently, we confirmed the location of the enhancer sequences for the fetal adrenal cortex in the fourth intron while those for the pituitary gonadotrope and ventromedial hypothalamic nucleus were located in the sixth intron.

In general, it is well known that functional genomic sequences such as exon, basal promoter, and splicing site are structurally conserved among animal species. We asked whether the functional enhancer sequences are conserved among vertebrate animal species. As expected, sequence comparisons revealed that the sequences were actually conserved in such mammalian species as human, mouse, rat, etc. Since the conserved region should contain core sequences to be recognized by certain transcription factors, the conserved regions were further analyzed by introducing nucleotide substitution. These constructs were subjected to the TG mouse assays and thus the functionally active core sequences composing the tissue-specific enhancers were finally identified.

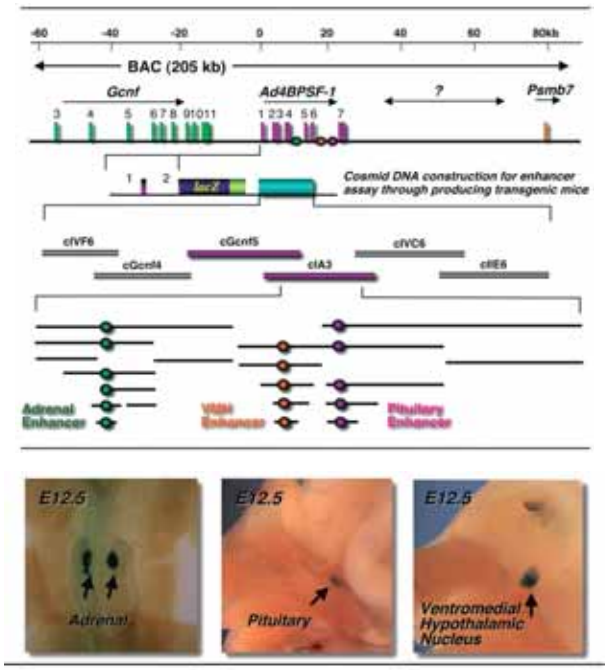

Figure 1, Identification of enhancers for the fetal adrenal, ventromedial hypothalamus (VMH), and pituitary of the mouse Ad4BP/SF-1 gene. (upper panel) The Ad4BP/SF-1 gene consists of seven exons (red boxes). Two other genes, Nr6a1 (green boxed) and Gpr144 (region indicated by?), are localized 5í upstream and 3í downstream of the Ad4BP/SF-1 gene, respectively. Positions and structures of these genes were obtained from the NCBI database (Entrez Gene). A 6-kb fragment of the Ad4BP/SF-1 promoter region from the initiation methionine in exon 2 was used to generate the basal vector by ligation to the bacterial lacZ gene followed by SV40 poly (A) signal. Various DNA fragments were inserted downstream of lacZ, and subjected to transgenic mouse assays. Among the clones covering the Ad4BP/SF-1 locus, cIA3 induced lacZ expression in fetal adrenal, ventral diencephalon (VMH), and pituitary, while cGcnf5 induced lacZ expression only in the fetal adrenal. cIVC6 induced no lacZ expression. These active fragments were further fragmented into smaller pieces and subjected to the TG mouse assays. Finally, the enhancers for the fetal adrenal was localized in the fourth intron, while those for the VMH and pituitary were in the sixth intron. (lower panel) Representative expression patterns of lacZ at embryonic day 12.5 are shown.

II. Two-Step Regulation by Fetal Adrenal Enhancer

In order to identify the sequences responsible for the fetal adrenal enhancer function, we compared the nucleotide sequences of the enhancer regions of mice and humans. Among the sequences conserved between the two animals, we noted the presence of two potential Ad4BP/SF-1 binding sites. When analyzed by electrophoretic mobility shift assays, Ad4BP/SF-1 associated with the potential binding sites. Since Ad4BP/SF-1 activates gene expression by binding to recognition sites, the presence of the Ad4 sites in the fetal adrenal enhancer suggested that Ad4BP/SF-1 has a role in the transcriptional regulation of gene expression in the fetal adrenal gland. To examine this hypothesis, we generated TG mice harboring a construct carrying mutations in the two Ad4 binding sites. When we examined TG mouse fetuses at E11.5, lacZ expression in the mutant construct was similar to the wild-type construct. However, at E17.5, the lacZ signals disappeared from the TG fetuses with the mutated construct. To determine whether Ad4 sites were responsible for lacZ expression at E17.5, mutations were introduced in both Ad4 sites. TG assays with these constructs revealed that the Ad4 sites are essential for this expression pattern, and we subsequently examined the enhancer activities of constructs with a single site mutation. Since neither mutation led to the disappearance of the lacZ signal, a single site is sufficient to drive lacZ expression in the fetal adrenal gland. These results strongly suggest that Ad4BP/SF-1 binding to the Ad4 sites in the fetal adrenal enhancer participate in the autoregulation of *Ad4BP/SF-1* gene expression in the adrenal cortex at later stages of fetal development.

The data above suggests that Ad4BP/SF-1 maintains its own expression at later stages of development, but it remains unclear how Ad4BP/SF-1 expression is controlled prior to the maintenance phase of transcription. To address this question, we attempted to identify functional *cis*-elements other than the Ad4 sites present in the fetal adrenal enhancer. When the sequence of this DNA fragment was examined in detail, we found potential binding sites for the Pbx/Prep and Pbx/Hox heterodimers present in the upstream region proximal to the Ad4 site (Figure 2). Since both sites are conserved in human and chick, we examined whether those factors are expressed in the adrenal primordium. RNAs prepared from the isolated adrenal primordium were used for RT-PCR analyses to examine candidate gene expression.

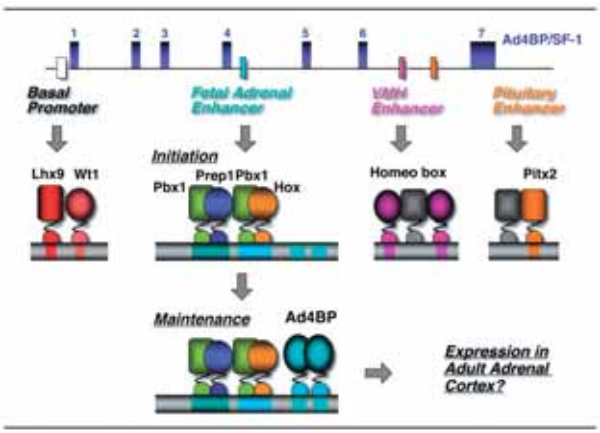

Figure 2, Schematic presentation of structures and functions of the tissue-specific enhancers and basal promoter of the Ad4BP/SF-1 gene. Transcription factors, Lhx9 and Wt1, are thought to bind the basal promoter, while Pbx1, Prep1, and Hox proteins are able to bind the fetal adrenal enhancer as a ternary complex to initiate transcription at the adrenal primodial cells. At a later stage, the gene product, Ad4BP/SF-1, binds to the enhancer to maintain the expression. Homeobox-containing proteins together with unidentified protein bind to VMH enhancer while Pitx2 acts as an essential factor to drive the pituitary enhancer. Although still unclear, a factor functionally correlated with Pitx2 is required for the enhancer function.

As expected, RT-PCR revealed that all candidate factors were successfully detected in the early adrenal primordium. Thus, we examined whether the Pbx/Prep and/or Pbx/Hox binding sites are functional or not. When either the Pbx/Hox or Pbx/Prep binding sites was mutated, weak lacZ signals were still observed in the fetal adrenal tissue. As expected, when both the Pbx/Hox and Pbx/Prep sites were mutated, lacZ expression was completely absent from the adrenal primordia. Importantly, this transcriptional activity driven by the Pbx/Hox and Pbx/Prep sites was active even though Ad4 sites were mutated.

Hox transcription factors direct the patterning of a variety of structures during the embryonic development of vertebrates and invertebrates through regulating numerous target genes. Based on the expression profile and binding specificity, *Hoxb5*, *Hoxb9*, *Hoxc5*, and possibly *Hoxc6* were thought to regulate the *Ad4BP/SF-1* expression in the fetal adrenal. The adrenal cortex is known to be derived from a certain part of the intermediate mesoderm lying along the anterior to posterior axis. To specify the adrenal region, these *Hox* gene products are thought to induce Ad4BP/SF-1 expression at that particular region of the mesoderm. As it has been established that *Hox* genes control anterior to posterior axial identity through regulating target gene expression, the location of the adrenal cortex could be determined by combined expression of the particular set of the *Hox* genes.

III. Asymmetric ovarian development in birds

Mammalian gonads develop bilaterally, and this is achieved through the orchestrated action of a number of genes. Many of these genes have been identified through the study of knockout mice as well as patients suffering from gonad developmental abnormalities. However, no identified gonad defects exhibit clear LñR asymmetry, and no genes involved in gonad development are expressed asymmetrically in the L-R axis. In contrast to the situation with mammals, most female birds develop ovaries only on their left side, while males develop bilateral testes. During the early sexually-indifferent stage, chick embryonic gonads show no obvious morphological LñR asymmetry, and they consist of two components, the cortex and medulla. After sexual differentiation, testicular development occurs bilaterally in the male (genetically ZZ). The testicular cords appear in the medulla where Sertoli and Leydig cells differentiate, while the cortex regresses and eventually disappears. In female birds (genetically ZW), the left cortex proliferates and develops into the ovary, while the right cortex disappears. Such asymmetric gonad development has not been described in other vertebrates, and the process regulating avian gonad development is interesting from both evolutionary and developmental perspectives. We have studied the molecular mechanisms for this asymmetric ovarian development in collaboration with Prof. Yoshioka (Hyogo Univ. of Teacher Education).

The study revealed that homeobox gene *PITX2* is expressed asymmetrically in the left presumptive gonad and this asymmetric expression induces the asymmetric expression of the retinoic-acid-catabolizing and -synthesizing enzymes, *CYP26A1* and *RALDH2*, respectively. Subsequently, retinoic acid suppresses the expression of *Ad4BP/SF-1* in the right ovarian primordium. Conversely, Ad4BP/SF-1 expressed in the left ovarian primordium asymmetrically upregulates *cyclin D1* to stimulate cell proliferation. Leftñright nodal asymmetry is transferred to the left lateral plate mesoderm through induction of *PITX2*. Interestingly, avian but not mouse *PITX2*/*Pitx2* expression expands throughout the left presumptive gonad derived from the lateral plate mesoderm, most likely ultimately leading to the observed ovarian asymmetry. We thus provided a mechanism linking early embryonic *PITX2* expression with subsequent asymmetric visceral organ development with particular emphasis on bird ovarian development.

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Zubair, M., Ishihara, S., Oka, S., Okumura, K., and Morohashi, K. (2006). Two-step regulation of *Ad4BP/SF-1* gene transcription during fetal adrenal development; initiation by a Hox-Pbx1-Prep1 complex and maintenance via autoregulation by Ad4BP/SF-1. Mol. Cell. Biol. *26*, 4111-4121.

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DIVISION OF MORPHOGENESIS

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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 and transcription factors. We address this problem using several model animals, including frogs, flies and ascidians, and by 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 the morphogenesis of early embryos, involving dynamic cell migration and change in embryo shape. 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 ìconvergent extension (CE)î. As convergent extension begins, cells are polarized and aligned mediolaterally, followed by the mutual intercalation of the cells that acquired planar cell polarity (PCP). In the regulation of convergent extension, several growth factor signaling pathways including Wnt/PCP pathway are implicated.

In addition to the components of the Wnt/PCP pathway, we have recently found that activin/nodal members of the TGF-β superfamily induce expression of two genes regulating cell adhesion during gastrulation: FLRT3, a putative type I transmembrane protein containing extracellular leucine-rich repeats, and the small GTPase Rnd1. Both loss- and gain-of-function analyses of FLRT3 and Rnd1 show that these proteins physically interact and modulate cadherin-mediated cell adhesion during early embryogenesis by regulating cadherinís subcellular localization. As numerous studies have linked aberrant expression of small GTPases, adhesion molecules such as cadherins and TGF-β signaling to oncogenesis and metastasis, it is intriguing that this FLRT3-Rnd1 pathway controls cell behavior and tissue morphogenesis in both embryos and adults.

More recently, we have found that a novel FGF-responsive gene encoding ankyrin repeats domain protein 5 (ANR5) has an essential role in regulating cell adhesion. In the *Xenopus* gastrula, reduced levels of xANR5 perturbed cell adhesion and tissue separation, processes that were regulated by a cadherin family protein, Paraxial Protocadherin (PAPC). We also showed that xANR5 physically interacted with PAPC and regulated PAPC-dependent morphogenetic processes and signaling pathways. Interestingly, the polarized localization of the xANR5 protein in dorsal mesodermal cells was completely disrupted by drugs that prevent intracellular calcium signaling, and mutations in xANR5ís predicted calcium-binding domains provoked its translocation into the nucleus. On the basis of these observations, we propose that the intracellular calcium signal regulates the localization of xANR5, which in turn regulates the adhesive properties of mesodermal cells during *Xenopus* gastrulation.

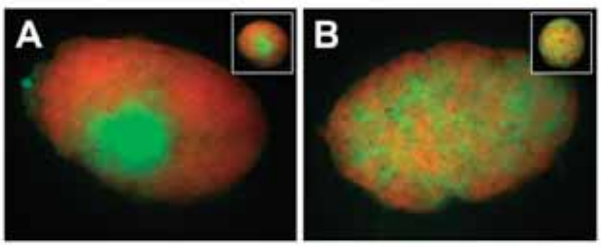

Figure 1. Cell sorting by PAPC requires ANR5 function. Expression of PAPC promotes homophilic cell-to-cell adhesion and resulting cell sorting of PAPC-expressing cells shown by red (**A**). However, depletion of ANR5 by an antisense Morpholino oligonucleotide disrupts the cell adhesion and thus PAPC-expressing cell are intermingled with non-expressing cells shown as green (**B**).

II. Protein ubiquitination involved in the regulation of gastrulation movements

Protein ubiquitination is an important mechanism to regulate the stability and/or subcellular localization of target proteins. It has previously been unknown whether or not ubiquitination is involved in the regulation of gastrulation movements in *Xenopus* embryos. We found that two distinct ubiquitination systems play crucial roles in this process. One is essential for the signal transduction

of the noncanonical Wnt pathway that regulates cell polarity and cell migration during gastrulation. We identified a novel ubiquitin ligase complex, consisting of Rab family GTPase (Rab40) and Cullin. This complex is localized in the Golgi apparatus and essential for the regulation of the localization of Dishevelled, which plays the pivotal role in the Wnt signaling pathway (Figure 2). Loss-of-function of this ubiquitin ligase resulted in the inhibition of the Wnt pathway and caused a severe gastrulation-defective phenotype in *Xenopus* embryos. We also identified the other ubiquitination system that ubiquitinates and destabilizes Paxillin, one of the focal adhesion complex components. We found that the focal adhesion plays an important role in convergent extension and its stability must be tightly regulated. Interestingly, the Wnt signaling pathway increased Paxillin ubiquitination and destabilized focal adhesions, indicating that the Wnt pathway regulates convergent extension movements through regulating the stability of focal adhesions. These findings implicate previously unidentified ubiquitin systems into the morphogenetic process during vertebrate embryogenesis.

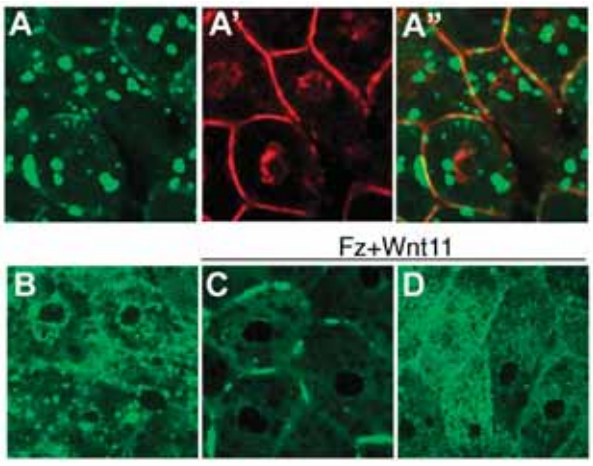

Figure 2. Localization and function of Rab40/Cullin ubiquitin ligase. **(A)** localization of Rab40-GFP in the *Xenopus* embryonic ectodermal cells. **(A')** the plasma membrane and the nuclei. **(A")** merged image. **(B, C and D)** Localization of Dishevelled-GFP **(B)** It is localized in the cytoplasmic vesicular structures without Wnt signaling, (**C**) Coexpression of Frizzled and Wnt11 activates Wnt signaling and translocates Dishevelled to the plasma membrane, (**D**) Coinjection of Rab40 antisense morpholono oligo inhibits the translocation of Dishevelled in response to Wnt signaling

III. Involvement of a fibrinogen-like protein in notochord-dependent dorsal patterning of the nervous system in *Ciona intestinalis* 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-fibrn*) of the *Drosophila Scabrous* gene is one of the downstream target gene of *Brachyury*. While the gene (*Ci-fibrn*) 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 (Figure 3). Knockdown of Ci-fibrn function resulted in failure of convergent extention of notochord cells and differentiation of neuronal cells and axon guidance. Correct distribution of Ci-fibrn protein is dependent on the Notch signal delivered by the overlying CNS. Disturbance of Ci-fibrn distribution caused ventral positioning of neuronal cells and abnormal track of axon extension. Therefore, it is highly likely that the interaction of the notochord-based fibrinogen-like protein and neural tube-based Notch signal is essential for the dorsal patterning of the nervous system or for the establishment of the chordate body plan.

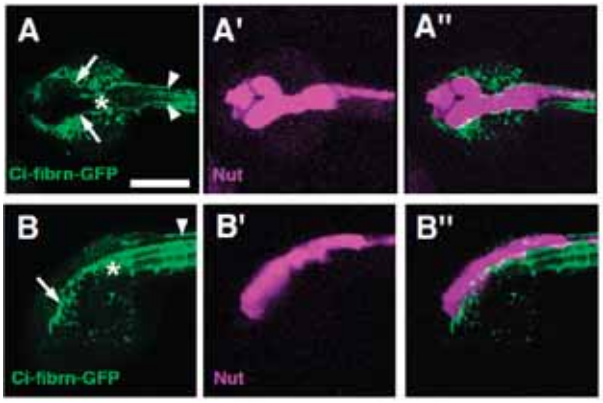

Figure 3. Expression and distribution of Ci-fibrn into anterior and dorsal regions of the trunk, surrounding the CNS, at the late tailbud stage in (**A**) dorsal and **(B)** lateral view. (**A, B**) Expression of GFP reporters in an embryo injected with *Ci-Bra*(notchord promoter):fibrn:EGFP (green), **(A', B')** *Ci-Nut*(CNS promoter):RFP (magenta), and **(A", B")** merged photomicrograph. Arrows indicate the border of the sensory vesicle; the asterisk indicates the anterior border of the notochord; and arrowheads indicate Ci-fibrn underneath elongated axons. Scale bar, 50 μm.

IV. Functional and genetical study of epigenetic regulator *tonalli* and a putative translation regulator *dNAT1* in *Drosophila* body patterning

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 confirmed that most of Hox genes expression is significantly reduced in the *tna* loss-of-function mutant. *tna* encodes 1109 amino-acid protein containing single SP-RING motif, which is thought to be involved in protein SUMO conjugation process. To visualize TNA protein we generate specific antibody to TNA. One of the antibodies to TNA specifically detects TNA protein in vitro and in vivo. TNA protein predominantly localized in the nucleus and we also observed TNA localization at substantial level in the cytoplasm. TNA localizes specific chromosomal region of the salivary gland chromosomes. This result is consistent with the hypothesis by which TNA participates in an essential role in the TRX-group function.

Translational regulation also participates in major roles the early patterning of *Drosophila* embryo. One of the eIF4G family proteins NAT1/p97/DAP5 has been identified as a novel translational repressor. To elucidate in vivo function of the *NAT1* we isolated *Drosophila NAT1* (*dNAT1*) mutant by reverse-genetical approach. We isolated four transposon insertion mutants as well as a 1.4 kb deletion alleles corresponding to the *dNAT1* locus. One of the P-element insertion lines $dNAT1^{GS1}$ shows severe embryonic lethality with abnormal germband extension defect. This lethality and morphological phenotype were completely rescued by introduction of the 12 kb *dNAT1* genomic DNA fragment by germ line transformation. Expression of some of the segement polarity genes were apparently abnormal in the $dNAT1^{GS1}$ mutant (Figure 4). We are currently trying to identify the molecule(s) that is regulated by dNAT1 at a level of tranlational regulation.

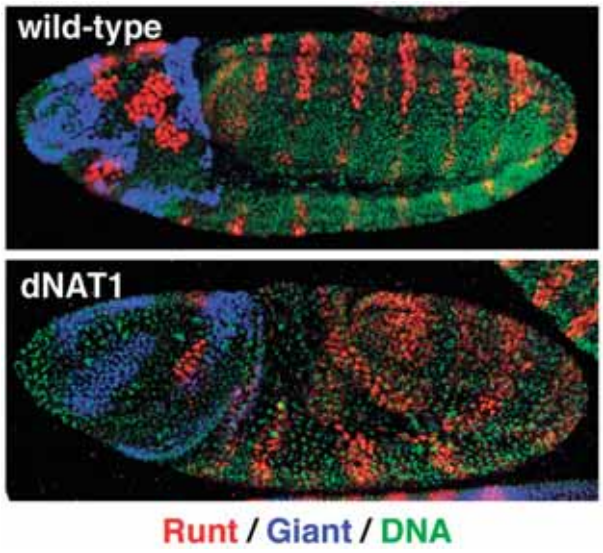

Figure 4. *Drosophila* stage-11 embryo stained with antibody to Runt (red), Giant (blue) and DNA dye (green). Embryo shows in anterior left and dorsal up. Germband is fully extended at this stage in the wild-type animals. $dNAT1^{GS1}$ mutant animal shows defective germband extension and abnormal expression in some of the segmentation marker proteins.

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Takada, R., Satomi, Y., Kurata, T., Ueno, N., Norioka, S., Kondoh, H., Takao, T., and Takada, S. (2006). Monounsaturated fatty acid modification of Wnt protein: its role in Wnt secretion. Dev. Cell. *11*, 791-801.

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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 of 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 development. In *Drosophila*, this cytoplasm is localized in the posterior pole region of eggs, and partitioned into the germline progenitors, or pole cells.

I. The role of maternal Nanos protein

In many metazoans, the germline forms early in development and is maintained until the differentiation of gametes in the adult gonads. Although genetic analyses have identified several mutations that eliminate pole cells, how pole cells are maintained during development is unclear.

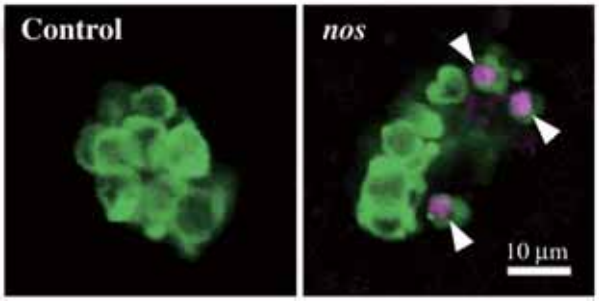

Figure 1. Nanos prevents apoptosis in pole cells. Confocal images of the pole cells in control (left) and *nos* (right) embryos at stage 13, stained with TUNEL labeling (magenta) and an antibody against Vas (green). Arrowheads show TUNEL-positive pole cells.

Several components of germ plasm have been identified. One of these components is maternal *nos* RNA, which is enriched in germ plasm during oogenesis and translated *in situ* to produce Nos protein after fertilization. While Nos is present transiently in the posterior half of embryos during the preblastoderm stage and is required for abdominal patterning, Nos in the germ plasm is inherited by pole cells at the blastoderm stage and is detectable in these cells throughout embryogenesis. In the absence of maternal Nos, pole cells undergo apoptosis during their migration to the embryonic gonads (Figure 1). Although Nos also plays important roles in repressing mitosis, somatic gene expression and somatic cell fate in pole cells, the primary role for Nos appears to be repressing apoptosis in the germline, because Nos is an evolutionarily conserved protein that is required for germline survival.

Nos is known to repress translation of specific RNAs that contain a discrete sequence called the Nos response element (NRE). In abdominal patterning, Nos represses the translation of maternal *hunchback* (*hb*) RNA. This repression requires NRE sequence. In pole cells, Nos represses translation of maternal *cyclin B* RNA that contains an NRE-like sequence within its 3'UTR. This repression results in the mitotic quiescence of pole cells during their migration to the gonads.

Apoptosis is mediated by caspases, a family of cysteine proteases that cleave diverse substrates to destroy cellular structure and integrity. Critical regulators of apoptosis function by antagonizing inhibitor of apoptosis protein (IAP) that directly blocks caspase action. In *Drosophila*, four proapoptotic genes, *reaper* (*rpr*), *head involution defective* (*hid*), *grim* and *sickle* (*skl*), encode members of a family of related proteins that bind to and inactivate the IAP. They are also referred to as RHG genes. Three of these RHG genes, *rpr*, *hid* and *grim*, are encompassed by a genomic region on the third chromosome, *H99*. Previously we have reported that deletion of *H99* region, *Df(3L)H99*, represses apoptosis of pole cells lacking Nos, consistent with a role for Nos in an apoptotic pathway that involves the RHG gene(s) from the *H99* region.

In this study, we demonstrate that maternal Nos represses apoptosis of pole cells by suppressing translation of *hid* RNA in an NRE-dependent manner. In the absence of Nos activity, translation of *hid* mRNA yields a protein product that induces apoptosis. In addition, we provide evidence that a maternally-provided protein kinase, Tao-1, is required to induce apoptosis in *nos* pole cells by promoting *skl* expression. Maternal *tao-1* RNA is enriched in the germ plasm and inherited by pole cells. Tao-1-dependent *skl* expression sensitizes pole cells to induction of apoptosis by *hid*. We propose that pole cells express the RHG genes *hid* and *skl*, and become competent to undergo apoptosis during normal development. However, maternal Nos represses *hid* translation to inhibit apoptosis of pole cells for their survival. These findings provide the first evidence that the germline is maintained through the regulated expression of RHG genes.

II. 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.

III. Molecular characterization of the embryonic gonads by gene expression profiling

Within the embryonic gonads, distinct cellular events associated with germline development occur, such as resumption of germline proliferation, selection of the germline-stem cell, gonad morphogenesis and cellular communication between the germline and somatic cells. Recent studies have also revealed that the male germline stem cell niche is already specified in the embryonic gonad. Despite the importance of the embryonic gonad in the germline development, only limited information is available regarding which genes are expressed in the embryonic gonad although transcriptome data of adult testes and ovaries has accumulated. Thus, we attempted to identify the genes expressed within the embryonic gonads by a direct and comprehensive approach. In *Drosophila*, transcriptome analysis of individual organs and cell types has been hampered by the smallness of their size. To overcome this problem, we have developed an efficient method to isolate embryonic gonads by flow cytometry.

First, we generated a cDNA library from gonads purified from *Drosophila* embryos by fluorescence-activated cell sorting (FACS). Using this library, we catalogued the genes expressed in the gonad by Expressed Sequence Tag (EST) analysis. A total of 17,218 high-quality ESTs representing 3,051 genes were obtained. This corresponds to 20% of the predicted genes in the genome. The embryonic-gonad transcriptome is unexpectedly distinct from that of adult gonads and includes an extremely high proportion of retrotransposon-derived transcripts. We verified 101 genes preferentially expressed in the embryonic gonads by whole-mount *in situ* hybridization (Figure 2). Within this subset, 39 and 58 genes were expressed predominantly in germline and somatic cells, respectively, while 4 genes were expressed in both cell lineages. The gonad-enriched genes encompassed a variety of predicted functions. However, genes implicated in SUMOylation and protein translation, including germline-specific ribosomal proteins, are preferentially expressed in the

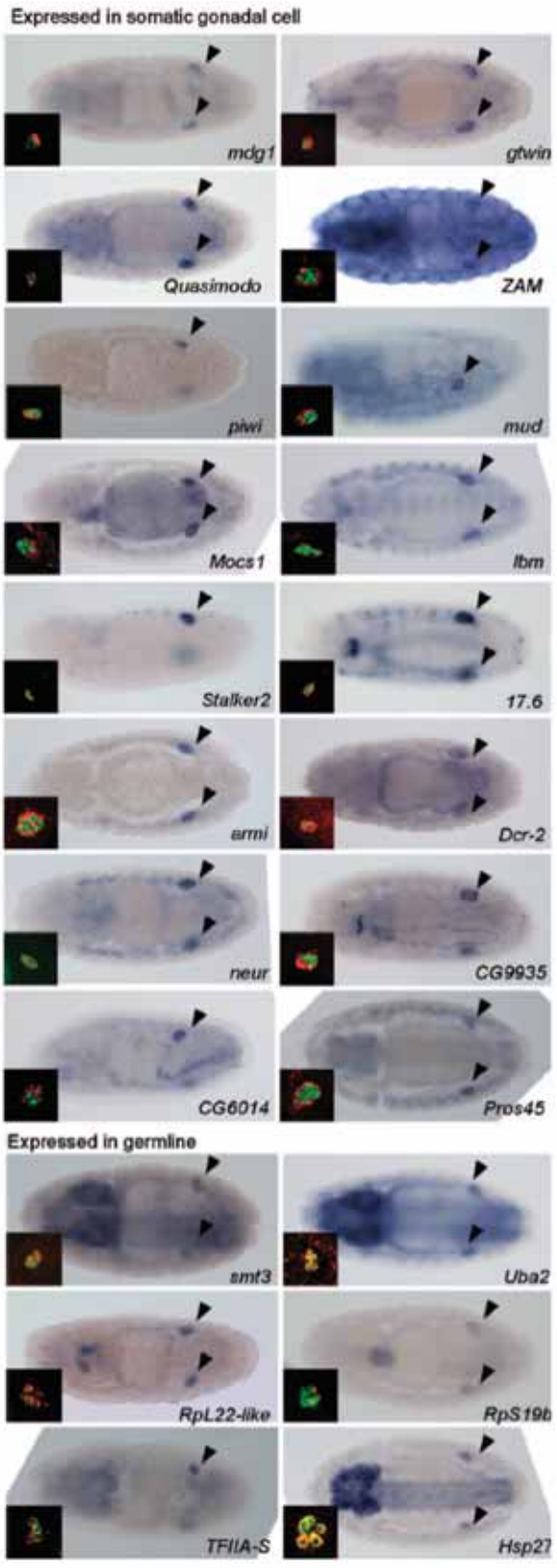

Figure 2. Spatial expression patterns of gonad-enriched transcripts. Embryos at stage 14-16 are shown with anterior to the left. Gonads are indicated by arrowheads. Inset in each panel provides the confocal microscopic image of the gonad double-stained with an antisense RNA probe for the indicated gene (red) and anti-Vasa (green), a germline marker.

germline, while the expression of various retrotransposons and RNA interference (RNAi)-related genes are more prominent in the gonadal soma. This transcriptome data is a resource for understanding the mechanism of various cellular events during germline development.

IV. Germline stem cell niche formation in male gonad

Stem cells possess the remarkable capacity to generate both daughter cells that retain a stem-cell identity and others that differentiate. Stem cells reside in dedicated cellular microenvironments, termed stem-cell niches, which dictate the stem cell identity, maintain the stem-cell population, and coordinate proper homeostatic production of differentiated cells. Recent studies have addressed molecular aspects of how niches define stem-cell identity and behavior through intercellular signaling. As these niches enable stem cells to maintain tissue homeostasis during development, growth, repair and aging, understanding the mechanisms that regulate formation of stem-cell niches during development is critical. These processes, however, remain largely uncharacterized.

The germline-stem-cell niche in *Drosophila* testes has emerged as a useful model system for studying stem cells. In the apical tip of the adult testes, the germline stem cells lie in intimate contact with somatic hub cells, known as the niche, which causes the stem cells to retain self-renewing potential. Germline stem cells divide to produce one daughter cell that remains associated with hub cells, while the other daughter cell detaches and initiates spermatogenesis.

Hub cells are derived from a subset of somatic gonadal cells (SGCs) that are located in the anterior region of male embryonic gonads. It has been reported that the antero-posterior cellular identities within the gonads is regulated by the homeotic genes, *abdominal-A* (*abd-A*) and *Abdominal-B* (*Abd-B*). However, how the formation of hub progenitors is restricted in the anterior of embryonic gonads remains elusive. Previous observations also suggest that the germline may be required for proper hub formation in male gonads. In the absence of germline cells, an expansion of the hub population is observed.

We demonstrate that a receptor tyrosine kinase, Sevenless (Sev), provides a cue to ensure that the niche develops in the anterior region of the male embryonic gonads. Sev is expressed by somatic cells within the posterior region of the gonads, and is activated by a ligand, Bride of sevenless (Boss), which is expressed by the germline, to prevent ectopic niche differentiation in the posterior gonadal somatic cells. Our findings provide the first evidence that signal transduction from germline to soma is essential for the proper development of a stem-cell niche.

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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 tightly regulated gradient formation of secreted signal proteins, including machinery to secrete these proteins, 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 the 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 ìsegmentation clockî. To gain insight into the molecular mechanism underlying this specific regionalization, we are also characterizing genes involved in somite segmentation.

I. Molecular mechanism to secret Wnt proteins

The Wnt family of secreted signal proteins plays a key role in numerous aspects of embryogenesis, as well as in carcinogenesis. Most Wnt proteins transmit signals locally, presumably since their secretion and transport are under tight control (Figure 1). Although the molecular mechanism underlying their secretion and transport remains largely unknown, recent successes in identifying various molecules involved in these processes provide further clues.

One important step regulating the extracellular transport of various secreted signal proteins involves post-translational modification with lipid moieties. A fatty acid modification, i.e. acylation, occurs with Wnt, Hedgehog (Hh), and Spitz (Drosophila Transforming Growth Factor α). In the case of Wnt, Nusse and co-workers reported that murine Wnt-3a is S-palmitoylated at a conserved cysteine residue at the 77th residue (Cys77). A mutant form of mouse Wnt-3a, in which the palmitoylated Cys77 is substituted with alanine (C77A), shows diminished ability to activate Wnt signaling, but is secreted normally into the culture medium. Thus, the authors proposed that palmitoylation of this cysteine residue may be required to produce an increased local concentration of Wnt on the plasma membrane. However, although their mass spectrometry analysis covered 85% of the primary amino acid sequence of Wnt-3a, there remains the possibility of additional acylation sites.

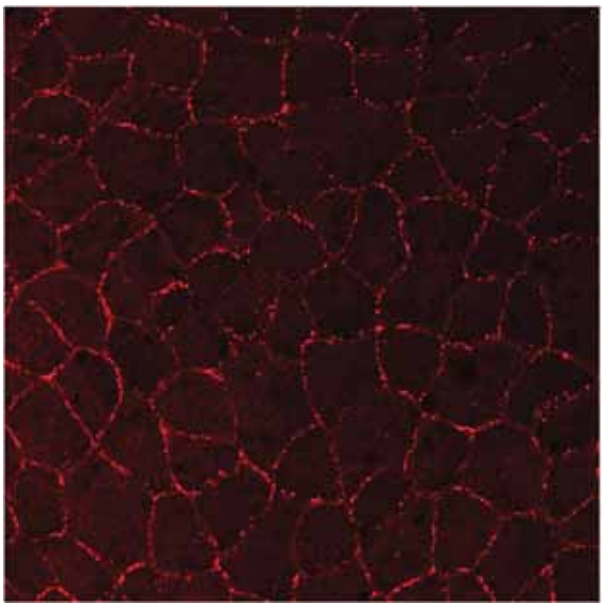

Figure 1. Secreted Wnt-3a proteins in epithelial sheet. Immunostaining of Wnt-3a proteins secreted from Xenopus epithelial cells. Wnt proteins are secreted in dot-like structure in the extra-cellular space.

There is strong evidence to suggest that acylation is involved in the processing and intracellular trafficking of Wnt prior to secretion. Genetic evidence suggests that Wnt-secreting cells require the action of specific genes, e.g., *porcupine* (*porc*) in *Drosophila* or its ortholog, *mom1,* in *C. elegans*, both of which encode proteins with structural similarities to those of a family of membrane-bound O-acyl transferases (MBOAT), which transfer acyl groups, such as a palmitoyl group, to substrates. Porc is localized at the endoplasmic reticulum (ER) and its over-expression in culture cells enhances the intracellular processing, for example, N-glycosylation, of Wingless (Wg: the Drosophila Wnt-1 ortholog). In addition, treatment with a chemical inhibitor of acyl-transferases produces defective intracellular

rafficking of Wg. Thus, *porc*-dependent acylation may regulate the processing and intracellular trafficking of Wnt, although acylation at Cys77 does not appear to be involved in these processes.

To resolve inconsistencies between studies examining the roles of Wnt acylation and to better understand the biological significance and molecular mechanism of Wnt function of acylation (Figure 2)[Ö] We show that murine Wnt-3a is also acylated at a conserved serine residue (Ser209). Significantly, we demonstrated that this residue is modified with a mono-unsaturated fatty acid, palmitoleic acid (Figure 3). Wnt-3a defective in acylation at Ser209 is not secreted from cells in culture or in Xenopus embryos, but is retained in the endoplasmic reticulum (ER). Furthermore, Porcupine, a protein with structural similarities to membrane-bound O-acyltransferases, is required for Ser209-dependent acylation, as well as for Wnt-3a transport from the ER for secretion (Figure 2). These results strongly suggest that Wnt protein requires a particular lipid modification for proper intracellular transport during the secretory process.

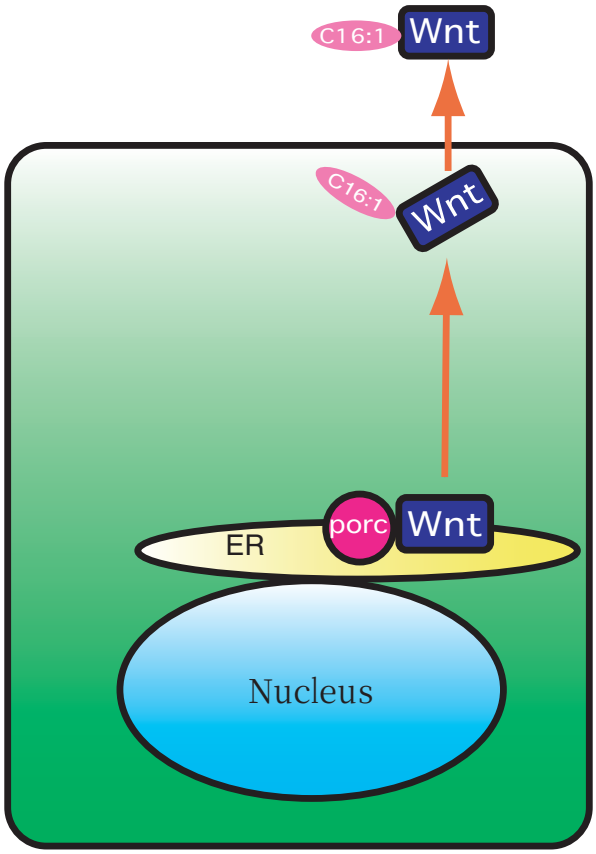

Figure 2. The function of palmitoleic lipid modification of Wnt protein. Wnt proeins are modified with palmitoleic acid (C16:1) by acytransferase, Porcupine (porc), in the ER. This modification is required for trafficking of Wnt proteins from the ER.

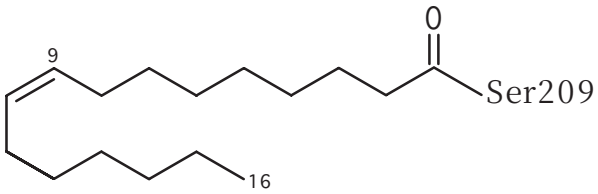

Figure 3. Structure of the lipid chain, palmitoleoyl moiety, bound to Ser209 of murine Wnt-3a protein

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 trapped gene, CP2L1, which encodes a transcriptional regulator, was mainly expressed in the ductal epithelium of several developing organs, including the kidney and a number of exocrine glands. The spatial and temporal expression of this gene coincided well with that of several *Wnt* genes. Furthermore, the expression of this gene was significantly decreased in cultures of embryonic tissues treated with a Wnt signal inhibitor, indicating that the in vivo expression of this gene is dependent on Wnt signaling.

We have further analyzed the developmental role of CP2L1 by generating mutant embryos defective in its function from the gene-trapped ES cell line. In *CP2L1*-deficient mice, the expression of genes directly involved in functional maturation of the ducts was specifically reduced in both the salivary gland and kidney, indicating that *CP2L1* is required for the differentiation of duct cells. Furthermore, the composition of saliva and urine was abnormal in these mice. These results indicate that *CP2L1* expression is required for normal duct development in both the salivary gland and kidney.

Interestingly, the identification of a gene essential for the maturation of the exocrine ducts and kidney should help to elucidate their underlying molecular mechanisms. Duct maturation includes both duct formation and the acquisition of physiological function. Because CP2L1 is a transcriptional regulator, genes whose expression is directly or indirectly regulated by this factor may be involved in these processes. In *CP2L1*-deficient embryos, the expression of genes involved in the physiological function of the ducts was reduced (Figure 4). In addition to the genes directly involved in physiological function, we found that the expression of *keratin* genes was also abnormal in the ducts of the mutants. Thus, CP2L1 participates in establishing the function of ducts by coordinating the expression of several genes that are involved in physiological function and generate the appropriate cellular architecture.

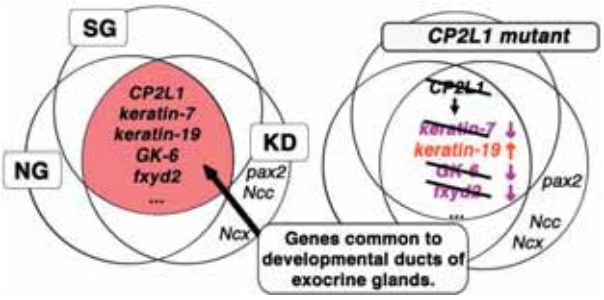

Figure 4. CP2L1 may play a common regulator in development of exocrine ducts. Several exocrine ducts express a common set of genes, and dysfunction of CP2L1 causes defects in the expression of these genes.

III. Identification and characterization of genes required for somite development

3-1 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.

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. 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.

We then examined the role of ripply gene famity in the mouse. Its function was also analyzed in an *in vitro* system.

3-2 Functional analysis of other genes involved in somite development

The segmental pre-pattern established in the anterior PSM leads 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.

To gain insight into the mechanism underlying somite development, we performed an ENU mutagenesis screening of zebrafish, in addition to the *in situ* hybridization screening described above. We found that *integrinα5* and *fibronectin* were mutated in embryos showing defective boundary formation in their anterior somites. Detailed analysis with these mutantas indicated that Integrinα5-directed assembly of Fibronectin appears critical for epithelialization and boundary maintenance of somites.

These results indicate that our strategies are effective for the identification of the 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.

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Laboratory Scope

Our laboratory aims to reveal the molecular mechanisms of formation of the gonads and sex differentiation. We are using medaka fish (*Oryzias latipes*) for these purposes.

Medaka has been recently established as a model vertebrate. The entire genome sequence was determined and a variety of inbred strains with a large polymorphic genome is available, which allows us to investigate biological phenomena by the means of molecular genetics. In addition, an exogenous gene can be introduced into medaka genome (transgenic medaka) and cells can be transplanted to host medaka to generate chimera medaka.

With these advantages, we have been generating transgenic medaka enabling us to identify the different cell lineages by fluorescence and to analyze the process of gonad formation and sex differentiation in vivo. Additionally, in order to identify the genes essential for gonadogenesis, we carried out a mutational screening of medaka with a defect in gonads and are performing a positional cloning. With these two unique analytical methods (visualising cells and mutants), we are attempting to unveil the fundamental mechanisms of sex differentation which are believed to be common to many organisms.

I. Identification of the gonadal fields that coordinate germ cell migration and development of gonadal somatic cells

Much research has focused on the function of the genes involved in sex differentiation using gene-disrupted mice. However, the functions of each cell type and the interactions between the different cell lineages are totally unknown during sex differentiation. It is important to understand the origin and the lineages of the gonadal precursor cells in order to analyze cellular events.

Since one of the important cell lineages is germline, we first characterized the migration of primordial germ cells (PGCs) towards the gonadal area. We found three different modes of PGC movement: 1) active migration towards the peripheral region at early gastrulation (*cxcr4*-dependent), 2) passive movement towards the embryonic body with convergent movement of somatic cells at early segmentation stages, and 3) active posterior migration towards the most posterior end of *sdf1a*

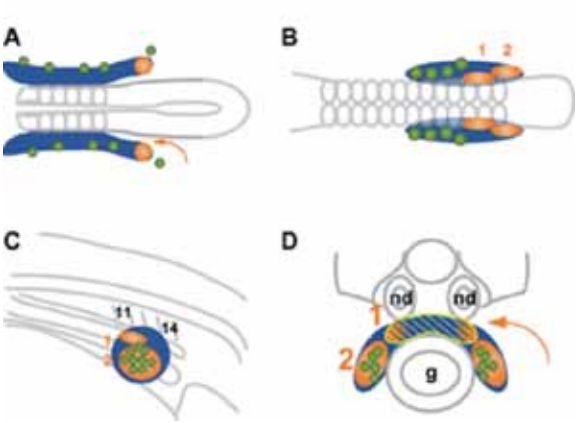

Figure 1. The two different precursors of gonadal mesoderm arise from the gonadal fields (orange). *sdf-1a* expression domain (blue). PGCs (green) (From reference, Nakamura et al, 2006).

expression domain at late segmentation stages (*sdf1a*-dependent) (Kurokawa *et al*, 2006). We characterized the most posterior region of *sdf1a* expression domain and showed that it has the same properties as gonadal fields where the precursors of gonadal mesoderm arise. This result indicates that the gonadal field is the place that coordinates PGC migration and the development of the gonadal mesoderm. We further demonstrated that two different populations with distinct gene expression are spatially organized from the gonadal precursors along the embryonic axis and are specified before the gonadal primordium forms (i.e., before the sex is determined) (Nakamura *et al*. 2006) (Figure 1).

II. Germ cells are essential for sexual dimorphism of the gonads

The nature of somatic cells and germ cells is a topic of broad and long standing interest. Many studies have therefore explored the interaction between germ cells and

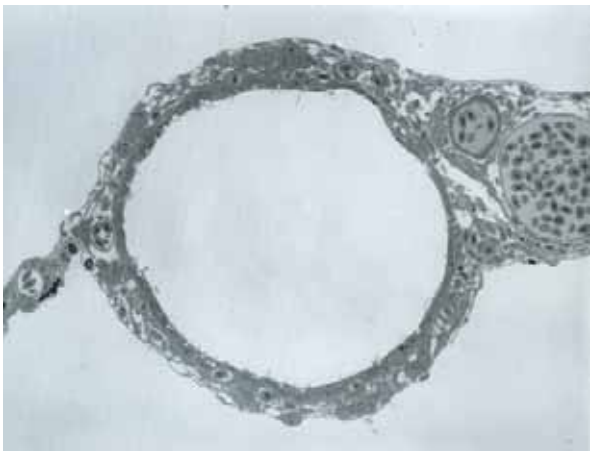

Figure 2. The germ cell-deficient gonad exhibits a single fundamental structure common to both ovary and testis. A large lumen is located in the middle and a single layer of supporting like cells encloses the lumen, which is separated by a basement membrane from an outer stromal region.

gonadal somatic cells. The dogma resulting from these studies is that germ cells do not significantly affect the sex differentiation of gonads.

We generated medaka embryos that completely lack germ cells in the gonadal primordium by impairment of PGC migration and found female to male sex reversal 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 (Figure 2). Novel *aromatase*-expressing theca cells that we identified in ovary did not develop properly and female supporting cells that control gametogenesis began to express male-specific genes as development of the gonad proceeded, suggesting trandifferentiation from female to male supporting cells. The cells producing male sex steroid hormone persisted in the germ cell-deficient medaka. All of this data indicated that the gonadal somatic cells are predisposed to adopting male development and the production of male steroid hormone results in sex reversal to male secondary sex characteristics. Thus, contrary to accepted dogma, we demonstrated that germ cells are essential for sexually dimorphic gonads (Kurokawa *et al.* submitted).

III. Generation of transgenic medaka to identify the cell lineages that constitute the gonads

To clarify the cell types that constitute the gonad, we are generating transgenic fish to visualize the cell lineages. We have established several lines of transgenic fish that allow us to analyze how they build up the gonad during the course of development. Crossing the transgenic fish

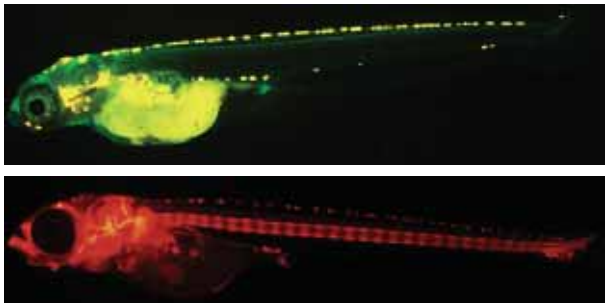

Figure 3. Different colored transgenics allowed us to reconstruct gonad structures composed of different cell types and to monitor cell movement in the structures.

with different colors (Figure 3) enabled us to successfully reconstitute novel units of structure that have not been reported yet in the gonad.

An attempt to monitor the process of development of each lineage has also been made in living embryos and larva using timelapse movies. In order to solve the difficulties in visualizing the cells located in the deep positions in the embryos and larva, 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. Identification of the genes of medaka mutants with defects in the gonads

In collaboration with SORST Kondoh team, 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 and 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 of particular interest because of the excessive number of germ cells that are arrested in early development of follicle growth and because male to female sex reversal occurs irrespective of their genetic sex. As a result of the positional cloning, we have identified a candidate gene and are now proving that mutation in the gene is responsible for all the phenotype of *totoro* (Morinaga *et al*, submitted).

Figure 4. Blue staining shows PGCs in the gonad. Left: *zenzai* mutant that cannot maintain gerrn 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. We again identified one possible candidate gene for the phenotype of *zenzai* mutant.

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

Publication List:

Original papers

Kurokawa, H., Aoki, Y., Nakamura, S., Ebe, Y., Kobayashi, D., and Tanaka, M. (2006). Time-lapse analysis reveals different modes of primordial germ cell migration in the medaka *Oryzias latipes*. Develop. Growth Differ. *48*, 209-221.

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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 systems of chicks and mice. This research covers many developmental events including the patterning of the retina, axonal navigation, branching and targeting, synapse formation, refinement and plasticity. 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 regional specification in the developing retina

Topographic maps are a fundamental feature of neural networks in the nervous system. The retinotectal projection of lower vertebrates including birds has been used as a readily accessible model system of the topographic projection. We have been studying the mechanisms for regional specification in the retina as the basis of the topographic retinotectal projection.

We can now present gene cascades for retinal patterning and region-specific expression of topographic molecules for retinotectal projection as in Figure 1. *FoxG1* and *FoxD1* are expressed in the nasal and temporal regions of the developing chick retina at an early stage (peaking at E3), respectively, and determine the regional specificity in the retina by their counteraction. Consequently, two homeobox transcription factors, *SOHo1* and *GH6*, are expressed specifically in the nasal region: it is known that the two control the retinotectal projection along the A-P axis by the repression of *EphA3* expression. Afterwards, *ephrin-A5* and *ephrin-A2* begin to show nasal-high expression and *EphA3* temporal-high expression in the retina. In this process, *FoxG1* controls *ephrin-A5* via a DNA binding-dependent mechanism, *ephrin-A2* via a DNA binding-independent mechanism (see below), and *FoxD1*, *SOHo1*, *GH6* and *EphA3* via dual mechanisms.

Along the D-V axis, counteraction between BMP4 and Ventroptin governs the regional specification in the retina. At the early stages of development from HH stage 11 to E5, dorsally-expressed *BMP4* determines the regional specificity of the dorsal retina and ventrally-expressed *Ventroptin* counteracts the activity of BMP4. Transcription factors *Tbx2/3/5* in the dorsal retina and *cVax* in the ventral retina begin to be expressed under the control of the BMP4 signal. At approximately E5, *BMP4* expression in the dorsal retina rapidly disappears. Concomitantly, *Ventroptin* turns to be expressed in an oblique-gradient fashion (V/N-high pattern from E6 onward). Then, instead of *BMP4*, *BMP2* begins to be expressed in an oblique-gradient fashion (D/T-high pattern), complementary to that of *Ventroptin,* to counteract it. The inhibitory effect of FoxG1 on the BMP signaling is thought to be responsible for turning the expression patterns of *Ventroptin* and *BMP2* about 30 degrees to the posterior side from the first D-V axis. Switching from *BMP4* to *BMP2* should occur owing to the difference in their genetic regulatory mechanisms, and this would be the basis of the tilting of the D-V axis in the developing retina. Expression of *EphBs*, *ephrin-Bs* and *ephrin-A2* are under the control of BMP2 signal. BMP2 thus appears to play an important role in the topographic projection along both axes.

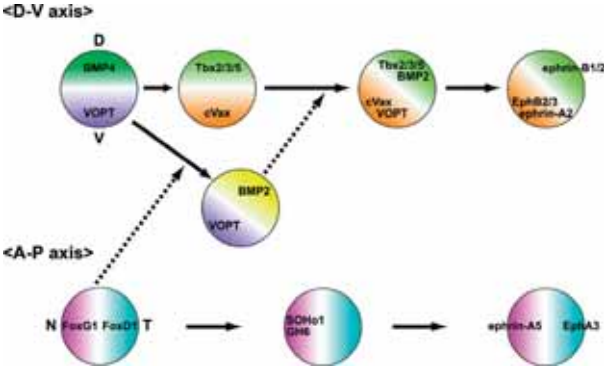

Figure 1. Developmental expression of topographic molecules in the chick retina. Regional specification along the nasotemporal (A-P) and dorsoventral (D-V) axes in the developing retina precedes the topographic retinotectal projection. The developing stage proceeds from left to right.

II. Mechanisms for the topographic retinotectal projection

Eph receptors are implicated in topographic projections in many regions of the developing nervous system, including the retinotectal (or retinocollicular) projection, where gradients of expression of Eph receptors and ephrins in the retina and tectum play essential roles. Eph receptors are activated by autophosphorylation of tyrosine residues upon the binding of their ligands, ephrins. The

protein tyrosine phosphatases (PTPs) responsible for the negative regulation of Eph receptors, however, have not been elucidated.

We identified protein tyrosine phosphatase receptor type O (Ptpro) as a specific PTP that efficiently dephosphorylates both EphA and EphB receptors as substrates. Biochemical analyses revealed that Ptpro dephosphorylates a phosphotyrosine residue conserved in the juxtamembrane region, which is required for the activation and signal transmission of Eph receptors. Ptpro thus appears to moderate the amount of maximal activation of Eph receptors. Using the retinotectal projection system, we showed that Ptpro controls the sensitivity of retinal axons to ephrins, and thereby plays a crucial role in the establishment of topographic projections. Our findings explain the molecular mechanism to determine the threshold of the response of Eph receptors to ephrins *in vivo*.

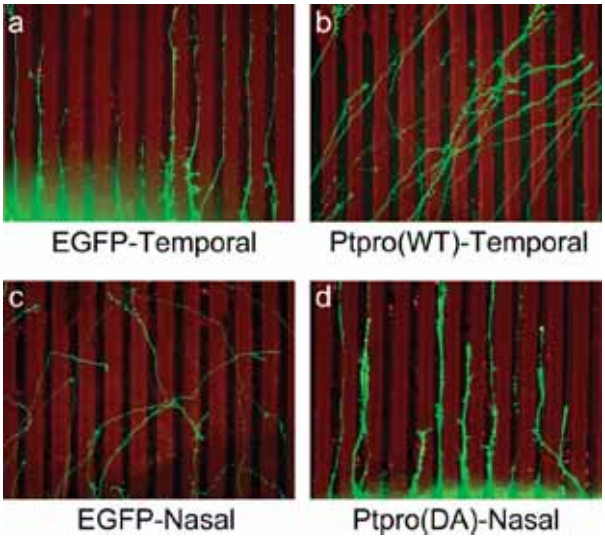

Figure 2. Regulation of the sensitivity of chick retinal axons to ephrin-A2-Fc by Ptpro. To visualize retinal axons, *egfp* was coelectroporated into the retina, and the retinal strips were subjected to the ephrin-A2-Fc stripe assay. (a) Control temporal axons preferred to grow on control Fc-containing lanes (dark) but not on ephrin-A2-Fc lanes (red). (b) Ptpro (WT)-overexpressing temporal axons randomly grew on both control Fc and ephrin-A2-Fc lanes. (c) Control nasal axons randomly grew on both control Fc and ephrin-A2-Fc lanes. (d) When the DA mutant of Ptpro, Ptpro (DA), was overexpressed, nasal axons showed preferential growth on control Fc lanes like the normal temporal axons.

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

Ptprz (also called PTPζ/RPTPβ) is a receptor-type protein tyrosine phosphatase (RPTP) predominantly expressed in the brain as a chondroitin sulfate proteoglycan. *Ptprz*-deficient mice exhibit an age (maturation)-dependent impairment of spatial learning in the Morris water maze test and enhancement of long-term potentiation (LTP) in the CA1 region in hippocampal slices. The enhanced LTP is canceled out by pharmacological inhibition of Rho-associated kinase (ROCK), suggesting that the lack of *Ptprz* causes learning impairment due to aberrant activation of ROCK.

We found that *Ptprz*-deficient mice exhibit impairments in hippocampus-dependent contextual fear memory (Figure 3) because of abnormal tyrosine phosphorylation of p190 RhoGAP, a GTPase-activating protein (GAP) for Rho GTPase. Phosphorylation at Y1105, a major tyrosine phosphorylation site on p190 RhoGAP, was decreased 1 h after the conditioning in the hippocampus of wild-type mice, but not of *Ptprz*-deficient mice. Pleiotrophin (PTN), a natural ligand for Ptprz, increased tyrosine phosphorylation of p190 RhoGAP in B103 neuroblastoma cells. Furthermore, Ptprz selectively dephosphorylated pY1105 of p190 RhoGAP *in vitro*, and the tyrosine phosphorylation at Y1105 controled p190 RhoGAP activity *in vivo*. These results suggest that Ptprz plays a critical role in memory formation by modulating Rho GTPase activity through dephosphorylation at Y1105 on p190 RhoGAP.

RPTPs are considered to transduce extracellular signals across the membrane through changes in their PTP activity, however, our understanding of the regulatory mechanism is still limited. We revealed that PTN inactivates Ptprz through oligomerization and increases the tyrosine phosphorylation of substrates for Ptprz, G protein-coupled receptor kinase-interactor 1 (Git1) and membrane associated guanylate kinase, WW and PDZ domain containing 1 (Magi1). Oligomerization of Ptprz by an artificial dimerizer or polyclonal antibodies against its extracellular region also leads to inactivation, indicating that Ptprz is active in the monomeric form and inactivated by ligand-induced oligomerization.

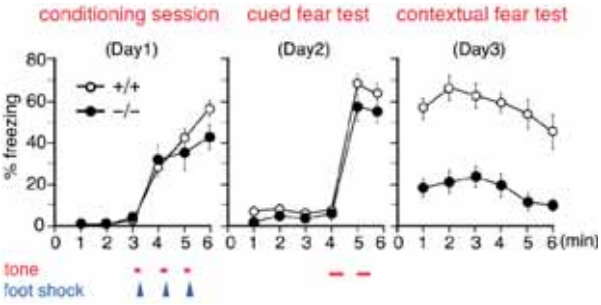

Figure 3. Impaired contextual fear memory in *Ptprz*-deficient mice. During the conditioning session, no differences were observed between the wild-type and *Ptprz*-deficient mice in freezing response on Day 1. *Ptprz*-deficient mice showed normal responses in the cued fear test on Day 2, but impairments in contextual fear conditioning on Day 3.

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 fluid. For Na homeostasis of the body, controls of Na and water intake and excretion are of prime importance. Although the circumventricular organs (CVOs) are thought to be involved in body-fluid homeostasis, the system for

sensing the Na level within the brain that is responsible for the control of Na- and water-intake behavior has long been an enigma.

We found that the Na_x channel is preferentially expressed in the CVOs in the brain and that *Na*$_x$-deficient mice ingest saline in excess under dehydrated conditions. Subsequently, we demonstrated that Na_x is a Na-level-sensitive Na channel. When *Na*$_x$ cDNA was introduced into the brain of the knock-out mice with an adenoviral expression vector, only animals which received a transduction of the *Na*$_x$ gene into the subfornical organ (SFO) among the CVOs recovered salt-avoiding behavior under dehydrated conditions. Based on these findings, we advocate that the SFO is the center for 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 level of Na in body fluids.

We recently found that Na_x channels are specifically expressed in the perineuronal processes of astrocytes and ependymal cells enveloping particular neural populations in the sensory CVOs (Figure 4). These Na_x-positive glial cells were sensitive to an increase in the extracellular sodium level, indicating that glial cells – not neurons – are the primary site of sodium-level sensing. The mechanism by which the sodium signal sensed by ìinexcitableî glial cells is transferred to neurons remains to be elucidated.

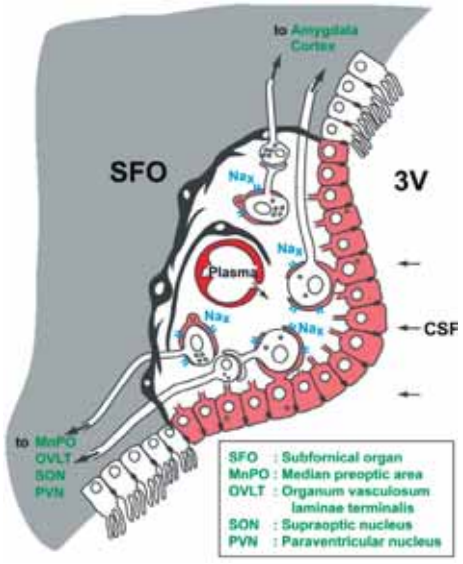

Figure 4. Schematic drawing of the SFO. Na_x is exclusively localized to perineuronal lamellate processes extended from ependymal cells and astrocytes. This suggests that glial cells bearing Na_x are the first to sense a physiological increase in the level of sodium in the body fluid, and they regulate the neural activity by enveloping neurons. 3V, 3rd ventricle.

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Original papers

Fukada, M., Fujikawa, A., Chow, J.P.H., Ikematsu, S., Sakuma, S., and Noda, M. (2006). Protein tyrosine phosphatase receptor type Z is inactivated by ligand-induced oligomerization. FEBS Lett. *580*, 4051-4056.

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Watanabe, E., Hiyama, T.Y., Shimizu, H., Kodama, R., Hayashi, N., Miyata, S., Yanagawa, Y., Obata, K., and Noda, M. (2006). Sodium-level-sensitive sodium channel Na_x is expressed in glial laminate processes in the sensory circumventricular organs. Am. J. Physiol. ñ Regul. Integr. Comp. Physiol. *290*, 568-576.

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In order to understand the formation and evolution of the brain and the mechanisms underlying memory, we are focusing primarily on two issues. 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 study the mechanisms underlying learning behaviors by examining gene expression.

I. Genes expressed in specific areas and layers of the neocortex

The neocortex is most remarkably evolved in the anatomical areas and it has been a matter of debate to 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,which are schematically illustrated in Fig. 1 and Fig. 2.

1) Examining 1088 genes by microarray analysis, most genes showed less than 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 expressed among the different areas of the human neocortex are very similar. However, the question remained whether or not 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

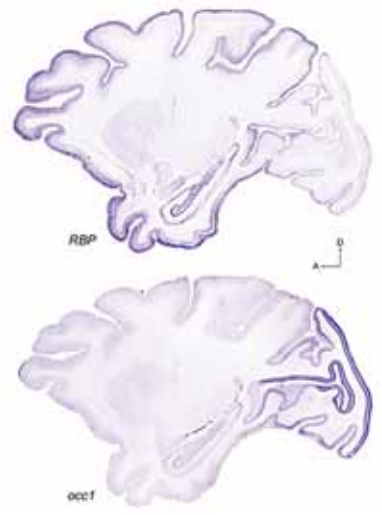

Figure1. Expression pattern of *occ1 and Rbp*. *occ1* RNA is markedly expressed in the visual cortex and expressed in primary sensory areas, particularly in visual cortex whereas primate and plays the major role in higher brain functions [?]. It is divided into distinct functional and Rbp is preferentially expressed in association areas (This Figure is cited from Komatsu et al., Cereb. Cortex, 15, 96-108, 2005)

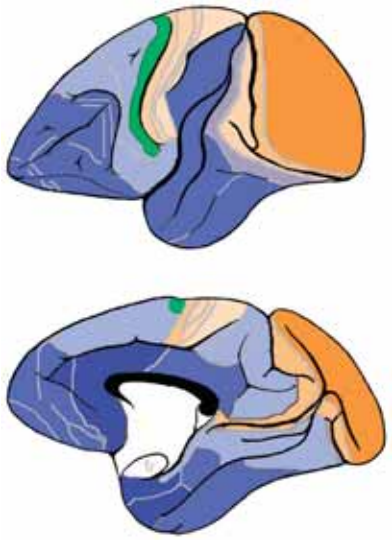

Figure 2. The expression of occ1 (orange color)m Rbp (blue) and gdf7 (green) are schematicaly illustrated in Brodmannís area figure in the guenon monkey. Top and bottom views are medial and laetral surfaces, respectively. (The figure is cited from Yamamori & Rockland, Neurosci. Res., 55, 11-27, 2006).

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

This year we reported other features of *occ1* expression in mammalian brains. Firstly, we compared the *occ1* expression in subcortical areas and found certain nuclei strongly expressed *occ1*. Interestingly, in most of the nuclei that *occ1* is strongly expressed in monkeys, the authologue of *occ1* is similarly expressed in mice, which suggests that the occ1 expression in subcortical nuclei is generally well conserved during mammalian evolution (except for a few nuclei such as LGN). Secondly, we examined activity dependent expression of occ1 in mice. occ1 is strongly expressed in the mouse LGN. We then monocularly deprived by enucleation or TTX injection and examined *occ1* expression in LGN. Contrary to the monkey primary visual cortex, *occ1* expression was not affected by monocular deprivation. This is a clear contrast with other well known activity dependent gene expressions such as c-Fos expression. This result thus suggests that activity dependency of *occ1* has been acquired during evolution of the primate brain (Takahata et al., Society for Neuroscience in North America, 2006).

As pointed out by Brodmann 100 years ago, all mammalian brains consist of six layers, which structure is a fundamental frame work of the mammalian neocortex. It thus may give us an important information for understanding evolution of the brain in mammals by comparing the layer specific gene expression pattern. We examined the expression patterns of four layer-specific genes in monkey and mouse cortices by fluorescence double in situ hybridization (Fig. 3). Based on their coexpression profiles, we were able to distinguish several subpopulations of deep layer neurons. One group was characterized by the expression of ER81 and the lack of Nurr1 mRNAs and mainly localized to layer 5. In monkeys, this neuronal group was further subdivided by 5-HT2C receptor mRNA expression. The 5-HT2C+/ER81+ neurons were located in layer 5B in most cortical areas but they intruded layer 6 in the primary visual area (V1). Another group of neurons, in monkey layer 6, was characterized by Nurr1 mRNA expression and was further subdivided as Nurr1+/CTGF- and Nurr1+/CTGF+ neurons in layers 6A and 6B, respectively. The Nurr1+/CTGF+ neurons coexpressed ER81 mRNA in monkeys but not in mice. On the basis of tracer injections in three monkeys, we found that the Nurr1+ neurons in layer 6A send some corticocortical, but not corticopulvinar, projections. Although the Nurr1+/CTGF- neurons were restricted to lateral regions in the mouse cortex, they were present throughout the

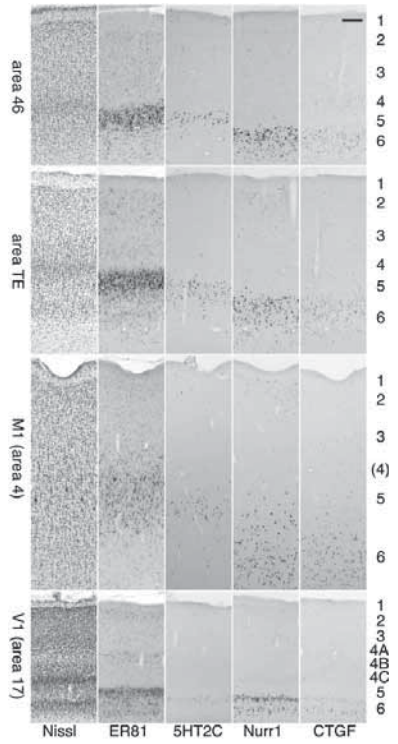

Figure 3. In situ hybridization patterns of layer-specific genes in various areas of monkey cortex. Adjacent sections of the monkey brain were processed for non-radioactive ISH with the probes indicated at the bottom of the panel. Here, we present data for area 46 on the bank of the principle sulcus, area TE on the inferior temporal gyrus, the primary motor cortex (M1 or Brodmann area 4) on the anterior bank of the central sulcus, and the primary visual cortex (V1 or Brodmann area 17) on the operculum of the occipital cortex. The numbers on the right indicate the layers determined by the adjacent Nissl staining. Scale bar on top right corner, 500 μm. (The figure is cited from Watakabe et al., Cereb Cortex. 2006 Oct 25; [Epub ahead of print])

monkey cortex. Thus, we revealed an architectonic heterogeneity across areas and species for the neuronal layer specific gene expression analysis (Watakae et al., Cereb Cortex. 2006 Oct 25; [Epub ahead of print Cereb]).

We also found another layer specific expression in mouse and monkey neocortex; that is, we showed that Semaphorin 3E (Sema3E), a class 3 member of the semaphorin family, exhibits highly layer specific expression in the mature neocortices of monkeys and mice. In macaque monkeys, Sema3E mRNA was restricted to layer VI across the entire neocortex. In all the areas examined, Sema3E-positive neurons were a subpopulation of VGluT1-positive excitatory neurons, but their percentages varied between 34% and 63% in the motor cortex and area TE, respectively. In the mouse cortex, Sema3E mRNA was also enriched in deeper layers, but, unlike the monkeys, it was expressed also in layer Vb. In addition, a subset of GABAergic interneurons in layers I-VI expressed Sema3E mRNA in mice but not in monkeys. In an in vitro binding assay, Plexin D1 bound to Sema3E but not to other members of class 3 semaphorins. Double ISH for Plexin D1 and Sema3E genes showed that these two genes exhibit

complementary patterns in mice and monkeys, although there are some species differences in lamina expression patterns. These results suggest that Sema3E and Plexin D1 may play a conserved role in regulating the interaction between upper and deep layer neurons in mice and monkeys (Watakabe et al., J. Comp. Neurol., 499, 258-273, 2006).

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 depends on the type of task. 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 for analyzing 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).

We studied multisensory processing further using a c-Fos mapping technique. We first developed a method to standerdize the cortex to quantitatively evaluate c-Fos expression by an automatic image analyzing system. Using this system, we found the secondary visual cortex (V2L) in rats is specifically activated under audiovisual multisensory stimulation. Injecting muscimol into V1, V2, V2L and superior colliculus (SC), we found that V2L is specifically involved in the stimulation of multisensory reaction (Hirokawa et al., 2006 Society for Neuroscience in North America).

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 in the other direction (Kitsukawa *et al*. SFN Meeting, 2002). The task required of the mouse can thus be regarded as representing a procedural learning. We examined various areas of the 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 progressed. However, their roles in behavioral tasks have remained obscure. We are currently examining the altered behavior [that appears/develops?] under the pharmacological treatments that affect the metabolism of the interneurons in the striatum.

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DIVISION OF BEHAVIORAL BIOLOGY (ADJUNCT)

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In mammals, several social behaviors are dependent on sex. These sex-dependent patterns of behavior 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 discrete brain areas responsible for the sex difference of the behaviors.

I. DNA methylation of sex steroid receptor genes

In mammals, DNA methylation, mainly occurring on CG dinucleotides, is a fundamental mechanism that differentiates the gene expression pattern in respective cells. 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 their sex status according to the environmental context. In rodents, endocrine disturbance at the fetal and/or postnatal stage irreversibly changes 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 behavior are not directly dependent on ìs ex-specificî genes but rather established through epigenetic processes. We have found the sex- and brain area-dependent DNA methylation pattern of rodent steroid receptor genes (estrogen receptor α, *ERα*; androgen receptor, *AR*; progesterone receptor, *PR*) that can be modulated by the novel noncoding RNAs, named *ERαas, ARas* and *PRas*. We report herein epigenetic, expressional, endocrinological, and behavioral analyses of transgenic (Tg) mice constitutively expressing these noncoding RNAs.

1-1 Association of the noncoding RNA with the coding transcripts through epigenetic mechanisms in the brain

The number of mammalian noncoding RNA genes is rapidly expanding, necessitating the evaluation of their RNA function. Neither of the noncoding RNAs we found showed obvious open reading frames or 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. These structural features led us to investigate the possible involvement of these noncoding RNAs on the establishment of the specific DNA methylation pattern *in vivo*.

We generated *ERαas*- and *ARas*-Tg mice. Most of the hypermethylated CG sites located on the sex-dependent differentially methylated region of *ERα* in intact adult females became hypomethylated in sex-matched *ERαas*-Tg mice (Figure 1). This effect seemed gene-specific, because DNA methylation status of *AR* did not differ between intact and *ERαas*-Tg females. Similarly, specific reduction of DNA methylation level in AR promoter region was observed in *ARas*-Tg mice (Figure 1). These findings conform to the previous *in vitro* experiment in which transient overexpression of *ERαas* or *ARas* fragment forced demethylation in a sequence-specific manner.

In general, the binding affinity of transcription factors is reduced by the hypermethylation that is frequently observed on the promoter region of either *ERα,* or *AR* in tumor cells. Therefore, we analyzed the expression of these coding transcripts. The results showed that *ERα* was expressed significantly higher in *ERαas*-Tg mice, but not

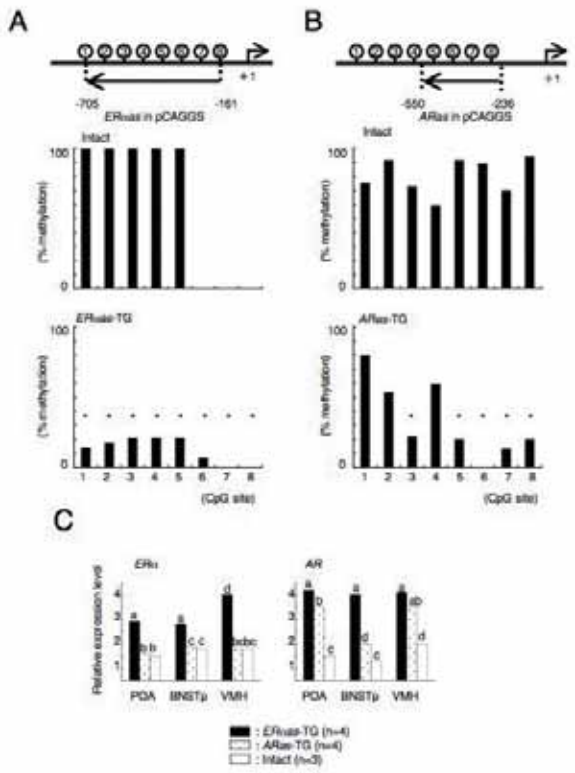

Figure 1. Deregulated epigenetic control of sex steroid receptor genes in *ERαas*- and *ARas*-Tg mice. (A) DNA methylation status of the *ERα* promoter in the BNSTp of intact and *ERαas*-Tg females. (B) DNA methylation status of the *AR* promoter in the VMH of intact and *ARas*-Tg females. BNSTp and VMH are the target brain areas for sex-dependent DNA methylation of *ERα* and *AR*, respectively. Asterisks denote the significant difference between intact and Tg mice. (C) *ERα* and *AR* expression in intact and Tg females analyzed by quantitative RT-PCR. Values with different letters indicate significant difference ($p<0.01$). POA, preoptic area; BNSTp, principal portion of bed nucleus of the stria terminalis; VMH, ventromedial hypothalamus.

in *ARas*-Tg mice, when compared with sex-matched intact mice (Figure 1), strongly suggesting that induction of DNA demethylation by promoter noncoding RNA upregulated the associated coding transcript. For *AR*, the results were a little complicated. *AR* was expressed higher in both *ERαas*- and *ARas*-Tg mice than in sex-matched intact mice. Since estrogen signaling has been realized to predominate the androgen receptor-mediated pathways in the masculinization of the rodent brain, this could explain the observed upregulation of *AR* not only in *ARas*-Tg but also in *ERαas*-Tg mice.

1-2 Prevailing effect of promoter noncoding RNA expression *in vivo*

ERαas- and *ARas*-Tg mice were anticipated to show the deregulated endocrinological and behavioral status. We investigated the female- and male-specific sexual behaviors by analyzing lordosis quotients and the number of mounts in these mice. Both Tg mice showed a significant reduction of lordosis quotients. Furthermore, females of *ARas*-Tg mice did mounting even though exogenous androgen treatment was not performed. We also measured the circulating testosterone and estrogen in these Tg mice and found that the level of these steroid hormones altered when compared with intact animals. We now postulate that the observed effects caused by the forced expression of *ERαas* and *ARas* reflect the impact of the corresponding endogenous noncoding RNAs. We are currently investigating the developmental aspect of the neural cells expressing/repressing these noncoding RNAs.

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LABORATORY OF NEUROPHYSIOLOGY

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NIBB Research Fellow: *YAMADA, Misuzu*

When the correct balance between water and sodium levels in the body fluid has been disrupted, 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 long eluded discovery. 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 the 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 using a 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 the sensing of sodium concentration and osmosity in the body fluids.

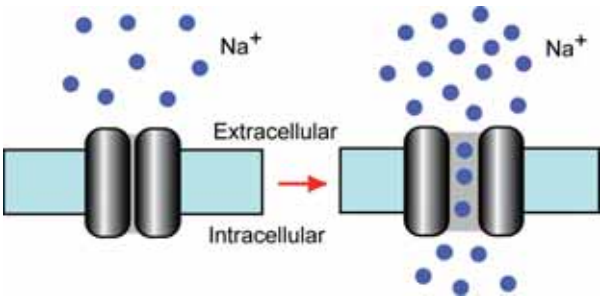

Figure 1. Na_x is a sodium channel sensitive to extracellular sodium level. When the extracellular sodium concentration increases, Na_x channel opens the gate pore and generates the sodium ion influx into the cells. This view was hypothesized by ion-imaging studies.

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 the 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. Yamamotoís 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.

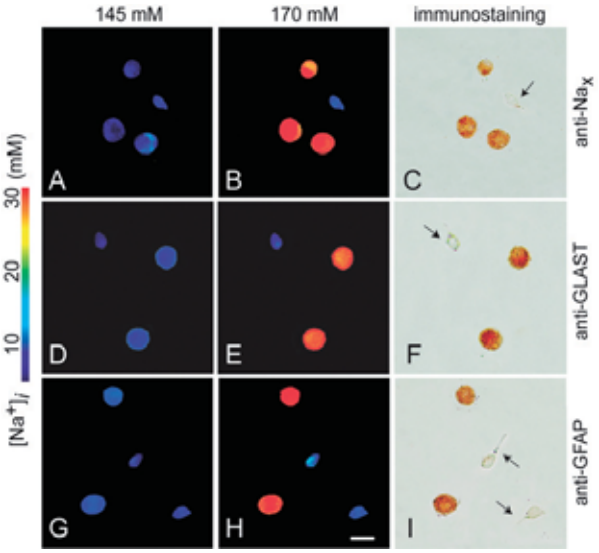

Figure 2. Glial cells isolated from the SFO express Nax channel and show sensitivity to the extracellular sodium level. Sodium imaging study using the dissociated SFO cells. Pseudocolor images of the intracellular sodium concentration ([Na+]i) of SFO cells in the control solution (the extracellular sodium concentration = 145 mM, A , D and G) and in the high sodium solution (170 mM, B, E and H). A, D, G and B, E, H are images 5 min before and 20 min after stimulation with the hypertonic 170 mM [Na+] solution, respectively. After sodium-image recordings, cells were fixed and stained with anti-Na_x (C), anti-GLAST (F) or anti-GFAP (I) antibodies. All the sodium-sensitive cells are immunopositive for Na_x, GLAST and GFAP. Arrows in C, F and I indicate small neurons bearing short neurites, which are all insensitive to the extracellular sodium increase. Scale bar: 20 μm.

In 2004, we developed 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 those animals that 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 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 and immuno-electronmicroscopic 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 the ion-imaging method (Figure 2). In addition, we found that the Na_x-expressing glial cells enveloped multiple kinds of neurons including GABAergic interneurons in the organs (Figure 3). 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 are 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.

In 2006, we tried to construct functional expression systems of Na_x sodium channel using various heterologous cell lines. Attempts to express functional Na_x channels in cultured cell lines have been long unsuccessful. We developed glial cell lines in which the expression of Na_x channel is inducible under the control of the tetracycline responsive element. Several experiments using the cell lines showed that cellular signals via Na_x channels are coupled with a metabolic cascade of the glial cells, suggesting that the information of physiological increase of the sodium level in body fluids sensed by Na_x channel in glial cells is transmitted to neurons by the altered metabolic state of the glial cells.

In addition, we are studying the involvement of Na_x sodium channel in the regulation of hormone release, using a 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 with useful information on the physiological function of Na_x channel.

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 recent years, we newly demonstrated that the primary subcellular locus sensing sodium-level is perineuronal glial processes. This finding suggests that the neuron-glia complex plays a key role in sodium sensing in the circumventricular organs.

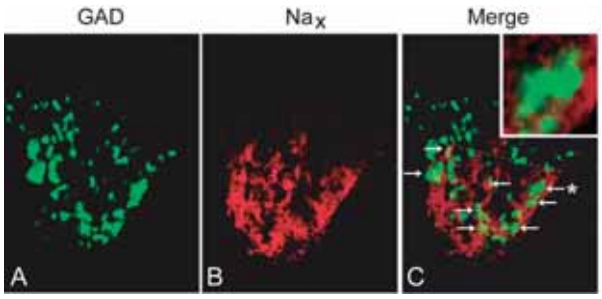

Figure 3. Nax-positive glial cells associate with multiple neurochemical circuitries of the subfornical organ. GFP fluorescence of GAD (A), Texas-Red fluorescence of Nax (B), and merged images (C). Tissue sections derived from GAD67-GFP mice were stained with anti-Na_x antibody and visualized with Texas-Red. Tissue sections 50 μm thick were penetrated with a detergent to enhance Na_x-signals. White arrows in C indicate GAD67-positive neurons enveloped with Na_x-positive glial cells. The area indicated by a white arrow with an asterisk is magnified in the inset of C. Dashed line in C indicates the boundary between the fornix and SFO. Scale bars: 50 μm.

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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. (2006). Sodium-level-sensitive sodium channel Nax is expressed in glial laminate processes in the sensory circumventricular organs. Am. J. Physiol. (Regul. Integr. Comp. Physiol.), *290*, R568-R576.

LABORATORY OF NEUROCHEMISTRY

Associate Professor: *SASAOKA, Toshikuni*

Our major research interest is to understand the physiological role of the 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 substitute the amino acid sequence of the target gene in particular cells. We analyze the physiological roles of the components of the dystrophin complex on the skeletal muscle membrane using genetically modified mice.

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 neurons. The dopaminergic system is also implicated in the control of emotion, motivation and cognition. Dysfunction of dopaminergic system can result in several neurological and psychiatric disorders such as Parkinsonís disease and schizophrenia.

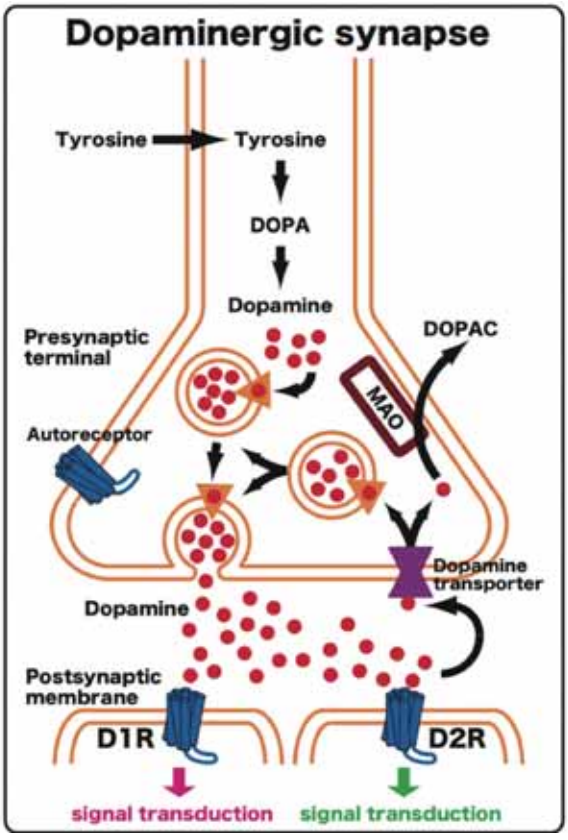

Figure 1. Schematic drawing of dopaminergic synapse

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 synergistic role. D1R has an opposite property to D2R with respect to the intracellular signal transduction.

In collaboration with the Laboratory of Director General we have been investigating the involvement of dopaminergic transmission via D1R and D2R in the regulation of locomotion and eating behavior. 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 the 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 the amount of transgene expression after Dox administration. We are analyzing these results to identify the mechanism of the relationship between the *D1R* expression and altered behavior. In addition we are investigating whether or not there is a critical period in development for the 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.

We develop conditional mutagenesis method in mice using Cre-loxP recombination. 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. This clearly indicates that the NMDAR activation by the critical amino acid substitution leads to the neurological phenotype.

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

III. Analysis of roles of the sarcoglycan complex, dystroglycan complex and caveolin-3

Sarcoglycans (SGs) are trans-sarcolemmal glycoproteins that associate together to form sarcoglycan complex (SGC) and are present in the sarcolemma. SGC, together with dystrophin and the dystroglycan complex, comprises the dystrophin complex, which is considered to be 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.

Dystroglycan (DG) is a transmembrane glycoprotein complex which plays an important role by connecting the intracellular cytoskeleton and the extracelluler matrix. DG is expressed as an 895 amino acid precursor and cleaved between amino acid residues 653 and 654 to generate α- and β-DG subunits. In collaboration with Dr. Torahiko Tanaka of Nihon University School of Medicine, Tokyo, in order to clarify the mechanisms involved in DG cleavage, we performed mutation analyses to determine which amino acid residues and which regions of DG are critical for cleavage. We transfected HEK 293 cells with wild-type and various mutant DGs, and confirmed the DG cleavage. We found that deletions in the upstream (within residues 550 to 645) and downstream (within residues 660 to 722) regions of the cleavage site abolished the cleavage. In contrast, deletion in the more upstream region (520-550) or downstream region (723-742) did not affect the cleavage. Because the critical regions comprise the epitopes for the association of α- (550-585) and β-DG (691-719), DG cleavage seems to be linked with the subunit association. By coexpression of α- and β-DG (not as a precursor), we confirmed the association of α- and β-DG subunits and found that deletions in the N-terminal region of β-DG deteriorate the subunit association. Furthermore, by alanine scanning around the cleavage site, we found that a mutation Trp659Ala completely abolished the cleavage as Ser654Ala did. We suggest that a relatively large portion of precursor DG (residues 550 to 722) forms a specific tertiary structure, in which the α and β-DG domains interact and the cleavage site becomes accessible.

Caveolin-3 is an integral membrane protein and a component of the dystrophin complex that serves as a scaffold of various molecules and is expressed in striated muscles. Its gene mutations cause limb-girdle muscular dystropy (LGMD1C or caveolinopathy) with mild clinical symptoms. In collaboration with Dr. Yasuko Hagiwara of the National Institute of Neuroscience, NCNP, Tokyo, we previously reported that caveolin-3 deficiency causes muscle degeneration and a decrease in sarcolemmal caveolae in *caveolin-3* gene-knockout (*Cav3*-/-) mice. To examine the pathogenic pathways and identify new or modifying factors involved in caveolinopathy, we examined the expression patterns of approximately 8,000 genes in the skeletal muscle of *Cav3*-/- mice using an oligonucleotide array. This data was compared to data from wild-type mice. The fold-ratio analysis suggested that approximately 400 genes, including the unknown genes examined, differ in expression levels. We focused on the *osteopontin* (*OPN*) gene that was under-expressed to the level of expression of the *Cav-3* gene. To examine the mechanism under-expression of *OPN*, we generated *dystrophin* and *Cav-3* double-deficient mice. Although *OPN* was overexpressed in the *dystrophin*-deficient *mdx* mice, simultaneous *Cav-3* deficiency resulted in a decrease in the *OPN* gene expression.

Publication List:

Original papers

Imai, F., Hirai, S., Akimoto, K., Koyama, H., Miyata, T., Ogawa, M., Noguchi, S., Sasaoka, T., Noda, T., and Shigeo, S. (2006). Inactivation of aPKC lambda results in the loss of adherens junctions in neuroepithelial cells without affecting neurogenesis in mouse neocortex. Development *133*, 1735-1744.

Ishii, Y., Oya, T., Lianshun, Z., Gao, Z., Kawaguchi, M., Sabit, H., Matsushima, T., Tokunaga, A., Ishizawa, S., Hori, E., Nabeshima, Y., Sasaoka T., Fujimori, T., Mori, H., and Sasahara, M. (2006). Mouse brains deficient in neuronal PDGF receptor-beta develop normally but are vulnerable to injury. J. Neurochemistry *98*, 588-600.

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 ìmi nd.î There are many approaches to elucidating the mechanism of ìm indî function, including research into human 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. The promotion of brain research is considered an important issue in many countries including Japan.

Our approach to understanding the brain is to explore the molecular mechanism of higher brain function regulating animal behavior 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 the dopaminergic system can result in neurological and psychiatric disorders such as Parkinsonís 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 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 a tetracycline controllable expression system and generated transgenic mice harboring conditional *D1R* expression on the *D1R/D2R* DKO background (Figure 1).

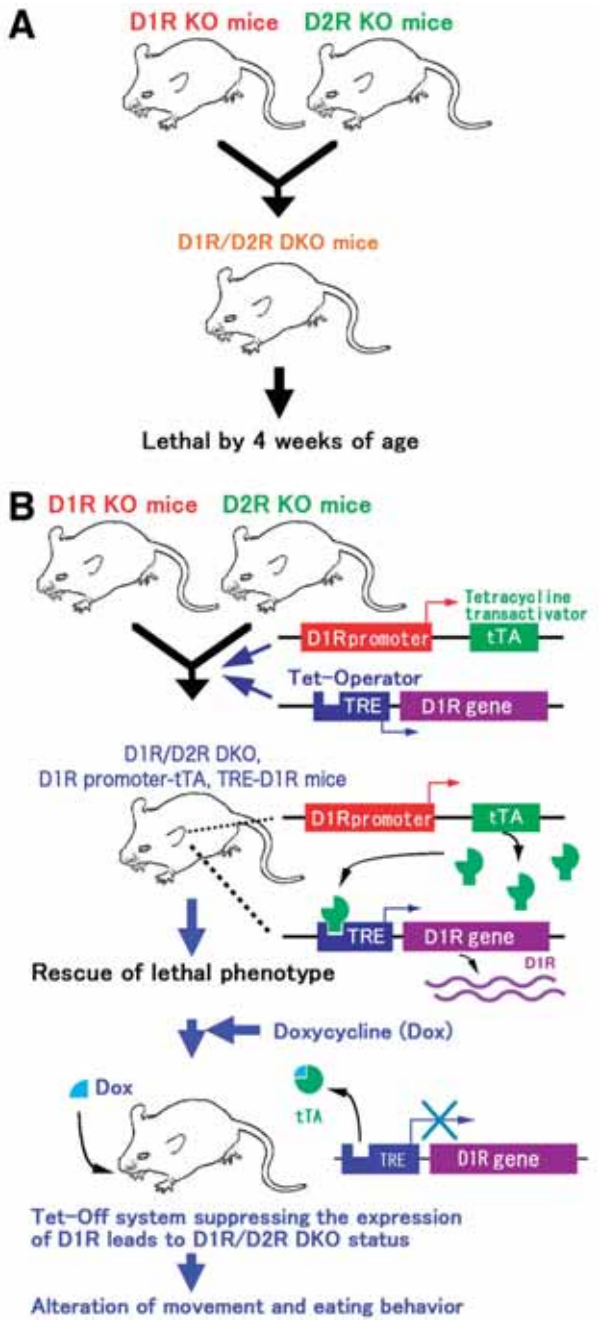

Figure 1. *D1R/D2R* DKO mice were rescued by conditional *D1R* expression. (A) *D1R/D2R* DKO mice showed impairment in locomotion and feeding and died prematurely. (B) To rescue *D1R/D2R* DKO mice, transgenic mice harboring conditional *D1R* expression on the *D1R/D2R* DKO background were generated by 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 (Figure 2) and decrease in locomotion and food/water intake by doxycycline (Dox) administration (Figure 3). These results indicate that areas harboring Dox-controllable *D1R* expression are responsible for the regulation of locomotion and eating behavior.

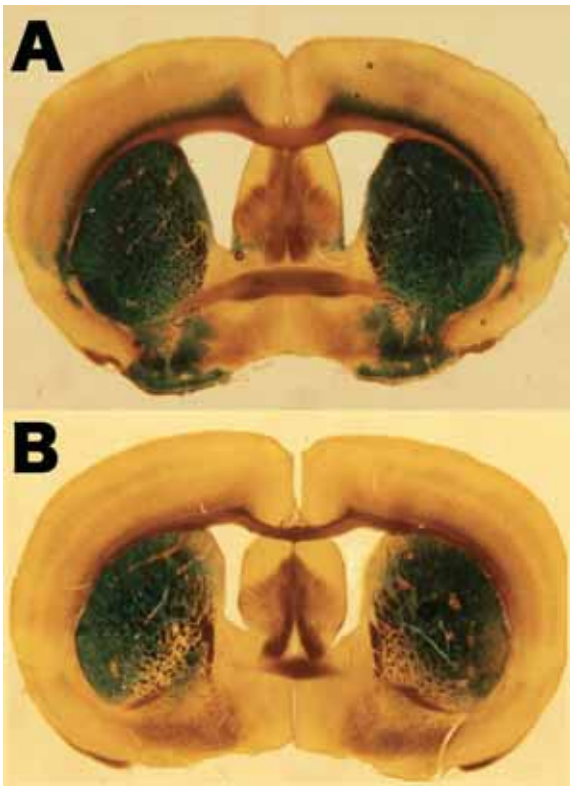

Figure 2. (A) Before doxycycline (Dox) administration the intensive expression of transgene was seen in the striatum of the *D1R/D2R* DKO-*D1R* rescued mice. (B) The amount of transgene expression was suppressed in the striatum by Dox administration. Frontal sections of mouse brains with X-gal staining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

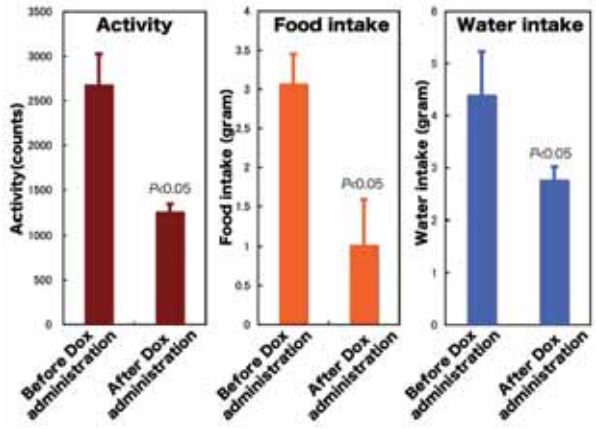

Figure 3. Alteration of locomotion, eating and drinking of *D1R/D2R* DKO-*D1R* rescued mice by Dox administration. Locomotive activity, food intake and water intake of the mice before and after Dox administration are shown.

II. Analysis of the function of NMDARs

The NMDARs are widely expressed in the nervous system, are 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 in order to assess behavioral alteration of the $NR2A^{-/-}$,$NR2B^{+/-}$ mice and study the molecular mechanism relationship between the mutation of NMDAR genes and altered behavior.

III. Analysis of roles *ras* family 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. We generated H-, N-, K-*ras* KO mice, and discovered that H-*ras* was involved in synaptic transmission and plasticity in the hippocampus and that K-*ras* was essential for normal development and involved in the survival of embryonic motoneurons. 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

Isono, K., Nemoto, K., Li, Y., Takada, Y., Suzuki, R., Katsuki, M., Nakagawara, A., and Koseki, H. (2006). Overlapping roles for homeodomain-interacting protein kinases hipk1 and hipk2 in the mediation of cell growth in response to morphogenetic and genotoxic signals. Mol. Cell. Biol. *26*, 2758-2771.

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DIVISION OF MOLECULAR GENETICS

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The main interest of this division is 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. Of these, spontaneous mutants with various flower colors of *I. nil* and *I. purpurea* have been isolated and cultivated since the 17th century in Japan and Europe, respectively. The wild-type *I. nil* and *I. purpurea* display blue and dark-purple flowers, respectively, both of which contain polyacylated and polyglycosylated cyanidin-based anthocyanins, and both plants exhibit red stems and dark-brown seeds (Figure 1). Almost all structural genes that encode enzymes to produce anthocyanidin 3-*O*-sophorosides for their flower pigmentation have been characterized, and the majority of their spontaneous mutations have been shown to be caused by insertions of DNA transposons. The transcriptional regulators for anthocyanin biosynthesis,

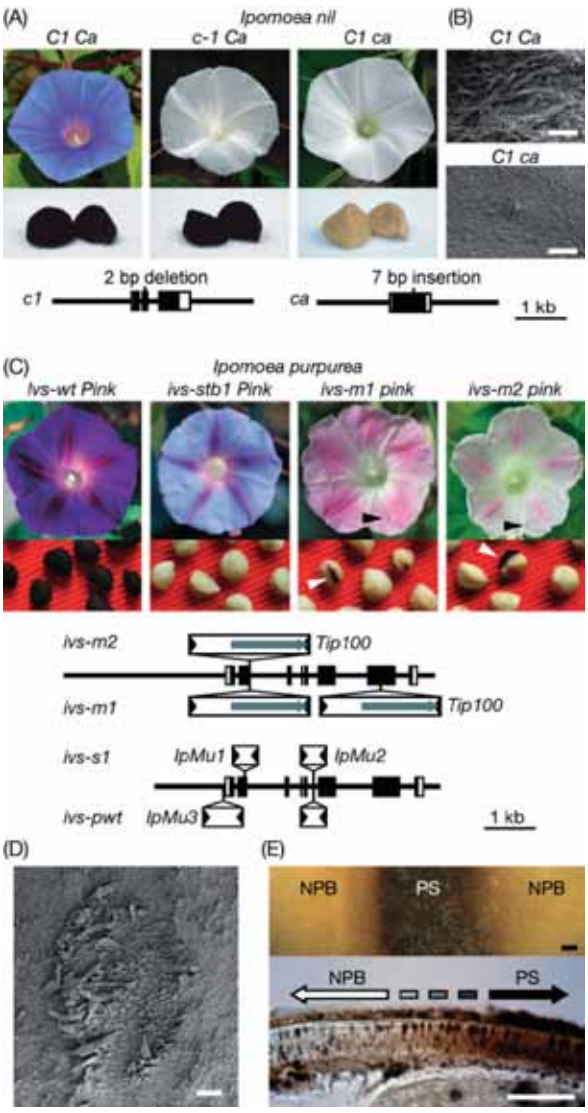

Figure1. Flower and seed phenotypes. (**A**) *I. nil*. (**B**) Scanning electron microscope (SEM) photographs of seeds. (**C**) *I. purpurea*. The black and white arrowheads indicate pigmented spots in flowers and seeds, respectively. (**D**) SEM photograph of small dark-brown reversion spot of a mature *ivs-m2* seed. (**E**) Magnified view (upper panel) and histological observation (lower panel) of the boundary region of a dark-brown reversion spot in the seed coats of the mutable PR43 *ivs-m2* line. The filled and open arrows with PS and NPB or grey broken bars indicate a pigmented spot and a non-pigmented background or a pigment-diffused area(s) on the seed coat, respectively. The scale bars indicate 50 μm.

which activate the structural genes, are known to include members of protein families containing R2R3-MYB domains, bHLH (basic helix-loop-helix) domains, and conserved WDR (WD40 repeats). Spacial and temporal expression of these structural genes encoding the enzymes for anthocyanin biosynthesis is determined by combinations of the R2R3-MYB, bHLH, and WDR factors and their interactions. In addition, their combinations and interactions also determine the set of genes for certain epidermal traits to be expressed.

In *I. nil*, the *c* mutants display white flowers with red stems and colored seeds, whereas the *ca* mutants exhibit white flowers with green stems and ivory seeds (Figure 1).

We showed that the recessive *c-1* and *ca* alleles are frameshift mutations caused by a 2-bp deletion and 7-bp insertions in the genes for the R2R3-MYB and WDR transcriptional regulators designated as InMYB1 and InWDR1, respectively. In the 2-bp deletion, dinucleotide AG was removed from a simple repeat sequence, AGAGAG. Regarding the 7-bp insertions, it is likely that they are footprints generated by independent excisions of a DNA transposon because the wild-type sequence, TAC, had changed into TAC(GGAG/TCCG)TAC. In addition to defects in flower, stem, and seed pigmentations, the *ca* mutants showed reduced trichome formation in seeds. Except for *CHS-E* in *ca* mutant, all structural genes tested coordinately reduced in both *c-1* and *ca* mutant flower limbs. However, slight but significant expression of *CHS-D*, *CHI*, and *F3H* for flavonol biosynthesis was detectable in *c-1* and *ca* mutants, whereas no such residual expression could be observed in other genes involved in the later anthocyanin biosynthesis pathway.

Spontaneous *ivory seed* (*ivs*) mutants of *I. purpurea* displaying pale pigmented flowers and ivory seeds are caused by insertions of DNA transposons in the *hAT* and the *Mutator* families into the *bHLH2* gene encoding a bHLH transcriptional regulator (Figure 1). A partial reduction in the expression of all structural genes for anthocyanin biosynthesis was observed in the young flower buds of these *ivs* mutants, whereas no reduction of *GST* was observed. The *bHLH2* expression appears to precede the expression of the structural genes for the proanthocyanidin biosynthesis in the seed coats. Interestingly, *CHS-E* rather than *CHS-D* is predominantly expressed in the seed coats, indicating that *CHS-E* is the first committed step of the proanthocyanidin biosynthesis pathway. The *DFR-B* and *ANS* transcripts were completely abolished in the *ivs* seed coats, whereas the early biosynthetic genes for flavonol biosynthesis remained active. The production and accumulation of both proanthocyanidin and phytomelanin pigments in their ivory seed coats were drastically reduced. Moreover, the unbranched trichomes in their ivory seeds were smaller in size and fewer in number than those in the wild-type dark-brown seeds, and the surface of the epidermis without trichomes in the dark-brown seeds looked rougher, due to the protruding tangential walls, than that in the ivory seeds. Combined with the results obtained in *I. nil* and *I. purpurea*, both bHLH2 and WDR1 transcriptional regulators in *Ipomoea* must control the biosynthesis and/or accumulation of anthocyanin, proanthocyanidin, and phytomelanin pigments as well as the formation of seed trichomes.

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 worldís population and a model plant for other cereal species. We have developed efficient and reproducible gene targeting by homologous recombination with a large-scale *Agrobacterium*-mediated transformation and a strong positive-negative selection and succeeded in modifying the *Waxy* gene without the concomitant occurrence of ectopic events. While *Waxy* is a unique gene in the rice genome, 4 copies of the *Adh* gene are present, and *Adh1*, *Adh2* and *Adh3* reside on chromosome 11 in the same orientation, and highly repetitive *Copia*- and *Gypsy*-like retroelements are present adjacent to *Adh1* and *Adh2* (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. We are attempting to modify the *Adh1* and *Adh2* genes, the coding sequences of which are similar to each other. While we were able to obtain transgenic plants having either *Adh1* or *Adh2* modified without the concomitant occurrence of undesirable ectopic events, the targeting frequency of *Adh1* appeared to be about one magnitude lower than those of *Waxy* and *Adh2,* indicating that *Adh1* appears to be a cold spot(s) for homologous recombination. To examine whether the repetitive *Gypsy*-like sequence precludes efficient homologous recombination-promoted gene targeting due to the occurrence of ectopic recombination, we used a vector for *Adh1* modification with short homologies lacking the *Gypsy*-like sequence and found no significant effects on targeting efficiency. The obtained *adh1* disrupted mutants showed very similar phenotype to the previously isolated *adh1* mutant. We also characterized structure and expression of the newly identified *Adh3* gene.

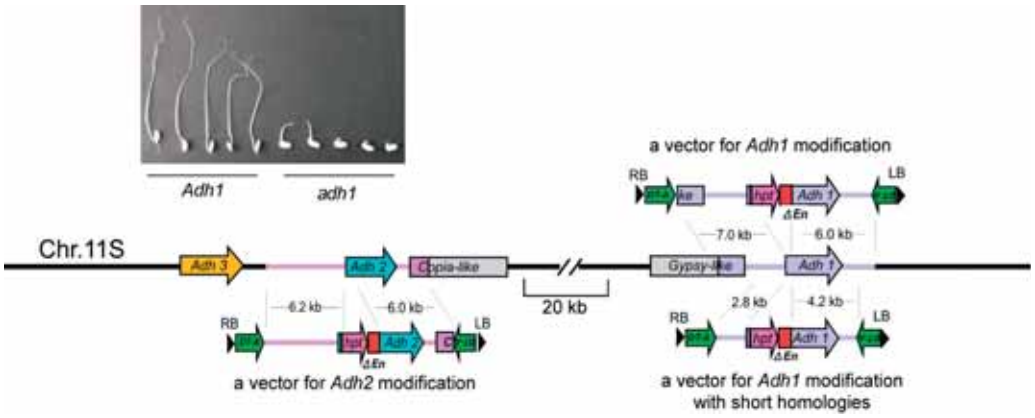

Figure 2. Strategy for gene targeting of the *Adh1* and *Adh2* genes in rice. The gene symbols *hpt* and *DT-A* on the T-DNA regions of the vectors used indicate the positive and negative selection markers, respectively. The sheedlings of the wild type and *adh1* mutants under the submerged condition are shown above the map.

III. Characterization of mutable *virescent* allele in rice

We have identified an active rice transposon *nDart1* as a causative transposon of a mutable *virescent* allele *pyl-v* conferring pale yellow leaves with dark green sectors in its seedlings (Figure 3A). The transposition of *nDart1* can be controlled under natural growth conditions; its transposition can be induced by crossing with a line containing an active autonomous element *aDart* and stabilized by segregating *aDart*. While the cultivar Nipponbare does not carry the active *aDart* element, 5-azaC treatment induces transposition of the *nDart1* elements, suggesting that a dormant or an epigenetically silenced autonomous element(s) is present in the genomes. While tissue culture is necessary in all of the currently available rice reverse genetic approaches including transposon tagging systems employing exogenous or endogenous transposons, no somaclonal variation should occur in our *nDart/aDart* system because no tissue culture is involved in its activation. We examined the *nDart*-related elements in the Nipponbare genome with the latest 4.0 pseudomolecules (Figure 3). The *nDart*-related elements can be classified into three subgroups of about 0.6 kb nonautonomous elements (*nDart1-3*, *nDart1-101*, and *nDart1-201*) and four subgroups of elements longer than 2 kb (*iDart1/dDart1*, *iDart2*, *iDart3*, and *iDart4*) on the basis of their lengths and sequence characteristics. The copy numbers of the small *nDart1* elements of about 0.6 kb and longer *iDart/dDart* elements are 18 and 63, respectively, and both elements are distributed throughout chromosomes. We are currently developing an *nDart*-mediated transposon tagging system in rice and obtaining interesting mutants with new mutable alleles.

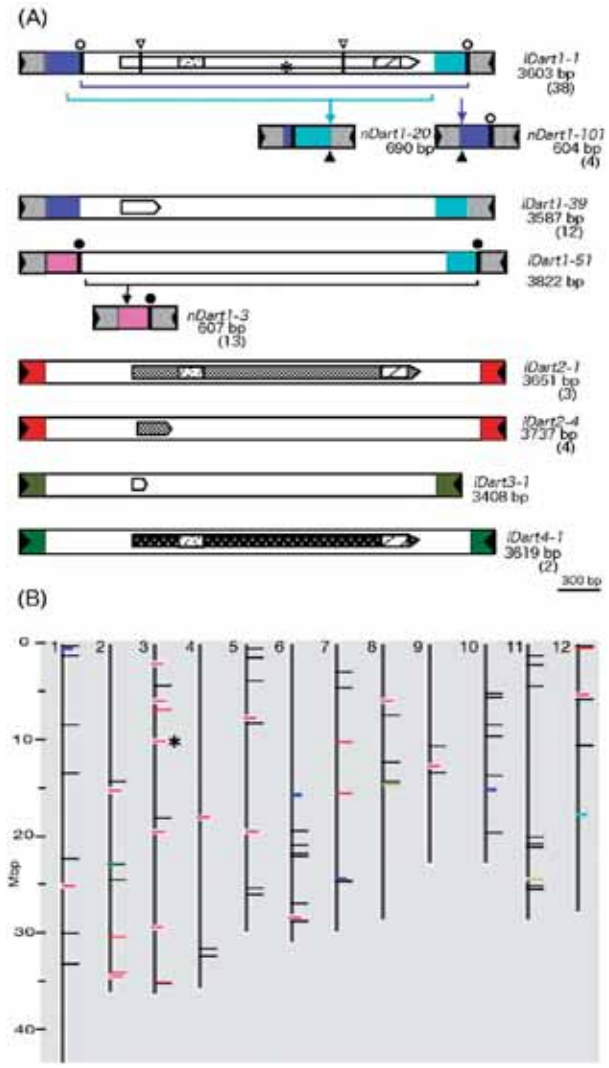

Figure 3. (**A**) Structures of *nDart*-related elements. Only typical structures in each representative element with its length are shown, and the numerals in parentheses indicate the copy numbers of a group of elements with closely related sequences. The horizontal filled arrowheads and the shadowed boxes at both ends indicate the terminal inverted repeats (TIRs) and subterminal regions. (**B**) Localization of *nDart*-related elements in Nipponbare. The short bars with pink-, dark-, and light-blue represent *nDart1* elements in the subgroups *nDart1-3*, *nDart1-101*, and *nDart1-201*, respectively, whereas the long bars with black, red, lime, and green indicate *iDart1/dDart1*, *iDart2*, *iDart3*, and *iDart4*, respectively. The asterisk indicates *nDart1-0* at the *pyl-v* allele. The open circles indicate the position of centromere.

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Original papers

Tsugane, K., Maekawa, M., Takagi, K., Takahara, H., Qian Q., Eun, C.H., and Iida, S. (2006). An active DNA transposon *nDart* causing leaf variegation and mutable dwarfism and its related elements in rice. Plant J. *45*, 46-57.

Morita, Y., Saitoh, M., Hoshino, A., Nitasaka, E., and Iida, S. (2006). Isolation of cDNAs for R2R3-MYB, bHLH, and WDR transcriptional regulators and identification of *c* and *ca* mutations conferring white flowers in the Japanese morning glory. Plant Cell Physiol. *47*, 457-470.

Furukawa, T., Maekawa, M., Oki, T., Suda, I., Iida, S., Shimada, H., Takamure, I., and Kadowaki, K. (2006). The *Rc* and *Rd* genes are involved in proanthocyanidin synthesis in the rice pericarp. Plant J. *49*, 91-102.

Review articles

Chopra, S., Hoshino, A., Boddu, J., and Iida, S. (2006). Flavonoid pigments as tools in molecular genetics. In The Science of Flavonoid, E. Glotewold ed., (Springer), pp. 147-173.

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The genomes of higher organisms contain significant amounts of repetitive sequences which are, in general, unstable. At present, neither the physiological function(s) of these repeated sequences, nor the mechanisms responsible for their production or for controlling instability are fully understood. To clarify these aspects, we are pursuing several lines of investigation using *Escherichia coli*, *Saccharomyces cerevisiae* and Chinese Hamster Ovary (CHO) cells. In 2006 we discovered a new role of condensin in maintaining a long repeated structure of rDNA. We also addressed the question of why almost all genomes in prokaryotes are linear. To approach this, we attempted to linearize the circular *E. coli* genome by utilizing the linearization system of a lysogenic *E. coli* phage N15. Following the success of this endeavor, we are now comparing the properties of the linear and the circular genome strains.

I. Analysis of mechanisms maintaining the repeated structure of ribosomal RNA genes

In most eukaryotes, the rDNAs are clustered in long tandem repeats on only one or a limited number of different chromosomes. Although the total number of these rDNA repeats appears to be maintained at a level appropriate for each organism, gene 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 *Saccharomyces cerevisiae*, 200 copies on average are tandemly arrayed in a central position on the longest 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 replication fork blocking activity at replication fork barrier (RFB) sites, rDNA region-specific recombination and expansion/contraction of rDNA repeats. The latter two activities are likely to be triggered by double-strand breaks at the RFB sites and repair of the breaks via gene conversion. Thus, this *FOB1*-dependent recombination mechanism apparently contributes to the maintenance of average copy number of rDNA. However, in *Δfob1* cells, the repeats are still maintained without any fluctuation of copy number. This suggests that there is another, so far unidentified system acting to prevent contraction of the number of repeats.

In order to explore such a putative second system, we collected a number of mutants in which the rDNA copy number decreased drastically under *Δfob1* conditions. Among these, we found condensin gene mutants, suggesting that, in addition to condensation and separation of chromosomes in M phase, condensin plays an important role in maintaining the repeated structure of rDNA. Each of the genes encoding a condensin subunit is known to be essential for growth, but here isolated condensin mutations are not strict but are of the leaky type. Analyzing double mutants and examining specific interactions between condensin and rDNA regions revealed that (1) in the double mutants, the copy number of rDNA was dramatically decreased, (2) condensin complexes associated with the RFB region in a *FOB1*-dependent manner (Figure 1B and C), (3) the association between condensin and the RFB was established during S phase and was maintained until anaphase, (4) double mutants showed slow growth which may be caused by defects in the separation step of the long rDNA array in anaphase. These results strongly suggest that *FOB1*-dependent condensin association with the RFB region is required for efficient segregation of rDNA repeats.

Recently we found that an RNA polymerase I (PolI) defective mutation suppressed the dramatic reduction of

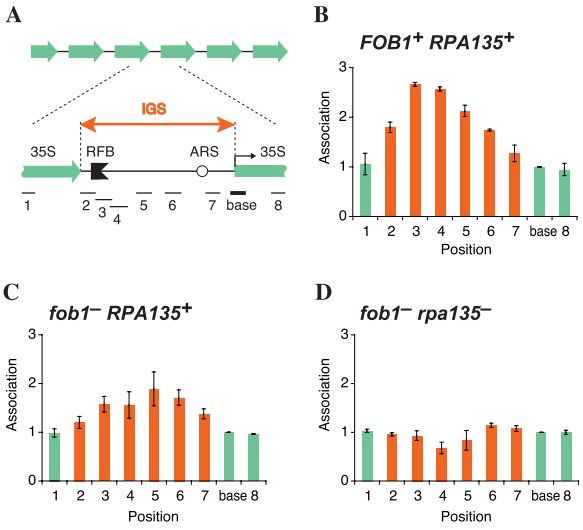

Figure1. Condensin relative association patterns with rDNA in a wild-type strain, *fob1*, and *fob1 rpa135* (PolI defective) mutants.

(A) The structures of the rDNA tandem array and an enlarged intergenic spacer sequences (IGS) between the 35S coding regions are shown. Within the IGS, the replication fork barrier (RFB) site and autonomous replication sequence (ARS) are located, respectively. The positions of the PCR fragments (1~8 and base fragment) used for chromatin immuno- precipitation (ChIP) assay are indicated as short bars under the map. Condensin association with rDNA in (B) wild-type strain, (C) *fob1* strain and (D) *fob1 rpa135* (PolI defective) double mutant.

the rDNA copy number in the condensin and *fob1* double mutant. Because PolI is an rDNA specific transcription enzyme, ΔpolI defective mutants are non-viable. However, if the defective cells carry a plasmid in which rDNA is inserted downstream of a Gal-dependent promoter, they become viable in the presence of galactose. Using this ΔpolI mutant, we examined effect of PolI enzyme on condensin association with rDNA. Under *fob1* conditions, Figure 1 C and D show that PolI enzyme seems to force condensin from transcribing to non-transcribing (IGS) region. This characteristic change of pattern suggests that constant PolI transcription throughout the cell cycle prevents condensin from associating with the transcribing region of rDNA. Thus, it would be expected that in the triple (*fob1*, condensin and PolI) mutant, the partially defective condensin which is uniformly associated with the rDNA region, would facilitate successful separation between the replicated long rDNA region in anaphase.

In higher eucaryotes, it is well known that M phase-specific repression of gene expression (mitotic repression) occurs, though the reason for this remains unknown. Our results suggest that mitotic repression allows condensin to associate uniformly with whole chromosomes, which ensures their successful condensation and subsequent separation. In any event, genetic and molecular analyses of highly specific chromosomal regions, such as the rDNA regions, provide useful data which help us to understand the nature of normal (non-rDNA) chromosomal regions..

II. *E. coli* with a linear genome

There are two types of chromosomal structure, linear and circular. While all chromosomes in eukaryotes are linear, those in almost but not all prokaryotes are circular. Thus, we can readily understand why almost all mitochondrial and chloroplast genomes are also circular, as both are inferred to be derived from some kind of bacteria. However, the reasons why the two main biological kingdoms have so distinctly different genome structures, or why there are, although extremely rare, exceptions (bacteria with linear genomes) remains unresolved. In order to address this, linearization of the circular genome of bacteria and comparison between circular and linear bacteria with identical genetic backgrounds seems a rational strategy.

We therefore attempted to linearize the circular genome of *Escherichia coli,* as there are in fact two types of exception bacteria with naturally linear genomes. One type, represented by the filamentous soil bacteria *Streptomyces* species, possesses a protein-designated ìt erminal proteinî (TP) that is covalently joined to the 5í ends of both termini of the genome. The second type, exemplified by the spirochete *Borrelia burgdorferi*, has telomeres with covalently closed hairpin structures at their termini. The ends of linear *Borrelia* chromosomes are similar to those of the linear *Borrelia* plasmid, the *E. coli* phage N15 and certain animal viruses such as the poxvirus. Especially N15 attracted our attention, because this phage is very similar to the λ phage in many aspects,

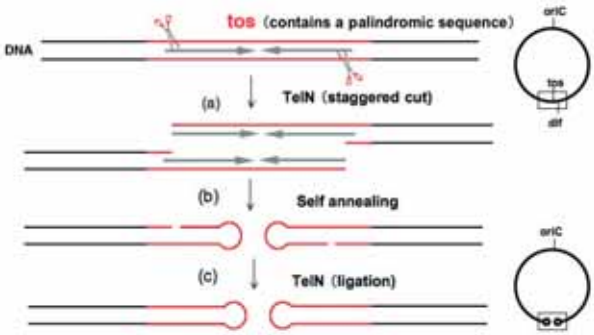

Figure2. Linearization of *E. coli* circular genome by TelN protein.

Locations of the *tos*, *oriC* and *dif* sites are shown on the *E. coli* circular genome in the right side figure. A palindrome sequence in the *tos* is represented by a pair of long arrows. (a) TelN cleavages the *tos* sequence at staggered positions, shown by two pairs of scissors. (b) Each single-stranded sequence exposed at the termini is self-annealed and (c) each gap left is sealed by TelN, producing a terminus with a hair-pin structure.

such as genome size, in having cohesive ends and so on. However, the lysogenization process is different; while λ phage lysogenizes by integration of the circular genome into the host chromosome, N15 lysogenizes by converting a circular genome into a linear one. For the linearization, the mechanism of which has been analyzed mainly by Russian investigators, two components - the *tos* site and protelomerase (TelN) protein - are required. Thus, in order to linearize the *E. coli* chromosome, we inserted the *tos* sequence into a replication termination region of the *E. coli* genome and then supplied TelN protein by introducing a plasmid with the telN gene or lysogenizing the N15 phage itself. In either case, the *E. coli* circular genome was confirmed to be linearized probably by the mechanism shown in Figure 2. Thus, *E. coli* with a linear genome is viable. In addition, we found that the linear state and whole genome structure are very stable, and that the linear genome clone is pure, probably not contaminated with circular genome cells. There were no appreciable differences between cells with linear and circular genomes in growth rates, cell and nucleoid morphologies, genome-wide gene expression (with a few exceptions), or DNA gyrase- and topoisomerase IV-dependency.

One structural difference to be expected is that the circular, but not the linear genome can form a dimer. With a circular chromosome, a dimer is formed through an odd number of recombination events between the sister chromosomes, but with linear genomes no dimer can be produced in this way even if sister-chromosomal recombination occurs. In *E. coli*, the dimer chromosome is monomerized by *dif* site-specific recombination catalyzed by *cer*-specific recombinase C and D (XerCD) and FtsK proteins. Thus, on a wild-type background, *dif* or *xerCD* mutants show slower growth, producing more elongated cells. We found that under *dif*-defective conditions, only cells with a circular genome but not cells with a linaer genome developed abnormal phenotypes.

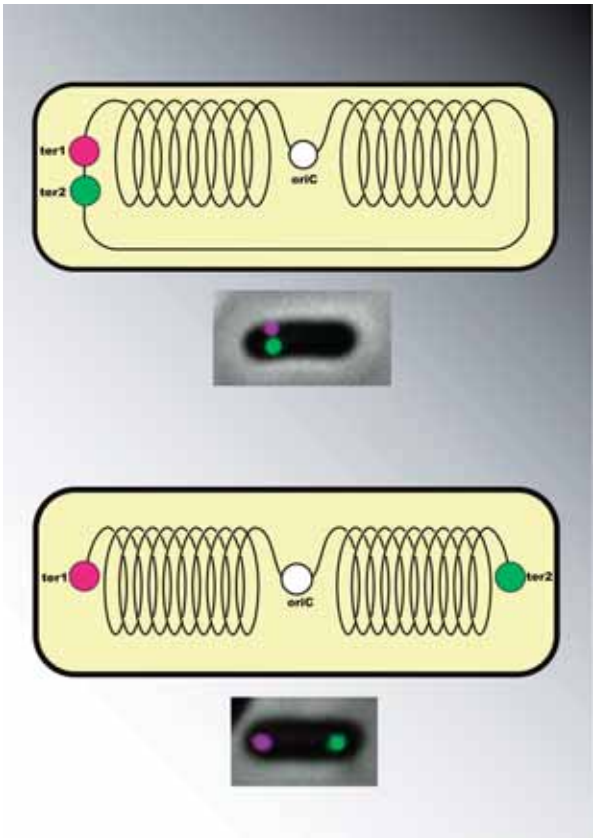

Figure3. Models showing locations of the two terminus sites (*ter1* and *ter2*) on the circular and the linear genomes in new born cells.

The *ter1* and *ter2* sites are located at ñ 20 kb and + 20 kb remote from the *tos* site.

In *Streptomyces* cells, each end of the linear genome was found to be associated with the other, probably through the two TPs. Thus, we tried to locate the position of each terminus of the *E. coli* linear genome in cells. To this end, we investigated the position of fluorescence foci of cyan and yellow fluorescent protein derivatives of the LacI and TerR repressors binding to their operator arrays inserted into two sites corresponding to the two termini of the linear genome. Microscopy indicated that while the two sites of the circular genome were close or overlapping in any cell cycle phase, those of the linear genome were separated and located at each end of a new born cell (Figure 3).

The termini of the linear gnome we constructed here are much closer to the *dif* site, the site directly opposite to the replication origin *oriC* site. If the ends are farther away from *dif*, does any phenotypic change occur? To examine this, the *tos* site was moved to five different positions on the chromosome and the growth of these cells was examined after N15 lysogenization (linearization). Those strains with genome termini the more remote from the *dif* site showed greater growth deficiencies and in an extreme case where the terminus was closest to *oriC* (approximate distance 500kb), lethality. This correlation might be caused by unbalanced replication of a pair of chromosome arms of different lengths.

From above results, the following conclusion can be drawn. (1) There were no obvious distinct differences between *E. coli* strains with circular or linear genomes. Therefore, there are at least two further conclusions from this: (a) presence of circular and linear genomes may not be inevitable, but accidental; and (b) *E. coli* with a linear genome as constructed here can survive in nature. (2) These findings raise the possibility that the topology of linear and circular genomes is not so different within the cell. (3) Two terminus ends of linear genomes separate and locate at terminus ends of the cell. Because the DNA molecule cannot condense itself, certain protein(s) must be involved. (4) The linearization system developed here could be a powerful technique in genome technology.

These results will be published in EMBO Reports (Cui et al., 2007, *8*, 181-187).

Publication List:

Original papers

Hayashi, K., Morooka, N., Yamamoto, Y., Fujita,K., Isono, K., Choi, S., Ohtsubo, E., Baba, T., Wanner, B.L., Mori H., and Horiuchi, T. (2006). Highly accurate genome sequences of *Escherichia coli* K-12 strains MG1655 and W3110. Mol. Systems Biol. doi:10.1038 msb100049:E1-E5

Inagaki, S., Suzuki, T., Ohto, M., Urawa, H., Horiuchi, T., Nakamura, K., and Morikami, A. (2006). *Arabidopsis* TEBICHI, with helicase and DNA polymerase domains, is required for regulated cell division and differentiation in meristems. Plant Cell *18*, 879-892

Johzuka, K., Terasawa, M., Ogawa, H., Ogawa,T., and Horiuchi, H. (2006). Condensin loaded onto the replication fork barrier site in ribosomal DNA (rDNA) repeats during S-phase in a *FOB1*-dependent fashion to prevent contraction of a long repeats in *S. cerevisiae*. Mol. Cell. Biol. *26*, 2226-2236.

Riley, M., Abe, T., Arnaud, M.B., Berlyn, M.K., Blattner, F.R., Chaudhuri, RR., Glasner, J.D., Horiuchi, T., Keseler, I.M., Kosuge1, T., Mori, H., Perna, N.T., Plunkett III, G., Rudd, K.E., Serres, M.H., Thomas, G.H., Thomson, N.R., Wishart, D., and Wanner, B.L. (2006). *Escherichia coli* K-12: a cooperatively developed annotation snapshot ñ 2005. Nucleic Acids Res. *34*, 1-9.

DIVISION OF EVOLUTIONARY BIOLOGY

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	WATASE, Masahiro
Secretary:	*KOJIMA, Yoko*

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. 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* was performed. 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. We identified 58 cDNAs whose overexpression caused the defects in asymmetric cell divisions in two repeated experiments. We knocked in a cytrin gene just before the stop codon of each candidate gene and examined the cellular localization of a fused protein under its native promoter. Thus far, we have examined 32 of 58 candidates and nine fused proteins were detected to be specifically localized in an apical meristematic cell. Further characterization of these genes by the overexpression of the genes in protoplasts with GFP-tubulin or GFP-talin and the loss-of-function experiments using RNAi are now in progress. Functional analyses of these genes should help us to understand the molecular mechanisms of how plants generate distinct meristematic cell lineages to build their multicellular bodies. This work was performed as a collaborative work with Dr. Tomomichi Fujita (Hokkaido University).

II. 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 determine the morphology of differentiated tissues and organs.

2-1 Mechanism of cytokinesis

The cells of land plants and their sister group charophycean green algae divide by the insertion of cell plates at cytokinesis. This is in contrast to other green algae, in which the invagination of plasma membrane separates daughter cells at cytokinesis. The cell plate appears in the middle of daughter nuclei, expands centrifugally towards a cell periphery, and finally fuses to a parental cell wall. Cell wall materials are transported to the expanding cell plate with a phragmoplast, which is mainly composed of microtubules. A centrifugal expansion of the phragmoplast is a driving force for that of the cell plate, although the molecular mechanism for the centrifugal expansion of the phragmoplast was a challenge. We observed tobacco BY-2 cells expressing GFP-α-tubulin with live imaging technique and found that a new microtubule at the margin of the phragmoplast appeared at the side of an existing phragmoplast microtubule. We further confirmed the microtubule appearance site using a live imaging of tobacco BY-2 cells expressing GFP-AtEB1b, a marker of growing microtubule plus ends. γ-tubulin, an essential component of a protein complex for microtubule nucleation, was concentrated at the base of newly formed microtubules. We propose a hypothesis that cytosolic γ-tubulin complexes are recruited onto existing phragmoplast microtubules and nucleate new microtubules as branches by a similar mechanism to the cortical microtubule formation at interphase (Fig.1). Detailed analysis of

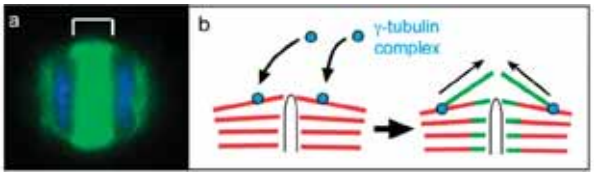

Figure 1. Phragmoplast development in flowering plants. a). A phragmoplast of tobacco BY-2 cells visualized with anti-α-tubulin antibody (green). Daughter nuclei are stained with DAPI (blue). A schematic explanation of a microtubule formation in a white bracket is shown in b. b). A scheme of microtubule nucleation during phragmoplast expansion. Red: preexisting microtubules. Green: newly formed microtubules. Blue: γ-tubulin complexes.

microtubule dynamics and functional analyses of γ-tubulin are in progress. T. Murata mainly performed this study.

III. Evolution of molecular mechanisms in plant development

3-1 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 focus of 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-like protein (API1) and ubiquitin-like protein (PUBL1), as well as an unknown protein. Functional analyses of these genes are currently under investigation, mainly by Y. Hiwatashi. A distortion of phragmoplast was observed in double disruptants of *API1* and its sister gene. This suggests that these kinesin-like proteins are indispensable for the proper formation of phragmoplast. On the other hand, double disruption of *PUBL1* and its sister gene, *PUBL2,* retarded the collapse of phragmoplasts, suggesting that these ubiquitin-like genes likely regulate the stability of phragmoplast microtubules. Further analyses are in progress.

3-2 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 were probably co-opted 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 *Arabidopsis thaliana* pollen, male gametophyte, were analyzed, mainly by N. Aono. Four of eight genes belonged to MIKC*-type MADS-box genes and quadruple disruptants of these genes were formed by multiple crossings of four single disruptants. The quadruple disruptants showed a defect in pollen germination both in vivo and in vitro.

3-3 Function of class 1 KNOX genes in a moss *Physcomitrella patens*

We performed a functional analysis of class 1 KNOX genes to investigate the co-option hypothesis on the evolution of body plans in sporophyte generation.

The shoot apical meristem repeatedly forms stem and lateral organs, such as leaves, in vascular plant sporophytes. The sporophyte of a moss forms a single sporangium without lateral organs, while leafy shoots are formed in the gametophyte generation. We aimed to reveal whether or not the homologues of vascular plant genes are involved in shoot apical meristem formation in gametophytic shoot formation in the moss *Physcomitrella patens* and whether or not the homologues are involved in sporophyte development of *P. patens* without leafy shoots. Recent genetic analyses in *Arabidopsis thaliana* showed that *SHOOT MERISTEMLESS*, which is a member of the class 1 KNOX subfamily of the homeobox gene superfamily, is involved in the formation and maintenance of the shoot apical meristem. Three class 1 KNOX genes, *MKN2*, *MKN4*, and *MKN5,* were isolated from *P. patens*. We inserted a GUS reporter gene at the end of every one of the three genes to investigate the expression patterns.

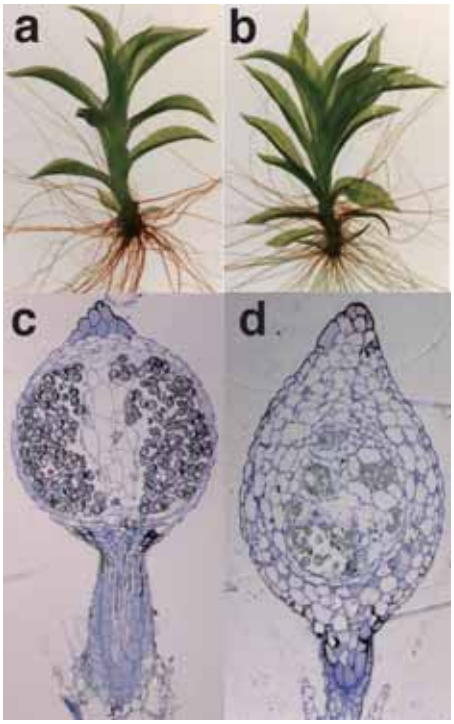

Figure 2. A leafy shoot (gametophore) of a wild type (a) and a triple class 1 KNOX disruptant (b). Longitudinal sections of sporophytes in the triple disruptant (c) and a wild type (d). Photos by Dr. Keiko Sakakibara.

The transgenic *P. patens* showed GUS activity in sporophytes but not in gametophytes, including leafy shoots. GUS activity was detected in an apical cell until the apical cell stops dividing. Later, GUS activity was detected in a seta meristem until a sporangium matured. We constructed triple disruptants of the three genes. They formed normal gametophytes, but aberrant sporophytes (Fig. 2). These results shows that the three class 1 KNOX genes function in the sporophyte development, but not in gametophytic shoot development. In spite of the significant morphological difference, KNOX class 1 genes are involved in the development of diploid generations in the moss and vascular plants, suggesting that the molecular mechanisms of gametophytic shoot formation are different from sporophytic shoot formation. We need to revisit the co-option hypothesis. This work is a collaboration with Dr. Keiko Sakakibara and Prof. Hironori Deguchi of Hiroshima University.

3-4 nuclear genome project of the moss *Physcomitrella patens*

A comparison of developmental genes among major land plant taxa would facilitate our understanding of their evolution, although it was not possible because of the lack of genome sequences in basal land plants. We established an international consortium for a genome project of the basal land plant; the moss *Physcomitrella patens* and its entire genome has been mostly sequenced as a collaborative work with the Joint Genome Institute at the U.S. Department of Energy. To facilitate the contig assembling and the gene annotation, we performed (1) the EST analyses of several libraries of cDNAs isolated from different developmental stages, (2) the construction of full-length cDNA libraries and sequencing in their full length, (3) the construction of BAC libraries and their end-sequencing, and (4) 5'-end serial analysis of gene expression (5' SAGE) as collaborative works with Dr. Tomoaki Nishiyama (Kanazawa Univ.), Prof. Asao Fujiyama (National Institute of Informatics), Prof. Sumio Sugano (Univ. Tokyo), and Prof. Yuji Kohara (National Institute of Genetics) groups. This work was mainly performed by T. Tanahashi.

We developed a system to construct phylogenetic trees efficiently with whole genome shotgun sequence data in public databases before their assembly. We collected homologs of approximately 700 *Arabidopsis thaliana* genes involved in development, and their phylogenetic analyses are in progress.

3-5 functional characterization of polycomb genes in the moss *Physcomitrella patens*

Polycomb group (PcG) proteins regulate chromatin modification and function as a cellular memory system to maintain the repressed state of developmental genes in both animals and plants. PcG genes are involved in phase changes of *Arabidopsis thaliana* development, such as vegetative to reproductive and haploid to diploid transitions. Bryophytes have dominant haploid generation, while sporophyte generation is dominant in angiosperms. The change of dominant generations was one of most conspicuous evolutionary aspects of land plants. To elucidate the molecular mechanisms underlying the evolution in alteration of generations, we characterized functions of PcG genes in *P. patens*. *A. thaliana CLF*, *MSI1*, *EMF2,* and *FIE* homologs were cloned in *P. patens*. We inserted a GUS reporter gene at the end of every one of the PcG genes to investigate the expression patterns. Disruptants for each gene were established and their characterization is in progress. This work was mainly performed by N. Aono.

IV. Molecular mechanisms of speciation

Sexual isolation is an important step for speciation, although the molecular mechanisms governing the isolation in plants are mostly unknown. A proper pollen tube guidance is essential for reproduction in angiosperms, and sexual isolation is often related to the arrest of guidance. In spite of the long history of studies on the pollen tube guidance, few guidance factors have been reported because of the difficulty of genetic analyses. We focused on receptor like kinases (RLKs), which function to receive extra cellular ligands and transmit the signal into a cell. We postulated that RLKs involved in pollen tube guidance are likely expressed more abundantly in pollen and/or pollen tube than in other tissue. Gene expression profiles between *A. thaliana* pollen and pollen tube were compared with those of other tissue using microarray. Pollen and Pollen tube expression profiles were similar to each other and 95 % of expressed genes were overlapped within the 4 fold differences. We focused on 45 RLKs predominantly expressed in pollen or germinating pollen to characterize signaling mechanisms during fertilization. Characterization of single and double T-DNA insertion lines are in progress. This work was mainly done by S. Miyazaki.

Publication List:

Original papers

Machida, M., Takechi, K., Sato, H., Chung, S.J., Kuroiwa, H., Takio, S., Seki, M., Shinozaki, K., Fujita, T., Hasebe, M., and Takano, H. (2006). Genes for the peptidoglycan synthesis pathway are essential for chloroplast division in moss. Proc. Natl. Acad. Sci. USA *103*: 6753-6758.

Shigyo, M., Shindo, S., Hasebe, M., and Ito, M. (2006). Phylogenetic analysis of AP2 domain-containing genes. Gene *366*: 256-265.

DIVISION OF SPECIATION MECHANISM

(ADJUNCT)

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Research Associate (Adjunct): *TAKAHASHI, Kazuhiko*
NIBB Research Fellow: *SASAKI, Takeshi*
Postdoctoral Fellow: *MIZOIRI, Shinji*
Technical Assistant: *SUZUKI, Tokiko*
Technical Assistant: *MIURA, Seiko*

During their long evolutionary history,, vertebrates 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. Speciation, therefore, is an important factor 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, particularly on a molecular level. The aim of our groupís research is to propose the processes and mechanism of the speciation of vertebrates using a molecular approach. To accomplish this aim, we chose the East African cichlid fishes as the model animals for our study of speciation (Figure 1).

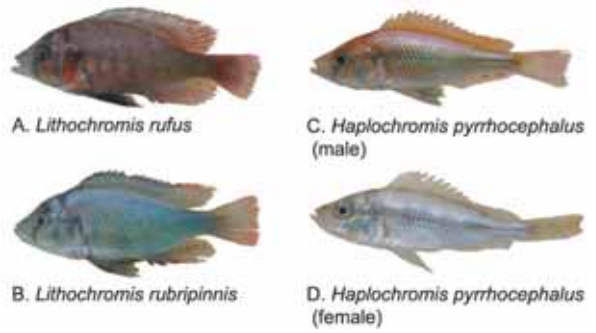

Figure 1. Cichlid fishes in Lake Victoria. These were caught in the field by our group.

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

Although cichlid fishes are broadly distributed in tropical regions throughout the world, our group focuses on the species that are endemic to three great lakes ñ Tanganyika, Malawi, and Victoria - and their drainages in East Africa. One thousand or more species of cichlid fishes live in the lakes. It is believed 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. The endemic cichlids of Lake Victoria diversified to 700 or more species from a small number of ancestors during its short history. This 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, the 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 the elucidation of their evolutionary history, we have seen that most of the selectively-neutral polymorphic alleles (presence/absence of retroposons at orthologous site in the genome) are retained both within and among the species of this lake, which is to say that 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 the 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

The lacustrine environment of Lake Victoria is highly diverse, thanks to area differences 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 habitats, our group and Dr. N. Okadaís laboratory (Tokyo Inst. Tech.) began conducting field investigations around Mwanza Gulf on the southern shore of Lake Victoria in August, 2004 (Figure 2). In 2006, two investigations were conducted, and we collected approximately 3,000 individual Victorian cichlids from various ecological habitats. These expeditions increased the number of species of these cichlids available for our study to approximately 150 (rough estimation). Pictures were taken of each individual, immediately after collection, to record their live colors. For the purpose of genetic analyses, fin clips from representative individuals were preserved in ethanol.

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 a visual system when they choose their mating partner, and such color variations are considered to affect the femaleís 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 the lake water. In a collaborative work with Dr. N. Okadaís laboratory at the Tokyo Institute of Technology, our group proposed that the 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 actual research in the field, our group focused on the evolution of opsin genes for the visual system in several Victorian cichlid species. *Lithchormis rufus* and *L. rubripinnis* (Figure 1A and B) inhabit shallow water near 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 an 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 the vision system to various light conditions, we are analyzing the six types of the opsin gene family. A more extensive analysis of the molecular evolution of opsin genes in Victorian cichlids is in progress in our division.

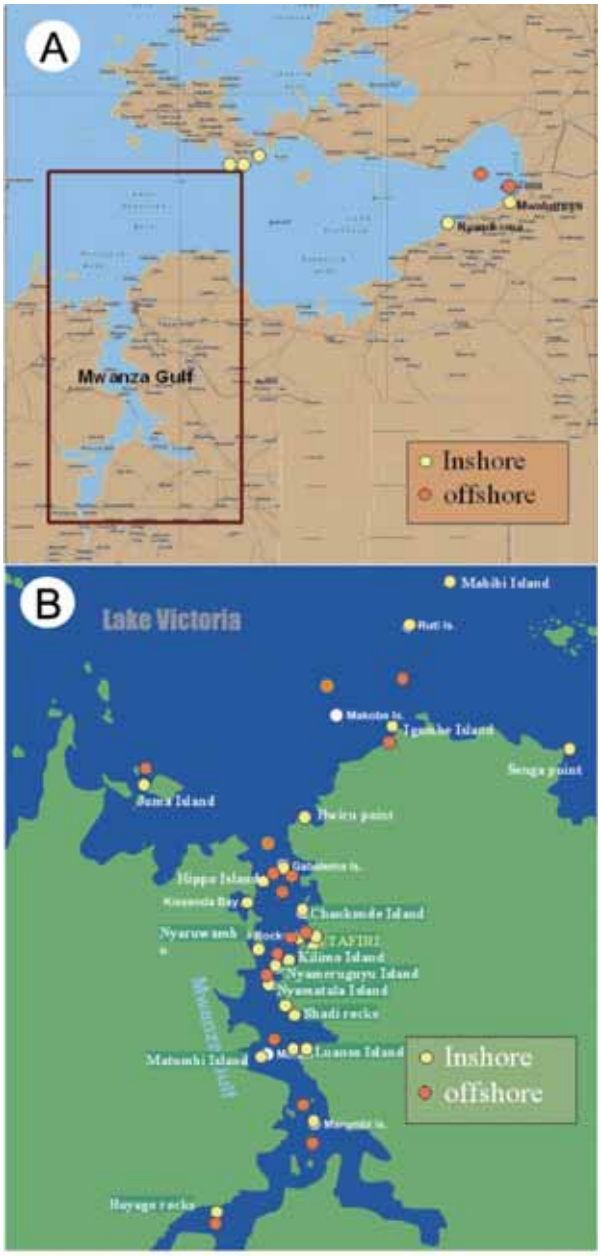

Figure 2. Localities of collection of cichlids in Lake Victoria. Panel A: Sampling localities in southern shore of Lake Victoria. The area surrounded by a brown rectangle in this panel corresponds to the region shown as a magnified map of panel B. Panel B: Sampling localities in Mwanza gulf. Sampling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

Publication List:

Original papers

Kobayashi, N., Watanabe, M., Kijimoto, T., Fujimura, K., Nakazawa, M., Ikeo, K., Kohara, Y., Gojobori, T., and Okada, N. (2006). *Magp4* gene may contribute to the diversification of cichlid morphs and their speciation. Gene *373*, 126-133.

Nikaido, M., Hamilton, H., Makino, H., Sasaki, T., Takahashi, K., Goto, M., Kanda, N., Pastene, L.A., and Okada, N. (2006). Baleen whale phylogeny and a past extensive radiation event revealed by SINE insertion analysis. Mol. Biol. Evol. *23*, 866-873.

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Terai, Y., Seehausen, O., Sasaki, T., Takahashi, K., Mizoiri, S., Sugawara, T., Sato, T., Watanabe, M., Konijnendijk, N., Mrosso, H.D. J., Tachida, H., Imai, H., Shichida, Y., and Okada, N. (2006). Divergent selection on opsins drives incipient speciation in Lake Victoria cichlids. PLoS Biol. *4*, e433.

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 organisms and to analyze the mechanisms of such processes, mainly by morphological methods. The 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 our morphological studies.

The 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 for studying 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".

Cell death in the degeneration region proceeds very rapidly and finishes in a half to one day period in *Pieris rapae* and in several other species examined. It was shown that the dying cells in the degeneration region have characteristics in 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 the 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. This realized concentration of macrophages seems to accelerate the shrinkage of the degeneration region.

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 previous 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 scanning and transmission electron microscopy (Figure 1) to describe the exact pathway and time course of the formation of the elaborate pattern of trachea and tracheoles and to establish the cytological and developmental relationship between the formation of the tracheal pattern and the epithelial cell pattern..

This laboratory also conducts morphological observation of various animal tissues by scanning and transmission electron microscopy and immuno-electron microscopic analyses. Training in 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.

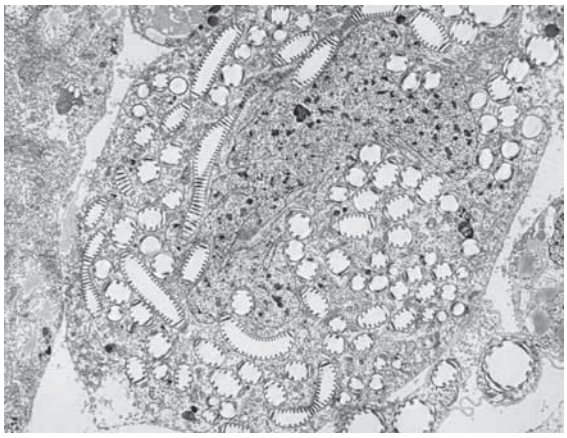

Figure 1. Transmission electron micrograph of the tracheole cell. Many cross-sections of the tracheoles can be observed within the cytoplasm of the tracheole cell. As the cell migrates, the tracheole is laid behind.

Publication List:

Original paper

Watanabe, E., Hiyama, T.Y., Shimizu, H., Kodama, R., Hayashi, N., Miyata, S., Yanagawa, Y., Obata, K., and Noda, M. (2006). Sodium-level-sensitive sodium channel Nax is expressed in glial laminate processes in the sensory circumventricular organs. Am. J. Physiol. (Regul. Integr. Comp. Physiol.), *290*, R568-R576.

DIVISION OF MOLECULAR ENVIRONMENTAL ENDOCRINOLOGY

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	TAKAHASHI, Eri
	HINAGO, Megumi
	TANIGUCHI, Ena
Secretary:	*IMAIZUMI, Taeko*

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.

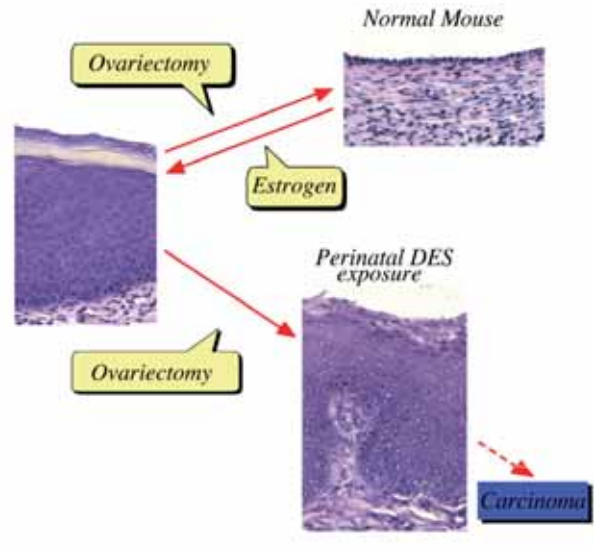

Figure 1. Scheme of estrogen-dependent and -independent vaginal epithelial cell proliferation in mice induced by perinatal estrogen exposure.

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.

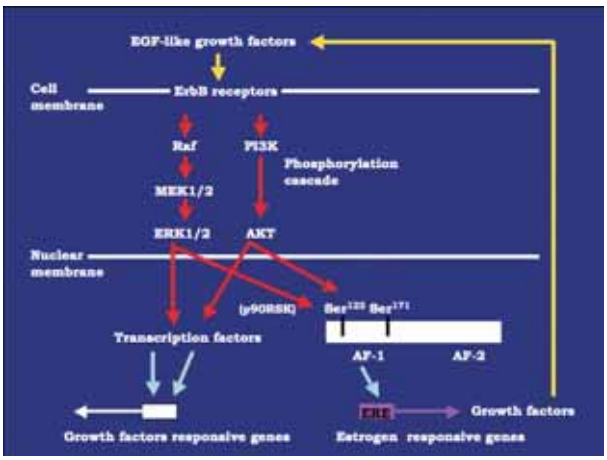

Figure 2. A hypothetical model for the estrogen-independent ER activation pathway in mouse vaginae.

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. 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.

II. Microarray analysis of estrogen responsive genes

cDNA microarray methods have been 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. Most of the estrogen-modulated genes were regulated in a dose-dependent manner and their expression was not altered by estrogen treatment in ERα knockout mice. This confirms that the 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. 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 are 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 that 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.

We identified the estrogen response element in the promoter regions of the adrenomedulin gene and aquapolin 5 gene using the chromatin-immunoprecipitation method and confirmed that these genes are estrogen responsive genes.

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. Using differential display we have found genes possibly related to the ovary-independent changes. We have also clustered groups of genes that are responsive to estrogenic stimuli in the 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.

III. Steroid hormone receptors of reptiles, amphibians and fishes

Exogenous estrogen exposure during embryogenesis induces abnormal sex differentiation in animal species. To analyze the estrogen function, we isolated ERs cDNA from *Gambusia affinis affinis* and *Kryptolebias marmoratu*. Exposure of roach (*Rutilus rutilus* ñ 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, aromatases, StAR, Sox9, vasa, etc.. Furthermore, we have cloned steroid hormone receptors from the American alligator (*Alligator mississippiensis*), the Nile crocodile (*Crocodilus nicotilus*), the Florida red belly slider turtle (*Pseudemys nelsoni*) and the Japanese giant salamander (*Andrias japonicus*) with the aim of analyzing the ER evolution. We are also isolating estrogen-responsive genes to understand the molecular physiology of estrogen action with an aim toward understanding the temperature-dependent sex determination of alligators at the molecular level.

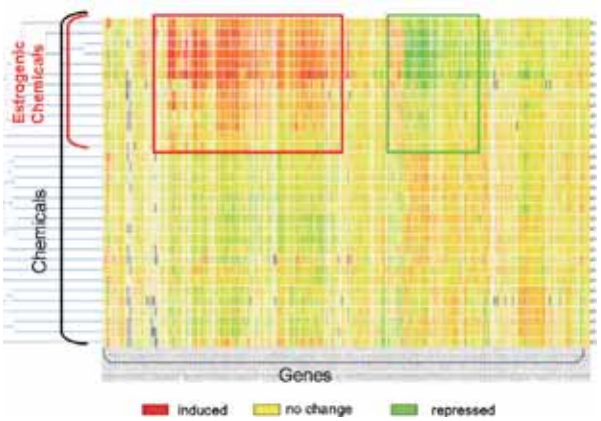

Figure. 3 Scatter plot of average expression levels in control and chemical-treated uterus

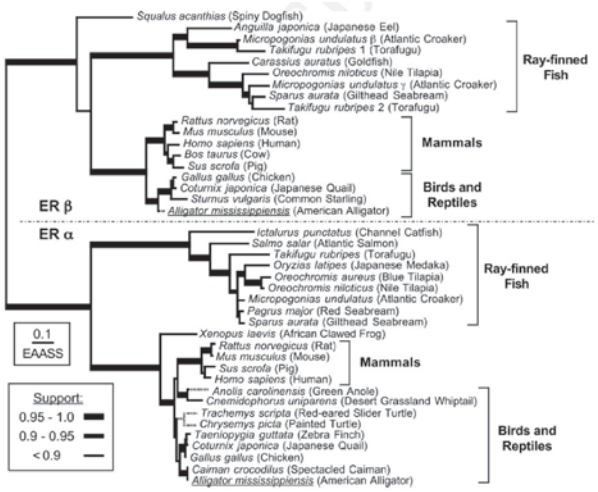

Figure. 4 Evolutionary relationships of estrogen receptor sequences

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 the 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

We found that the persistent and ubiquitous environmental contaminant, tributyltin chloride (TBT), induces the differentiation of adipocytes *in vitro* and increased adipose mass *in vivo*. TBT is a dual noanomolar affinity ligand for both the retinoid ëXí receptor (RXR) and the peroxisome proliferators activated receptor γ (PPARγ). TBT promotes adipogenesis in the murine 3T3-L1 cell model and perturbs key regulators of adipogenesis and lipogenic pathways *in vivo*. Moreover, *in utero* exposure to TBT leads to strikingly elevated lipid accumulation in the adipose depots, liver, and testis of neonate mice and results in increased epididymal adipose mass in adults. In the amphibian *Xenopus laevis*, ectopic adipocytes form in and around gonadal tissues following organotin, RXR or PPARγ ligand exposure. TBT represents the first example of an environmental EDC that promotes adipogenesis through RXR and PPARγ activation. Developmental or chronic lifetime exposure to orgaotins may therefore act as a chemical stressor for obesity and related disorders.

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Mitsui, N., Fujii, T., Miyahara, M., Oka, T., Kashiwagi, A., Kashiwagi, K., Handa, H., Urushitani, H., Santo, N., Tooi, O., and Iguchi, T. (2006). Development of metamorphosis assay using *Silurana tropicalis* for the detection of thyroid hormone disrupting chemicals. Ecotoxicol. Environ. Safety *64*, 281-287.

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Iguchi, T., Watanabe, H., and Katsu, Y. (2006). Application of ecotoxicogenomics for studying endocrine disruption in vertebrates and invertebrates. Environ. Health Perspect. *114 Suppl.1*, 101-105.

Watanabe, H., and Iguchi, T. (2006). Using ecotoxicogenomics to evaluate the impact of chemicals on aquatic organisms. Marine Biol. *149*, 107-115.

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. The genetic control of development of leaf shapes, however, 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 the model plant, *Arabidopsis thaliana* (L.) Heynh. (Tsukaya 2006).

I. Mechanisms of leaf development

Focusing on the mechanisms that govern polarized growth of leaves in *Arabidopsis thaliana,* we have identified four genes for polar-dependent growth of leaf lamina: the *ANGUSTIFOLIA* (*AN*) and *AN3* genes regulate width of leaves and the *ROTUNDIFOLIA3* (*ROT3*) and *ROT4* genes regulate length of leaves. *AN* and *ROT3* genes control cell shape while *AN3* and *ROT4* gene regulate cell number in leaves (reviewed in Tsukaya 2006a, b; Yamaguchi and Tsukaya 2006). In addition to the polar-dependent leaf shape control, we have focused on the mechanisms of organ-wide control of leaf size, reflected in the phenomenon ëcompensationí (Tsukaya 2006). On the other hand, accumulation of knowledge on basic mechanisms of leaf shape control have enabled us to conduct Evo/Devo studies of mechanisms behind leaf-shape diversity. Here we overview achievements during this year on the above research themes.

1-1 Polar growth of leaves in *A. thaliana*

The genome of *A. thaliana* encodes 23 members of RTFL peptides that regulate the number of cells along the proximo-distal polarity in lateral organs (Yamaguchi and Tsukaya 2006). Homology search revealed that rice genome has a number of RTFL homologs. Therefore, we constructed transgenic Arabidopsis that overexpresses one of such rice homologs. Our result clearly showed that the rice homolog also regulated the number of leaf cells along the proximo-distal polarity, suggesting that the basic function of RTFL is conserved among monocot and eudicot (Yamaguchi and Tsukaya 2006). Further mutation analyses have been carried out (Horiguchi et al. 2006a) and will provide us with more clues to understand how the polar-dependent growth of leaf lamina is controlled.

1-2 Evolution of establishment mechanisms of leaf polarities in monocots

On the other hand, we have recently started to try to understand the genetic basis of the development of unifacial leaves that are known from monocot clades. Our preliminary analyses suggested that the unifacial character might be due to overall changes in all polarities around leaves (*i.e.* adaxial-abaxial, distal-proximal, and central-lateral polarities). Moreover, genetic controls of leaf polarities were revealed to differ, at least in part, between eudicot and rice, a monocot species. Understanding the differences in the genetic mechanisms of establishment of leaf polarities in eudicot and monocot will provide good clues to how leaf shape is diversified from the Evo/Devo view point. For such purposes, comparative molecular-genetic and anatomical analyses between unifacial and normal bifacial leaf development have been recently undertaken using a number of monocot families such as Juncaceae, Liliaceae, Alliaceae, and Iridaceae.

1-3 Size control of leaves and mechanisms of compensation

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 2006; Horiguchi et al. 2006b). Thus, leaf size is, at least to some extent, regulated at an organ level by the compensatory system(s). To understand the details of such totally unknown regulatory mechanisms, we have conducted a large scale screening of leaf-size and/or leaf-shape mutants and have categorized these mutants in terms of number and size of leaf cells (Horiguchi et al. 2006a, 2006b). For this purpose, we have developed easy methods to visualize the precise number and size of leaf cells, namely, the centrifugation method and the dried-gel method (Fig. 1; Horiguchi et al. 2006 a).

As a result, we have succeeded in isolating specific mutants for number or size of leaf cells (Horiguchi et al. 2006a, b). Since our re-examination revealed that all known plant hormone-related mutants do not have specific defects in number or size of leaf cells, differing from previous reports (Horiguchi et al. 2006a), this is the first report on the isolation of mutants that have specific defects in the number or size of leaf cells. Moreover, we have isolated a number of new mutants that exhibit typical compensation syndrome, namely, decreased number of cells and increased cell volume. Combining

these mutants as a resource, mechanisms of compensation are now underway.

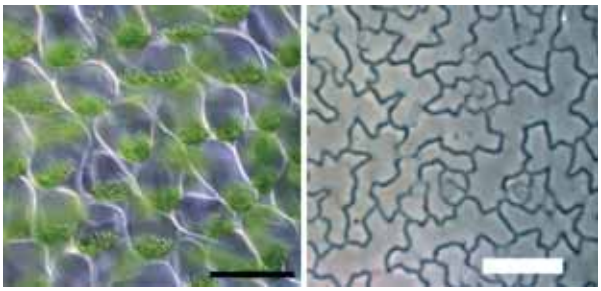

Figure 1. Easy methods to visualize size and shape of leaf cells in *Arabidopsis thaliana* (Horiguchi et al. 2006a). Left, Living palisade cells are visualized by the centrifugation method; right, contour of epidermal cells are shown by the dried gel method. Bars, 100 μm.

II. Environment adaptation of leaves

2-1 Gravitropism of leaves

How is leaf positioning controlled? Curiously, until now, no one had elucidated how rosette leaves are so elegantly arranged on the ground surface. Moreover, no one had elucidated whether or not leaves have gravitropism. Our analysis of *A. thaliana* revealed that leaves not only have negative gravitropism but hyponasty as well, both of which are suppressed by light (Mano et al. 2006). The finding of these curious features of leaf movement satisfactorily explained the positioning control of rosette leaves.

III. Biodiversity of leaf form

3-1 Biodiversity in leaf size

We are also interested in the biodiversity in wild plants. This year, we analyzed *Plantago asiatica* (Plantaginaceae) in Japan (Ishikawa et al., 2006). *P. asiatica* shows a wide range of size diversity and dwarf forms have been taken from temples and shrines, from deer habitats, and from Yakushima Island. Analysis of the molecular variations of this species revealed that the dwarf forms have evolved several times independently.

Similar dwarfism has also been seen in *Paederia foetida* (Rubiaceae). We re-examined the morphological and anatomical features of a small narrow leaf form of this species from Miyajima Island. We found that this form should be defined by the smallness of leaves but not by the leaf blade proportion, and that this variation in leaf size is attributable to the altered number of leaf cells without any change in the shape and size (Tsukaya et al. 2006).

3-2 Domatia formation on leaves

Some trees are known to make domatia on leaves that are thought to function as living rooms for mites. To understand how such additional structures are formed on leaf blades, we examined the developmental course of four types of domatia in *Cinnamomum camphora* (Lauraceae). The results clearly showed that the domatia are derived from ectopic, co-ordinated growths of parenchymatous cells after completion of the basic organogenesis of leaf blades (Nishida et al. 2006).

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DIVISION OF PHOTOBIOLOGY (ADJUNCT)

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Plants respond to light as an environmental factor to optimize growth and development and to 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 photomorphogenesis. One of our major subjects is chloroplast photo-relocation movement, which is thought to be one of the simplest model systems to study photomorphogenesis. Because the phenomenon is cell autonomous, whole processes from photo-perception to chloroplast movement can be accomplished in a single cell without any influence from surrounding neighbor cells. Moreover, gene expression is not involved in the signal transduction pathways, unlike in those of phy- and cry-mediated phenomena. Chloroplast movement is not real plant morphogenesis, but it is the reason that chloroplast movement and photomorphogenesis share the same photoreceptors.

I. Chloroplast relocation movement

We use the fern *Adiantum capillus-veneris* and the moss *Physcomitrella patens* as model plants for our cell biological and physiological approach to chloroplast movement since the gametophytes are very sensitive to light and the organization of the cells is very simple. We also use *Arabidopsis* mutants as well as wild type plants to identify the genes regulating chloroplast photo-relocation movement and for analyses of the genesí functions.

1-1 Velocity of signal transfer

Phototropins (phot1 and phot2, and neochrome which is a chimera photoreceptor of phytochrome chromophore binding domain and phototropin), were identified as photoreceptors for chloroplast movement (Kagawa *et al.* 2001, Kawai *et al.* 2003, Kagawa *et al.* 2004); however, a signal transferred from photoreceptors remained to be clarified. We therefore studied the velocity of signal transfer to find a clue to the identity of any possible candidates for the signal using long single cells of *Adiantum* protonemata. The velocity is different when the signal moves from the base to the tip (approximately 2.3 μm min^{-1}) and from the tip to the base (approximately 0.6 μm min^{-1}) of the protonemata. The reason for the difference is not clear but the cytoplasmic and/or water flow from the base to the tip to support tip growth of protonemata is the most plausible explanation.

II. Photoreceptor functions

2-1 Acneo1 as a blue light receptor

Fern phytochrome3 (phy3) (renamed *Ac*neochrome1 (*Ac*neo1)) is a chimera photoreceptor that consists of a phytochrome chromophore binding domain and a full-length phototropin (Nozue *et al.* 1998), so that it had been thought that it might be able to absorb red as well as

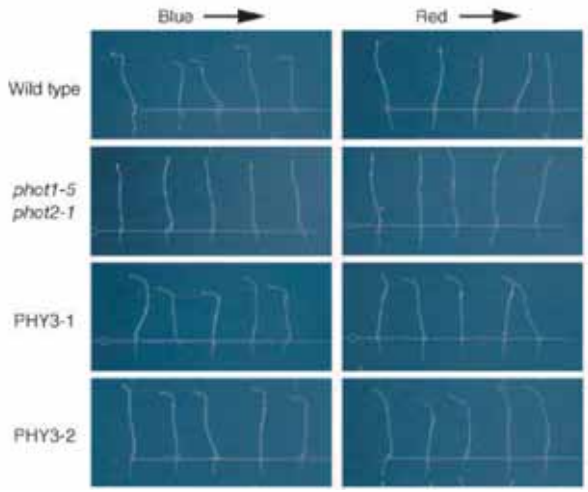

Figure 1. Phototropic response of *Arabidopsis* seedlings. *Ac*neo1 can mediate both red and blue light-induced phototropic response in *Ac*neo1 transgenic *Arabidoposis* seedlings of *phot1phot2* background. Blue and red lights were given from left hand side to the plants cultivated 3 days under darkness. Wild type plants show phototropic response in blue light but not in red light. *phot1phot2* mutant plants do not show phototropism either under red or blue light. Two independent transgenic lines phy3-1 and phy3-2 show tropistic response under both red and blue light. (see Kanegae *et al.* 2006 for more details)

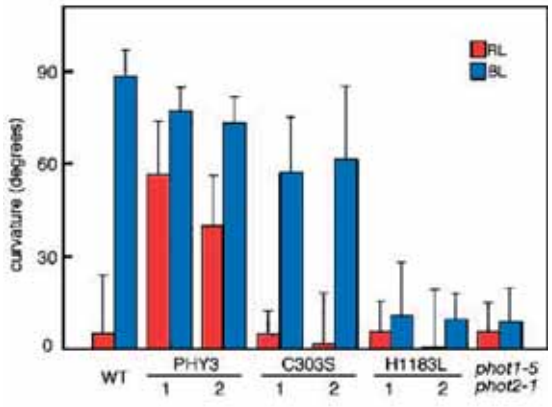

Figure 2. *Ac*neo1 can function as a blue light receptor. Even when phytochrome chromophore does not bind in C303S mutant phototropic response was observed under blue light. The blue light response transferred through kinase domain was shown in H183L mutant plants. (see Kanegae *et al.* 2006 for more details)

blue light. However, no evidence of red and blue light-absorption by *Ac*neo1 has ever been shown. In collaboration with Dr. Kanegae (Tokyo Metropolitan University) we recently demonstrated that *Ac*neo1 can absorb red as well as blue light; we also showed that red and blue light can function synergistically when given simultaneously, resulting in increased sensitivity ten times greater than when red or blue light is given independently (Kanegae *et al*. 2006). In these experiments the degree of phototropic response, but not chloroplast movement, was measured in order to evaluate the sensitivity to light in *Arabidopsis phot1phot2* double mutants transformed with *Ac*neo1.

2-2 Photoreceptors in fern stomata opening

Photoresponse of fern stomata opening was studied in collaboration with Drs. Doi and Shimazaki of Kyushu University. Fern stomata open under red light but not in blue light, although in seed plants stomata opening is redundantly mediated by blue light receptors phot1 and phot2 (Kinoshita *et al*. 2001). Therefore, *Ac*neo1 is a good candidate for this response because of its chimera structure made of red light receptor phytochrome and phototropin. Contrary to our expectations, *rap2* mutant lines deficient in *Ac*neo1 still showed red light-mediated stomata opening, indicating that the stomata opening in ferns is not mediated by phototropin family proteins but controlled by mechanisms completely different from those of seed plants. It is curious that blue light does not mediate fern stomata opening although phototropins are expressed in the fern guard cells.

III. Photomorphogenesis

3-1 Phototropism of Adiantum *rhizoid*

Phototropic responses in seed plants are induced by blue light and mediated by blue light receptor phototropins. In many cryptogam plants, including the ferns and mosses, however, red as well as blue light is effective in inducing a positive phototropic response in protonemal cells. In *A. capillus-veneris*, both protonemata and sporophytes show red light-induced phototropism and the photoreceptor for this response is *Ac*neo1 (Kawai *et al*. 2003). Because nothing precise has been reported so far concerning phototropism of fern rhizoid cells, we analyzed negative phototropism of *A. capillus-veneris* rhizoid cells. Mutants defective of *Ac*neo1 lacked red light-induced negative phototropism, indicating that under red light, *Ac*neo1 mediates negative phototropism in rhizoid cells, in contrast to its role in regulating positive phototropism in protonemal cells. *Acneo1* mutants were also partially deficient in blue light-induced negative phototropism in rhizoid, suggesting that *Ac*neo1, in conjunction with phototropin, redundantly mediates the blue light response.

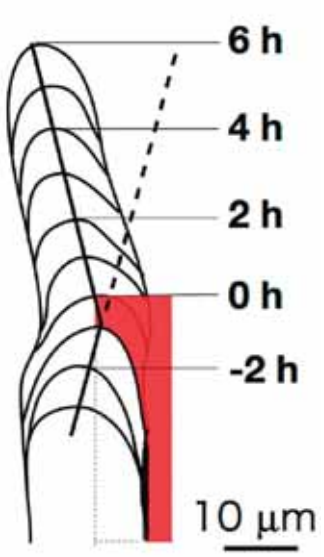

Figure 3. A half side of *Adiantum* rhizoid was irradiated with red microbeam light (5.5μmol m^{-2} s^{-1}. 1min) as shown in red rectangular at hour 0. Rhizoidal images were traced every 1 hour to follow the growth direction. The dotted line shows the original direction of rhizoid growth and the solid line shows the new direction. Note that the rhizoid showed negative phototropism. (see Tsuboi *et al*. 2006 for more details)

Original papers

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LABORATORY OF PHOTOENVIRONMENTAL BIOLOGY

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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 (Figure 1).

1-1 Kinetic properties of PAC photoactivation

Although PAC appeared to be a photoreceptor for the step-up photophobic response, 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*. Photochem. Photobiol. Sci. *4*, 727-731, 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 subsecondsí latency.

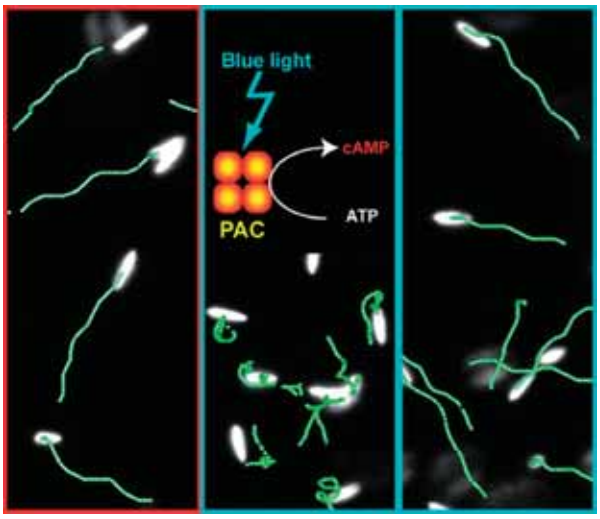

Figure 1. Tracks of *Euglena* cells before and after applying the step-up stimulus. The cells were observed using a phase contrast microscope equipped with a high-speed video camera (FASTCAM-PCI R2, Photron, Tokyo, Japan) under infrared illumination. Movement of each cell for 2 s was traced and plotted as green dots at 10 s before onset of irradiation (left, with a red border), just after onset of irradiation (center, with a blue border), 60 s (right, with a blue border) after onset of irradiation. Illustration of PAC activation by blue light is also shown.

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.

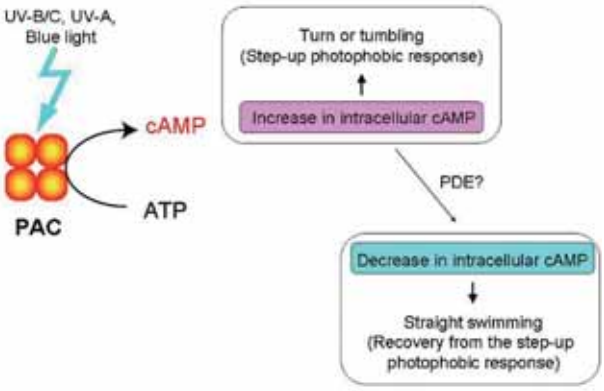

Figure 2. Schematic representation of PAC involvement in the step-up photophobic response of *Euglena*. PAC is activated by UV-B/C, UV-A and blue lights in a photon fluence-dependent manner to produce cAMP, which increases intracellular cAMP. The increase in intracellular cAMP triggers the step-up photophobic 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). (Ito *et al*. Photochem. Photobiol. Sci. *4*, 762-769, 2005)

1-4 Fast manipulation of cellular cAMP level by light *in vivo*

A six year collaboration with scientists at Max-Planck-Institut f¸r Biophysik (Frankfurt, Germany) et al. has yielded an exciting paper reporting functional expression of PACs in *Xenopus laevis* oocytes, HEK293 cells and in *Drosophila melanogaster*, where neuronal expression yields light-induced changes in behavior. The activity of PACs is strongly and reversibly enhanced by blue light, providing a powerful tool for light-induced manipulation of cAMP in animal cells (Schr^de r-Lang, S., Schwarzel, M., Seifert, R., Strunker, T., Kateriya, S., Looser, J., Watanabe, M., Kaupp, U.B., Hegemann, P., and Nagel, G. Fast manipulation of cellular cAMP level by light *in vivo*. Nat. Methods. Epub 2006 Nov 26.).

Publication List:

Original paper

Review article

Iseki, M., Matsunaga, S., Murakami, A., and Watanabe, M. (2006). Photoactivated adenylyl cyclase (PAC), the photoreceptor flavoprotein with intrinsic effector function mediating euglenoid photomovements. In Comprehensive Series in Photochemical & Photobiological Sciences, Flavins: Photochemistry and Photobiology, Silva, E. and Edwards, A. M., eds. (RSC Publishing), pp. 271-286.

LABORATORY OF STRESS RESPONSE

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Technical Assistant: *KINOSHITA, Chie*

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, α and β, 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.

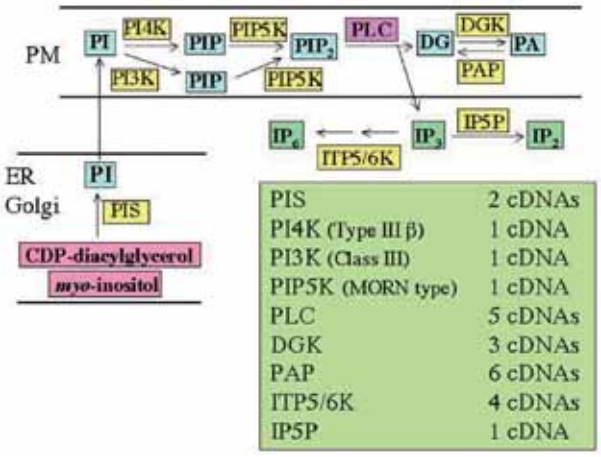

Figure 1. Schematic representation of the PI pathway. Light blue and yellow boxes indicate phosphoinositides and enzymes (PI synthase, lipid kinases or lipid phosphatases), respectively. Current situation of the cloning of *Physcomitrella* full-length cDNAs encoding enzymes involved in the PI pathway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

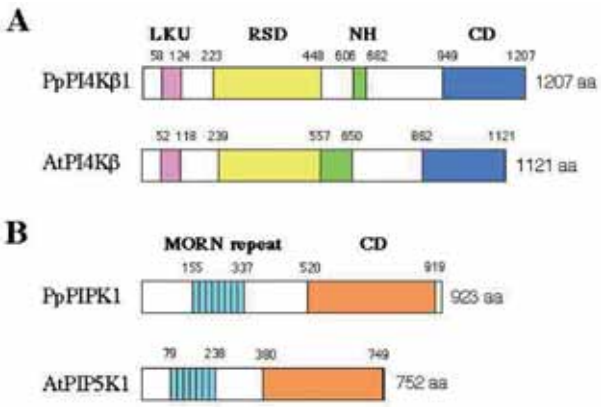

Figure 2. Structural characteristics of plant β form of type III PI4Ks and PIPKs. Protein domains represent in PpPI4Kβ1 (A) and PpPIPK1 (B), in comparison with *Arabidopsis* homologues. Numbers indicate amino acid positions. Accession nos. are as follows; AtPI4Kβ, AJ002685; AtPI5K1, Q56YP2. RSD, repetitive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

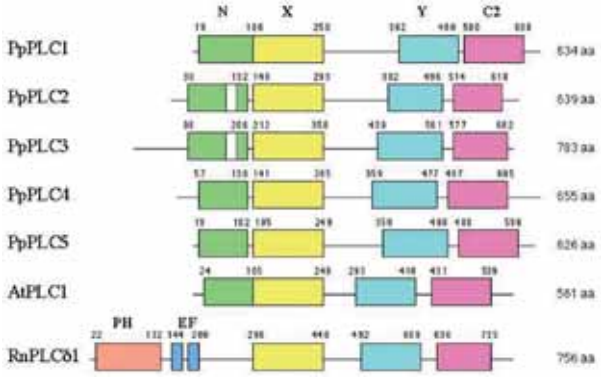

Figure 3. Structural characteristics of five *Physcomitrella* PI-PLCs. Protein domains represent in five *Physcomitrella* PI-PLCs, PpPLC1 to PpPLC5, in comparison with AtPLC1 from *Arabidopsis* and RnPLCδ1 from *Rattus norvegicus*. All PI-PLCs indicated have the X, Y and C2 domains, although the PH (pleckstrin 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 Fellow: *FUJITA, Hironori*
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Graduate Student: *IMAMURA, Hisako*
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 especially useful in understanding pattern formation in development. The study of the mechanisms responsible for morphological differences between species is an important research focus of current developmental biology.

I. Predicting regulation of the phosphorylation cycle of KaiC clock protein using mathematical analysis

Cyanobacteria are the simplest organisms exhibiting circadian rhythms. In the bacterium, clock genes kaiA, kaiB and kaiC have been characterized as the indispensable clock regulators. 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. It was recently revealed that periodic oscillation of the phosphorylation level of KaiC persists even under continuous dark conditions, where transcription and translation have almost ceased. The KaiC phosphorylation cycle was reconstituted even *in vitro*, thus confirming that the interaction between Kai proteins generates the cycle, although the specific mechanism that drives the clock remains unclear.

Using mathematical models, we investigated the mechanism for the transcription-less KaiC phosphorylation cycle. We developed a simple model based on the possible KaiC behavior, which was suggested by previous experimental studies. In the model the KaiC-KaiA complex formation followed by a decrease in free KaiA molecules may attenuate the KaiC phosphorylation rate, and it acts as negative feedback in the system. However, our mathematical analysis proved that simple dynamics based on the experimentally suggested model never show the KaiC phosphorylation cycle.

We then developed the generalized formulae of models and determined the necessary condition to generate the KaiC phosphorylation cycle. Linear stability analysis revealed that oscillations can occur when there is sufficient distance of feedback between the recipient reaction and the effector. Furthermore, we found that the negative feedback regulations in closed systems can be classified into two types: *destabilizing inhibition* and *stabilizing inhibition*.

Figure 1. Schematic representation of closed circuit model and the condition for the possible oscillation by inhibition of the transition from state V_1 to V_2. Red-colored state (V_1, V4-V_7) indicate that inhibition from the states can destabilize the system and possibly cause oscillation. Inhibition from the blue-colored state never induces oscillation.

# of states	inhibitor V_1	V_2	V_3	V_4	V_5	V_6	V_7	V_8	
3	○	×	×	-	-	-	-	-	× : always stable
4	○	×	×	○	-	-	-	-	○ : oscillation possible
5	○	×	×	○	○	-	-	-	
6	○	×	×	○	○	○	-	-	
7	○	×	×	○	○	○	○	-	
8	○	×	×	○	○	○	○	○	

Table 1. Summary of the results of the general state transition model with conservation of molecules. The system could oscillate when the inhibiting state is more than two steps ahead of the inhibited reaction (from V_1 to V_2). If the inhibiting state is less than three steps ahead of the reaction, the system is always stable. The necessary distance between the inhibiting state and reactant state does not depend on the system size.

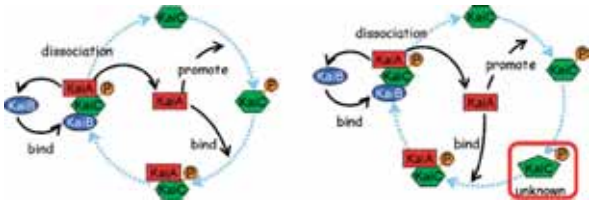

Figure 2. Schematic representations of "Basic model" (left) and "Multiple-phosphorelation-state model" (right). The basic model is determined from experimental results. It was proven that the model never shows oscillation. The multiple-phosphorelation-state model was developed based on the mathematical analysis. The model shows clear periodic oscillations. There are at least two different phosphorelated states. The time-delay caused by the transition between the states is essential for generating oscillation.

Based on this result, we predicted that, in addition to the identified states of KaiC, another unknown state must be present between KaiC phosphorylation and the complex formation. By incorporating the unknown state into the previous model, we realized the periodic pattern reminiscent of the KaiC phosphorylation cycle in computer simulation. This result implies that the KaiC-KaiA complex formation requires more than one step of posttranslational modification including phosphorylation or conformational change of KaiC.

II. Mathematical models for pattern formation of dendrites of neurons

Dendrite is a neuronal process which is specialized for receiving and processing synaptic or sensory input. A remarkable feature of dendrite is its morphological diversity. The shapes of dendritic trees are characteristic of individual neuronal types and they are highly variable from one neuronal type to another. This diversity contributes to differential processing of information in each type of neuron. Therefore, patterning neuronal class-specific dendrites is a process to produce forms that realize physiological functions of neurons. However, a comprehensive logic of dendrite development has not been formulated yet.

Previously proposed mathematical models to explain the pattern formation of dendrites assumed that dendrite development is a consequence of stochastic sprouting and subsequent growth arrest. Different forms of branching functions were postulated and modified so that simulated dendrograms fit dendritic arbors of real neurons. One of the problems of the previous models is that those dendrograms represent limited features of dendritic patterns such as order of branches and degree (the number of branches at each order) and do not reproduce the full range of the morphological features of the original dendrites. Other problems include the fact that many of the models cannot specify experimentally confirmed mechanisms to account for their assumptions. To overcome these problems, we developed a new class of dendrite growth model, which represents all extension, orientation of growth and branching of dendrites in a single scheme. In addition, this model has explicitly incorporated an underlying biological mechanism, that is, competitive interactions between neighboring dendrites.

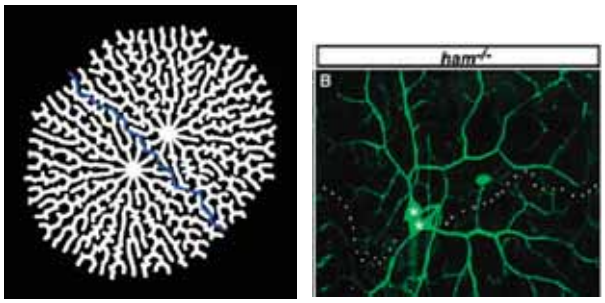

Figure 3. Examples of obtained dendrite patterns by computer simulation of the model (left) and the correspondence observed in an experiment (Grueber et al., 2003; Moore et al., 2002). Dendrites from the cells spread and cover the space. However, they never interfere with each other.

A key point in our modeling is to couple chemical dynamics to dendrite growth. In our model, we distinguish two spatial compartments: inside and outside regions of neurons. The cell compartment dynamically grows under the regulation of a chemical reactant activator. Thus we call our model a ìc ell compartment modelî. The activator reacts with another reactant suppressor in the way of the reaction-diffusion (RD) model of the so-called "Activator-Inhibitor type" (Turing, 1952; Gierer and Meinhardt, 1972). We set a restriction in the 2D space so that the activator only diffuses inside of the cells. These settings endow the system with feedback loop regulations at two different levels: one between two chemicals, and another between the dynamics of the chemicals and the expansion of the cell compartment. Using this formula, we study the dynamics of dendritic branch formation. Computer simulation showed that the cell compartment model developed dendritic branching autonomously and numerical analysis determined the conditions that allow it. This work is collabolation with Dr. T. Uemura and Dr. K. Sugimura in Kyoto University.

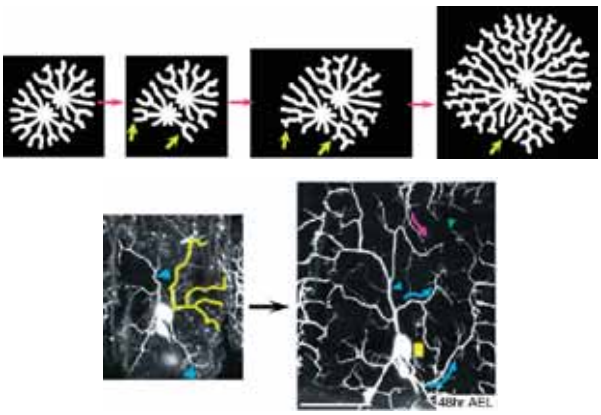

Figure 4. Regeneration after artificial severing. Patterns obtained by computer simulation of the model (up) and the corresponding pattern obtained in an experiment (down).

Publication List:

Original papers

Nakamura, T., Mine, N., Nakaguchi, E., Mochizuki, A., Yamamoto, M., Yashiro, K., Meno, C., and Hamada, H. (2006). Generation of robust left-right asymmetry in the mouse embryo requires a self-enhancement and lateral-inhibition system. Dev. Cell *11*, 495-504.

Takigawa-Imamura, H., and Mochizuki, A. (2006). Predicting regulation of the phosphorylation cycle of KaiC clock protein using mathematical analysis. J. Biol. Rhythms *21*, 405-416.

Takigawa-Imamura, H., and Mochizuki, A. (2006). Transcriptional autoregulation by phosphorylated and non-phosphorylated KaiC in cyanobacterial circadian rhythms. J. theor. Biol. *241*, 178-192.

Mochizuki, A., Yahara, K., Kobayashi, I., and Iwasa, Y. (2006). Genetic addiction: selfish gene's strategy for symbiosis in the genome. Genetics *172*, 1309-1323.

Fujita, H., and Mochizuki, A. (2006). The origin of the diversity of leaf venation pattern. Dev. Dyn. *235*, 2710-2721.

Fujita, H., and Mochizuki, A. (2006). Pattern formation by the positive feedback regulation between flow of diffusible signal molecule and localization of its carrier. J. theor. Biol. *241*, 541-551.

LABORATORY OF GENOME INFORMATICS

Research Associate: *UCHIYAMA, Ikuo*

The accumulation of biological data has recently been accelerated by various high-throughput ìomicsî 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 the genomic information of various species. The current focus of our research is on the comparative analysis of microbial genomes, the number of which has been increasing rapidly. Extracting useful information from such a growing number of genomes is a major challenge in genomics research. Interestingly, many of the completed genomic sequences are closely related to each other. We are now trying to develop methods and tools to conduct comparative analyses not only of distantly related genomes but also of closely related genomes, since we can extract different types of information about biological functions and evolutionary processes from comparisons of genomes at different evolutionary distances.

I. Microbial genome database for comparative analysis

The microbial genome database for comparative analysis (MBGD) is a comprehensive platform for microbial comparative genomics. The central function of MBGD is to create orthologous groups among multiple genomes from precomputed all-against-all similarity relationships using the DomClust algorithm (see Section II below). By this algorithm, MBGD not only provides the comprehensive orthologous groups among the latest genomic data available, but also allows users to create their own ortholog groups on the fly using a specified set of organisms. The latter feature is especially useful when the userís interest is focused on some taxonomically related organisms. 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.

The database now contains more than 300 published genomes and the number continues to grow. For researchers who are interested in ongoing genome projects, we have started a new service called ìMy MBGD,î which allows users to add their own genome sequences to MBGD for the purpose of identifying orthologs among both the new and the existing genomes (Figure 1). Furthermore, in order to make the rapidly accumulating information on closely related genome sequences available, we enhanced the interface for pairwise genome comparisons using the CGAT interface (see Section III below), which allows users to see nucleotide sequence alignments of non-coding as well as coding regions. MBGD is available at http://mbgd.genome.ad.jp/.

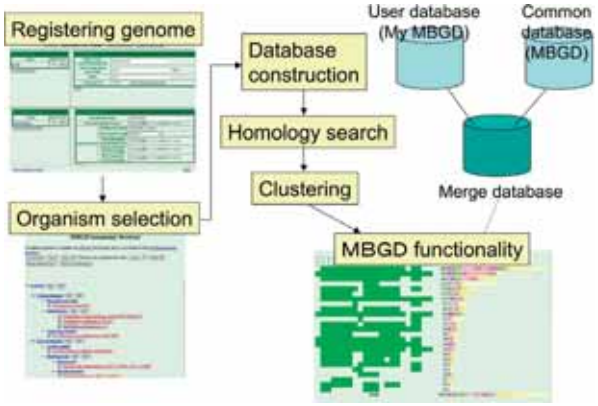

Figure 1. The My MBGD functionality, which allows users to add their own genome data to the MBGD database.

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 has yet to be established. In fact, the conventional approach to the identification of orthologs, called the bidirectional best-hit (BBH) criterion, is known to have several drawbacks, and the establishment of orthologous relationships in a database like the Clusters of Orthologous Groups (COGs) database requires additional complex procedures such 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, named DomClust, 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 in such a way 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.

We are now trying to enhance the algorithm so as to take partial phylogenetic relationships into account to identify orthologous relationships among a set of closely related genomes with some outgroup species.

III. Comparative genome analysis tool for the analysis of complex evolutionary changes between closely related genomes

The recent accumulation of closely related genomic sequences provides a valuable resource for the elucidation of the evolutionary histories of various organisms. However, although numerous alignment calculation and visualization tools have been developed to date, the analysis of complex genomic changes, such as large insertions, deletions, inversions, translocations and duplications, still presents certain difficulties.

We have developed a comparative genome analysis tool, named CGAT, which allows detailed comparisons of closely related bacteria-sized genomes, mainly through visualizing middle-to-large-scale changes to infer the underlying mechanisms (Figure 2). CGAT displays precomputed pairwise genome alignments on both dotplot and alignment viewers with scrolling and zooming functions and allows users to move along the pre-identified orthologous alignments. Users can place several types of information on this alignment, such as the presence of tandem repeats or interspersed repetitive sequences and changes in G+C contents or codon usage bias, thereby facilitating the interpretation of the observed genomic changes. In addition to displaying precomputed alignments, the viewer can dynamically calculate the alignments between specified regions; this feature is especially useful for examining the alignment boundaries. Besides the alignment browser functionalities, CGAT also contains an alignment data construction module that provides a general framework for the calculation of genome-scale alignments using various existing programs as alignment engines, which allows users to compare the outputs of different alignment programs.

In collaborative studies with Dr. Kobayashiís group (Tokyo Univ.), we were able to conduct several comparative analyses using the earlier versions of this program to infer the evolutionary history of apparently complex genome changes between closely related eubacteria and archaea.

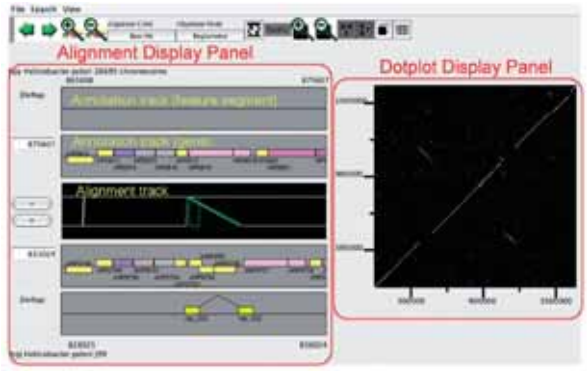

Figure 2. CGAT AlignmentViewer, which consists of an alignment display and a dotplot display. The example shown is ìinsertion with long target duplicationî, which was discovered during a comparison of two strains (26695 and J99) of *Helicobacter pylori* (Nobusato et al. 2000).

IV. Identification of core structures conserved among phylogenetically related genomes

It is known that both horizontal transfer and vertical transfer have 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 provide many clues for understanding evolutionary processes. Such data is now rapidly accumulating in our MBGD database.

We are trying to identify a common ìcore structureî 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 a common ancestor mainly through vertical transfer. For this purpose, we have developed a graph-based algorithm for aligning conserved regions of multiple genomes. The algorithm finds an order of pre-identified orthologous groups so as to retain, as much as possible, the conserved gene orders.

The method was applied to genome comparisons of the families *Bacillaceae* and *Enterobacteriaceae*. Using orthologous groups generated by the DomClust program, we constructed genome alignments and identified common core structures comprising about 1500 genes for *Bacillaceae* and 2000 genes for *Enterobacteriaceae*. It turned out that these core structures contain most of the essential genes identified in *Bacillus subtilis* and *Escherichia coli*, respectively. Further investigation for generalizing our approach is in progress.

Publication List:

Original papers

Kawai, M., Uchiyama, I., and Kobayashi, I. (2006). Genome comparison *in silico* in *Neisseria* suggests integration of filamentous bacteriophages by their own transposase. DNA Res. *12*, 389-401.

Kawai, M., Nakao, K., Uchiyama, I., and Kobayashi, I. (2006). How genomes rearrange: Genome comparison within bacteria *Neisseria* suggests roles for mobile elements in formation of complex genome polymorphisms. Gene *383*, 52-63.

Tsuru, T., Kawai, M., Mizutani-Ui, Y., Uchiyama, I., and Kobayashi, I. (2006). Evolution of paralogous genes: Reconstruction of genome rearrangements through comparison of multiple genomes within *Staphylococcus aureus*. Mol. Biol. Evol. *23*, 1269-85.

Uchiyama, I. (2006). Hierarchical clustering algorithm for comprehensive orthologous-domain classification in multiple genomes. Nucleic Acids Res. *34*, 647-658.

Uchiyama, I., Higuchi, T., and Kobayashi, I. (2006). CGAT: a comparative genome analysis tool for visualizing alignments in the analysis of complex evolutionary changes between closely related genomes. BMC Bioinformatics *7*, 472.

LABORATORY FOR SPATIOTEMPORAL REGULATIONS

Associate Professor: *NONAKA, Shigenori*
Technical Staff: *KAJIURA-KOBAYASHI, Hiroko*
NIBB Research Fellow: *ICHIKAWA, Takehiko*
Secretary: *KAMIYA, Akemi*

In spite of superficially bilateral symmetry, our bodies are highly asymmetric along the left-right (L-R) axis, such as the placement of internal organs. Our main aim is to clarify the mechanism by which mammalian embryos generate and establish the L-R asymmetry.

I. Initial step for the left-right asymmetry

The first L-R asymmetry in mammalian development arises on the embryonic surface. A gastrulating mouse embryo has a shallow hollow on its ventral surface, called as ëthe nodeí, with hundreds of cilia moving in a clockwise rotational manner (Figure 1; Nonaka et al., 1998). Sum of the vortical motions of the cilia however generates leftward flow of the surrounding fluid rather than a vortex. Because of posteriorly tilted rotation axis, the node cilia can generate leftward force without pre-existing left-right asymmetry. (Figure2; Nonaka et al., 2005).

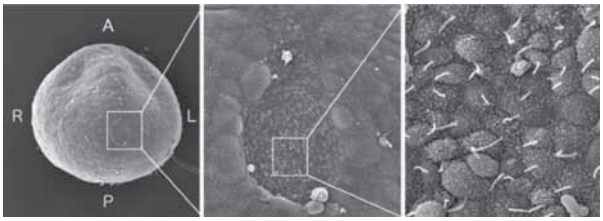

Figure 1. Left, ventral view of a 7.5-day mouse embryo. Middle, the node. Right, node cilia.

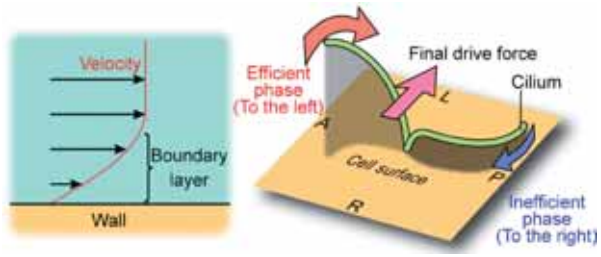

Figure 2. Mechanism to generate leftward flow by a rotating cilium. Left, fluid very close to the wall is generally reluctant to move (surface effect). Right, this constraint negatively affects water-dragging efficiency by the cilium. Given that the cilium rotates clockwise with a posteriorly tilted axis, fluid dragged to the right is less than that to the left, resulting leftward force production.

The leftward flow, called nodal flow, determines subsequent L-R development. This idea has been confirmed by several evidences: First, mutations without motile cilia in the node result in randomized L-R asymmetry. Second and more important, embryos raised in a rightward artificial flow of culture medium develop reversed L-R asymmetry (Figure3; Nonaka et al., 2002).

While it is clear that nodal flow conveys asymmetric information, the entity of the information remains unclear. We are now working to clarify the mechanism how the direction of nodal flow is converted to the subsequent step, asymmetric gene expression.

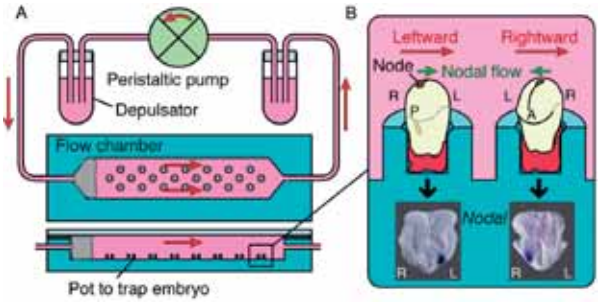

Figure 3. Flow culture exiperiment. A, A peristaltic pump and depulsators supply constant fluid flow in the chanber (red arrowheads). B, Embryos held in the pots receive the pump-driven flow on their surface. If the pump-driven flow reverse intrinsic nodal flow, expression of nodal, a master gene for the leftness, is reversed (Right).

II. New microscopy

Long-term live imaging techniques of early mouse embryos should be extremely useful for our analyses of L-R development and broader research interests. For this purpose, we are working to introduce Single Plain Illumination Microscope (SPIM) that has been developed by Dr. Ernst Stelzer in EMBL.

Publication List:

Review article

Marshall, W.F., and Nonaka, S. (2006). Cilia: tuning in to the cell's antenna. Curr. Biol. *16*, R604-614.

STRATEGIC PLANNING DEPARTMENT

Chair:	*NAGAHAMA, Yoshitaka*
Vice-Chairs:	*UENO, Naoto*
	MOROHASHI, Ken-ichirou
	NISHIMURA, Mikio
Professor:	*WADA, Masamitsu*
Associate Professor:	*KODAMA, Ryuji*
Postdoctoral Fellow:	*KURATA, Tomoko*
Technical Assistants:	*MAKIHARA, Nobuko*
	MUKOHDA, Yasuyo
	OTA, Misaki
	TANAKA, Megumi
	EMDE, Jason R.

The Strategic Planning Department was founded in April 2005 to be a central office for efficient management of cooperational activities with other organizations, distribution of scientific information to the public, planning and managing of conferences, workshops and other extramural activities and planning a long-range strategy of the institute.

The main activities of the Department in 2006

1) Supporting international conferences
On-line registration, web page construction, brochure editing, hotel reservation, meal and party arrangement, photographic recording, etc for international conferences held in NIBB (Table 1).

2) Management of education-related programs
Internship Program 2006
Preparations for the First International Practical Course (Jan. 2007)

3) Press Release
News on scientific achievement are sent to newspaper and magazine reporters via e-mail and password protected web page.

4) Editing of publications
Bulletin of NIBB 2006 (in Japanese)
Annual Report of NIBB 2006 (in English, with proof reading)

5) Production of posters and leaflets
Design and distribute posters for international conferences and advertisements for entrance examination of graduate school (Figure1)

6) Updating and maintenance of NIBB web page

7) Publication of "NIBB News"
(Intra-institutional newsletter in Japanese)

8) Maintenance of the achievement archives of NIBB

9) Assisting visitors (in collaboration with Technical Division)

Table 1. International conferences managed by Strategic Planning Department in 2006

Meetings	Date	Title	Organizer
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
53rd NIBB Conference	June 14-17, 2006	Dynamic Organelles in Plant	M. Nishimura
4th Okazaki Biology Conference	September 10-15, 2006	Terra Microbiology 2	K. Kato D. J. Arp
4th NIBB-EMBL Symposium	December 3-5, 2006	Biology of Protein Conjugation - Structure and Function -	Y. Ohsumi W. Weissenhorn

Figure 1. Examples of posters and abstract books produced by Strategic Planning Department in 2006

52nd NIBB Conference | 2nd NIBB-EMBL Symposium | 4th NIBB-EMBL Symposium | 3rd Okazaki Biology Conference | 4th Okazaki Biology Conference | 1st International Practical Course

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 Assistant: *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* ìCRC Handbook of Organic Photochemistry and Photobiology, 2nd ed.î. 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.

Publication List on OLS Collaboration:

Original papers

Arakawa, R., Terao, M., Hayashi, H., Kasai, H., and Negishi, T. (2006). Evaluation of oxidative damage induced by natural sunlight in Drosophila. Genes Environ. *28*, 153-159.

Kong, S.-G., Suzuki, T., Tamura, K., Mochizuki, N., Hara-Nishimura, I., and Nagatani, A. (2006). Blue light-induced association of phototropin 2 with the Golgi apparatus. Plant J. *45*, 994-1005.

Mano, E., Horiguchi, G., and Tsukaya, H. (2006). Gravitropism in leaves of *Arabidopsis thaliana* (L.) Heynh. Plant Cell Physiol. *47*, 217-223.

Negishi, K., Higashi, S., Nakamura, T., Otsuka, C., Watanabe, M., and Negishi, T. (2006). Oxidative DNA damage induced by 364-nm UVA laser in yeast cells. Genes Environ. *28*, 2, 74-76.

Teramoto, H., Ishii, A., Kimura, Y., Hasegawa, K., Nakazawa, S., Nakamura, T., Higashi, S-i., Watanabe, M., and Ono, T.-A. (2006). Action spectrum for expression of the high intensity light-inducible *Lhc*-like gene *Lhl4* in the green alga *Chlamydomonas reinhardtii*. Plant Cell Physiol. *47*, 419-425.

Watanabe, K., Yamada, N., and Takeuchi, Y. (2006). Oxidative DNA damage in cucumber cotyledons irradiated with ultraviolet light. J. Plant Res. *119*, 239-246.

Zsiros, O., Allakhverdiev, S.I., Higashi, S., Watanabe, M., Nishiyama, Y., and Murata, N. (2006). Very strong UV-A light temporally separates the photoinhibition of photosystem II into light-induced inactivation and repair. Biochim. Biophys. Acta *1757*, 123-129.

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 maintains several computers

to provide computation resources and the means of electronic communication within NIBB. Our main computer system, the Biological Information Analysis System (BIAS), consists of a shared memory parallel computer (SGI Altix 350; 8CPU, 48GB memory) with a disk array storage system (D-RAID; 1.6TB × 10), a high-performance cluster system (DELL PowerEdge 1850; 2CPU × (16+1) nodes), and a data visualization terminal (DELL Precision 370). 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 institute members.

The computer laboratory also provides network communication services. Most of PCs 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 services, file sharing services and printer services are provided through this network. We also maintain a public World Wide Web server that contains the NIBB home page (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.

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 the fundamental principles of various biological phenomena based on the integration of computational science and biology; (2) establishing new methodologies for integrative biology; and (3) providing 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 challenges, 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 challenges. 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 using mathematical or computational methods, and developing new methodologies 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 differences between species are an important research focus of 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 2006, we held two meetings on mathematical approaches in biology and enhanced the interaction between senior and junior reseachers. 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, Yoko
TAKAGI, Yukari
Supporting Staffs: *KOBAYASHI-NISHIMURA, Keiko*
YASUDA, Mie
NOGUCHI, Yuji
YOSHIDA, Etsuko
KAWAMURA, Motofumi
OKUDA, Tadayoshi

The worldwide genome project has almost been 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 the functional analysis of a gene of interest it is essential to utilize genetically altered model organisms, which are generated using genetic engineering technology, including gene deletion, gene replacement and 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. The 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 opened at the end of 2003 in the Yamate area.

The 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. Large scale autoclaves for sterilization

In 2006, 4,671 mice were brought into the CTAP in the Yamate area, and 26,451 mice (including pups bred in the facility) were taken out from the CTAP from November 1, 2005 to October 31, 2006.

A number of strains of genetically altered mice from outside the CTAP were brought into the mouse housing

area by microbiological cleaning using the *in vitro* fertilization-embryo transfer techniques, and stored using cryopreservation.

A new mouse facility in the Myodaiji area opened at the beginning of 2005. The facility provides research-supporting activities to researchers in the Myodaiji area. In 2006, 98 mice were brought into the CTAP in the Myodaiji area, and 1,475 mice (including pups bred in the facility) were taken out from the CTAP from November 1, 2005 to October 31, 2006.

Figure 3. Equipment for generating mutant mice under specific pathogen free conditions

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 the conditions desired for fish breeding in the aquarium systems. Currently, five or more mutant lines and ten or more transgenic lines of medaka and zebrafish 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 transgenic animals, allowing researchers to generate and maintain transgenic animals.

In 2006 (from November 1, 2005 to October 31, 2006), 3,788 medaka and zebrafish (1,455 embryos and 2,333 adults) were brought to the facility and 40,684 medaka and zebrafish (38,938 fertilized eggs, 1,310 embryos, and 436 adults, including animals bred in the facility) were taken out from the CTAP. In a laboratory for chick embryos 32,365 fertilized chicken eggs were brought in and 280 animals (135 fertilized eggs and 145 embryos) were taken out from the CTAP. These animals
were used for research activities in neurobiology and developmental biology

Figure 4. Breeding equipment for zebrafish

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 body fluid water and sodium homeostasis using gene-targeting mice, the Laboratory of Neurochemistry is studying the physiological role of the dopaminergic system using genetically altered mice, and the Laboratory of Molecular Genetics for Reproduction is studying the 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:	*IIDA, Shigeru*
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. Confocal Laser Scanning Microscope(OLYMPUS FV1000) and Fluorescence Microscope (KEYENCE BZ-8000) were newly installed in 2006. The Centerís 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-100Σ, PI-50α, PI-50,PI-200)
Automatic Nucleic Acid Isolation System (Kurabo NA-2000)
Genetic Anlyzer (ABI PRISM 3130xl, 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)
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)
Bio Imaging Analyzers (Fujifilm BAS 2000)
Luminescent Image Analyzer (Fujifilm LAS 3000 mini)
Fluorescence Bio Imaging Analyzer (Takara FMBIO)
Microscopes (Carl Zeiss Axiophot, Axiovert)
Environmental Scanning Electron Microscope (PHILIPS XL30 ESEM)
Confocal Laser Scanning Microscope (Leica TCS SP2, OLYMPUS FV1000)
Fluorescence Microscope (KEYENCE BZ-8000)
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 Staffs: *NISHIDE, Hiroyo*
NAKAMURA, Takanori
Technical Assistants: *SUZUKI, Keiko*
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*
KUROYANAGI, Asuka

Transgenic Animal Facility

Subunit Chief: *HAYASHI, Kohji*
Technical Assistants: *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: *KAJIURA-KOBAYASHI, Hiroko*

Cell Biology

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

Developmental Biology

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

Neurobiology

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

Evolutionary Biology and Biodiversity

Unit Chief: *FUKADA-TANAKA, Sachiko*
Subunit Chief: *YAMAGUCHI, Katsushi*
Technical Staffs: *MOROOKA, Naoki*
SUMIKAWA, Naomi

Environmental Biology

Subunit Chief: *MIZUTANI, Takeshi*

Reception

Supporting Staffs: *SAKAGAMI, Mari*
TSUZUKI, Shihoko
KATAOKA, Yukari
UNO, Satoko
KONDO, Yukie

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

The Technical 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 Technical 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*
KAMIYA, Kiyomi
KANEUJI, Kimie

The technical and supporting staff 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 and Kamiya work at the Yamate-Area.

The number of registrants and the number of users from January 2006 to December 2006 are presented in Table 1.

Users counted by the monitoring system going in and out of the controlled areas numbered 4,396 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.

In March 2006, the two systems for radiation safety at Myodaiji-Area - the monitoring system for users going in and out of the controlled areas, and the main control computers of the radiation monitoring system - were renewed. Both systems are shown in figure 3.

Table 1. Numbers of registrants and users at Myodaiji-Area and Yamate-Area in the 2006

	Myodaiji-Area	Yamate-Area
registrants	143	119
users	64	50

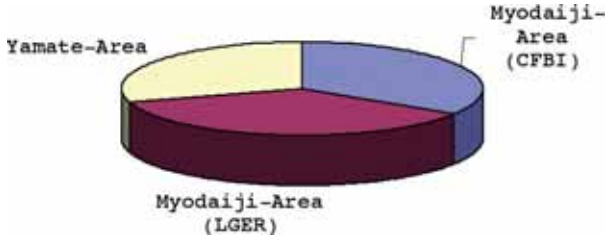

Figure 1. Percentage of users going in and out of each controlled area in 2006

Table 2. Users and visitors who entered each controlled area in 2006

	Myodaiji-Area		Yamate-Area	total
	CFBI-branch	LGER-branch		
users	1288	1462	1149	3899
visitors	208	128	161	497
total	1496	1590	1310	4396

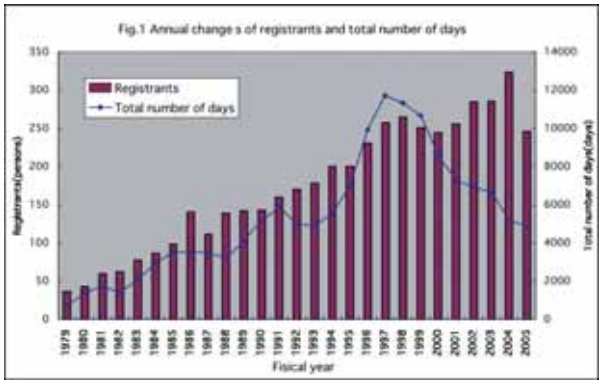

Figure 2. Annual changes of registrants and number of totals per fiscal year

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	1110.000	1110.000
^{125}I Used	0.000	0.000	1110.000	1110.000
^{45}Ca Received	37000.000	0.000	-	37000.000
^{45}Ca Used	18500.000	0.000	-	18500.000
^{35}S Received	18500.000	0.000	37000.000	55500.000
^{35}S Used	18500.000	0.000	16128.636	34628.636
^{32}P Received	244250.000	915750.000	860250.000	2020250.000
^{32}P Used	189250.000	801420.000	783660.000	1774330.000
^{14}C Received	18500.000	0.000	0.000	18500.000
^{14}C Used	10605.680	0.000	0.000	10605.680
^{3}H Received	268250.000	0.000	1110000.000	1378250.000
^{3}H Used	137614.840	0.000	862110.000	999724.840

Figure 3. Photographs show the renewal systems at Myodaiji area.

Left : The monitoring system for users going in and out of the radiation controlled area (upper : control computers; lower : an ID card and a card reader)

Right : The main control computer of the radiation monitoring system

The Third Okazaki Biology Conference
The Biology of Extinction 2

Organizing Chair: Tetsukazu Yahara (Kyushu University, Japan)
Callum Roberts (University of York, USA)
March 12 (Sun) ñ 17 (Fri), 2006

Extinction and speciation are two key processes that contributed to the formation of global patterns of biodiversity. Extinction is, therefore, a natural process. On the other hand, increasing human influence upon global ecosystems is accelerating species extinction. How does extinction occur? How do natural and anthropogenic factors interact upon extinction? What consequences are to be expected following mass extinction? These are critical questions from both basic and applied standpoints.

The purpose of the conference "The Biology of Extinction 2" was to discuss these questions by following up progress in biological sciences related to the extinction of species since the first OBC ìThe Biology of Extinctionî in 2004. The goal of this series of conferences was to explore the possibility of forming a new research field of basic biology centered on the theme of extinction.

We invited nine non-Japanese and five Japanese researchers to attend the first OBC conference and they made significant contributions to its success. In addition, we invited ten new members in order to broaden our perspectives and stimulate discussion.

Scientific topics:

Historic and Prehistoric Extinctions
Patterns and Genetics of Extinction
Population Perspectives for Extinctions
Community Perspectives for Extinction
Invasion and Extinction
Climate Change, Extinction and Management
Toward an Integrated Understanding of Extinction

There were two oral sessions, one in the morning and one in the afternoon, on each day of the conference, excepting Wednesday. These sessions constituted seven sets of papers with specific themes, each of which included three or four speakers. Wednesday afternoon was a time for poster presentations. On Friday morning, eight short talks were given by presenters selected from among the poster presenters. The quality of all of the posters and short talks was very high.

In the seven sessions listed below, we identified some critical progress in our understanding of extinction proneness, the dynamics of extinction, speciation and migration, climatic change effects upon extinction, the role of human psychology upon anthropogenic extinction, and other issues. After the sessions, we had a general discussion about how extinction biology can be developed and synthesized. All of the participants were enthusiastic about participating in the birth of a new research field.

In conclusion, we believe that the meeting was a successful one. This third OBC conference provided an opportunity for experts in different branches of biology to meet and interact productively.

Speakers

ARAUJO, Miguel B. (MNCN, Spain), BIELBY, Jon N. (Imperial College London, UK), BRADSHAW, Corey J. A. (Charles Darwin Univ., Australia), BROOK, Barry W. (Charles Darwin Univ., Australia), CALEY, Julian (AIMS, Australia), COURCHAMP, Franck (CNRS, France), DUFFY, David C. (Univ. Hawaii Manoa, USA), FRANKHAM, Richard (Macquarie Univ., Australia), GOLDBERG, Emma E. (Univ. California, San Diego, USA), HALLEY, John M. (Aristotle Univ. Thessaloniki, Greece), HAMER, Keith C. (Univ. Leeds, UK), HANSKI, Ilkka (Univ. Helsinki, Finland), HARRISON, Ian J. (AMNH, USA), HASTINGS, Alan (Univ. California, Davis, USA), HUME, Julian P. (Natural History Museum, England), JACKSON, Jeremy B. C. (Univ. California, San Diego, USA), JETZ, Walter (Univ. California, San Diego, USA), KOH, Lian Pin (Princeton Univ., USA), LANDE, Russell (Univ. California, San Diego, USA), MARTINEZ-MEYER, Enrique (UNAM, Mexico), OLSON, Storrs L. (Smithsonian Inst., NMNH, USA), ORME, David (Imperial College London, UK), PURVIS, Andy (Imperial College London, UK), RIPA, Jorgen (Lund Univ., Sweden), ROBERTS, Callum (Univ. York, USA), SALA, Enric (CMBC, USA), SEKERCIOGLU, Cagan H. (Stanford Univ., USA), SHAFFER, H. Bradley (Univ. California, Davis, USA), SODHI, Navjot S. (National Univ. Singapore, Singapore), STIASSNY, Melanie L. (AMNH, USA), VIE, Jean-Christophe (IUCN, Switzerland), WILMERS, Christopher C, (Univ. California, Davis, USA)
HAKOYAMA, Hiroshi (NRIFS, Japan), ISHIHAMA, Fumiko (NIES, Japan), IWASA, Yoh (Kyushu Univ., Japan), KUDO, Gaku (Hokkaido Univ., Japan), MASUDA, Michiko (NIT, Japan), MATSUDA, Hiroyuki (Yokohama National Univ., Japan), NATUHARA, Yosihiro (Osaka Pref. Univ., Japan), OTA, Hidetoshi, (Univ. Ryukyus, Japan), TAKAGAWA, Shinichi (Univ. Tokyo, Japan), TAKENAKA, Akio (NIES, Japan), TANAKA, Yoshinari (Chuo Univ., Japan), TOKITA, Kei (Osaka Univ., Japan), TOMIMATSU, Hiroshi (TMU, Japan), WASHITANI, Izumi (Univ. Tokyo, Japan), YAHARA, Tetsukazu (Kyushu Univ., Japan), YOKOMIZO, Hiroyuki (Kyushu Univ., Japan)

The Fourth Okazaki Biology Conference
Terra Microbiology 2

Organizing Chair: Kenji Kato (Shizuoka University, Japan)
Daniel J. Arp (Oregon State University, USA)
September 10 (Sun) ñ 15 (Fri), 2006

The conference was held at the Okazaki Conference Center and was attended by 54 invited participants from the USA (11), the UK (3), Switzerland (2), the Netherlands (2), Australia (2), Singapore (2), Germany (1), China (1), New Zealand (1) and Japan (29). Inspired by the achievements of the previous conference, the ìTerra Microbiology 2î conference was organized with the intention of embodying the results of the conference more directly by initiating several cooperative research programs. A fast developing field of the research on nitrogen metabolism from both metabolic sequence and genome analysis was focused on during Session 1. Session 2 and Session 3 focused on other important aspects of microbial ecology.

Session 1: Bio-geochemical cycling and microbial function.

Session 2: Gene hopping among microbes, from clinical and environmental evidence to the evolution of life.

Session 3: Bacterial cross talk.

In addition to the intensive discussions, the conference included two keynote lectures, 30 oral presentations, and 27 poster presentations. The development of meta-genomics was one of the principle topics of discussion during the conference.

Scientific topics:

Bio-Geochemical Cycling and Microbial Functions

Gene Hopping Among Microbes
-From Clinical and Environmental Evidences to Evolution of Life -

Bacterial Cross Talk

Speakers

AMINOV, Rustam (The Rowett Research Institute, UK), ARP, Daniel J. (Oregon State University, USA), BARKAY, Tamar (Rutgers University, USA), BAUER, Wolfgang D. (University of California, USA), EBERL, Leo (University of Zurich, Switzerland), GU, Ji-Dong (The University of Hong Kong, PR China), HENNECKE, Hauke H. (ETH, Switzerland), HETTICH, Robert L. (Oak Ridge National Laboratory, USA), KJELLEBERG, Staffan L. (The University of New South Wales, Australia), KLOTZ, Martin G. (University of Louisville, USA), LAANBROEK, Hendrikus J. (Netherlands Institute of Ecology, The Netherlands), LIU, Wen-Tso (National University of Singapore, Singapore), MURRELL, Colin (University of Warwick, UK), RAINEY, Paul B. (University of Auckland, New Zealand), RIVERA, Maria C. (University of California Los Angeles, USA), ROHWER, Forest (San Diego State University, USA), SALMOND, George P. C. (University of Cambridge, UK), SCHUSTER, Stephan C. (Penn State University, USA), SMALLA, Kornelia (Federal Biological Research Centre for Agriculture and Forestry, Germany), SOBECKY, Patricia A. (Georgia Institute of Technology, USA), SPIRO, Stephen (University of Texas at Dallas, USA), STROUS, Marc (Radboud University Nijmegen, The Netherlands), TIEDJE, James M. (Michigan State University, USA), ZHANG, Lian-Hui (Institute of Molecular and Cell Biology, Singapore), ZHOU, Jizhong (Institute for Environmental Genomics, USA)

ARITA, Masanori (The University of Tokyo, Japan), EDA, Shima (Tohoku University, Japan), FUJIWARA-NAGATA, Erina (Kinki University, Japan), IKEDA, Tsukasa (Utsunomiya University, Japan), KAMAGATA, Yoichi (National Institute of Advanced Industrial Science and Technology (AIST), Japan), KATAYAMA, Yoko (Tokyo University of Agriculture and Technology, Japan), KATO, Kenji (Shizuoka University, Japan), KATO, Junichi (Hiroshima University, Japan), KAWARABAYASI, Yutaka (National Institute of Advanced Industrial Science and Technology (AIST), Japan), KOBA, Keisuke (Tokyo Institute of Technology, Japan), KUGA, Yukari (Shinshu University, Japan), MINAMISAWA, Kiwamu (Tohoku University, Japan), MIYASHITA, Hideaki (Kyoto University, Japan), MORISAKI, Hisao (Ritsumeikan University, Japan), NANBA, Kenji (Fukushima University, Japan), NASU, Masao (Osaka University, Japan), OHTA, Hiroyuki (Ibaraki University, College of Agriculture, Japan), OHTOMO, Ryo (National Institute of Livestock and Grassland Science, Japan), OKABE, Satoshi (Hokkaido University, Japan), SAITO, Masanori (National Institute for Agro-Environmental Sciences, Japan), SENOO, Keishi (The University of Tokyo, Japan), SHOUN, Hirofumi (The University of Tokyo, Japan), SUNAMURA, Michinari (The University of Tokyo, Japan), SUWA, Yuichi (National Institute of Advanced Industrial Science and Technology (AIST), Japan), SUZUKI, Satoru (Ehime University, Japan), TAKAI, Ken (Japan Agency for Marine-Earth Science & Technology, Japan), UCHIYAMA, Ikuo (National Institute for Basic Biology, Japan), WADA, Minoru (The University of Tokyo, Japan), YOSHINAGA, Ikuo (Graduate School of Agriculture, Kyoto University, Japan), OHMORI, Masayuki (Saitama University, Japan)

52nd NIBB Conference
Reproductive Strategies

Organizing Chair: Motonori Hoshi (Keio University)
January 20 (Fri) - 23 (Mon), 2006

Reproduction is one of the most characteristic features of living organisms. Essential for preserving species, this ability to reproduce has maintained life since its beginnings 3.8 billion years ago. For mammals, including human beings, reproduction and sex (a mechanism for shuffling genes) are inseparable and each individual has a fixed sex. There exists, however, a great variety of reproductive strategies. Some living organisms reproduce asexually, while others use either sexual or asexual reproduction strategies depending upon the circumstances. Some organisms change their sex during their lifetime.

This conference brought together participants ranging in experience from veteran scientists whose names appear in the history of molecular biology to graduate students. The participants engaged in intense and spirited discussions on many aspects of sex and reproduction. Why does sex exist? How much diversity is there among reproductive strategies? How did such strategies evolve? What is the biological significance of such strategies? Discussions on these and other topics provided research training opportunities as well as opportunities to establish and develop friendships transcending age, experience, and nationality.

Scientific topics:

Origin and Evolution of Sexual Reproduction

Determination and Differentiation of Sex

Germ Differentiation and Meiosis

Gamete Interactions

Allo-Recognition in Sexual Reproduction

Epigenetics

Evolution and Adaptation of Embryos and Larvae

Conflict and Competition in Sexual Reproduction

Speakers

BIRKHEAD, Tim R. (University of Sheffield, UK), DARSZON, Alberto (Universidad Nacional Autonoma de Mexico, Mexico), DORRESTEIJN, Adriaan (University of Giessen, Germany), EPEL, David (Stanford University, USA), EXTAVOUR, Cassandra (University of Cambridge, UK), HEINZE, Jurgen (University of Regensburg Germany), HEYLAND, Andreas (The Whitney Laboratory for Marine Bioscience, USA), JOLY, Dominique (Centre National de la Recherche Scientifique, France), MESELSON, Matthew (Harvard University, USA), MICHIELS, Nico (University of Tuebingen, Germany), NIELSEN, Claus (University of Copenhagen, Denmark), NORMARK, Benjamin (University of Massachusetts, USA), OLSSON, Mats (University of Wollongong, Australia), PRUITT, Robert E. (Purdue University, USA), SCHARER, Lukas (University of Innsbruck, Austria), STEWART, James R. (East Tennessee State University, USA), VACQUIER, Victor D. (University of California, San Diego, USA), WAKE, Marvalee H. (University of California Berkeley, USA)

AIGAKI, Toshiro (Tokyo Metropolitan University, Japan), ABE, Shin-ichi (Kumamoto University, Japan), HASEBE, Mitsuyasu (National Institute for Basic Biology, Japan), HOSHI, Motonori (Keio University, Japan), INABA, Kazuo (Tsukuba University, Japan), ISHIKAWA, Fuyuki (Kyoto University, Japan), ISHINO, Fumitoshi (Tokyo Medical and Dental University, Japan), IWASA, Yoh (Kyushu University, Japan), KAKUTANI, Tetsuji (National Institute of Genetics, Japan), KISHIMOTO, Takeo (Tokyo Institute of Technology, Japan), KOBAYASHI, Ichizo (University of Tokyo, Japan), KOBAYASHI, Kazuya (Keio University, Japan), KOBAYASHI, Satoru (National Institute for Basic Biology, Japan), KUROIWA, Asato (Hokkaido University, Japan), MATSUI, Yasuhisa (Tohoku University, Japan), MOHRI, Hideo (Professor Emeritus, National Institute for Basic Biology, Japan), MOROHASHI, Ken-ichirou (National Institute for Basic Biology, Japan), NAGAHAMA, Yoshitaka (National Institute for Basic Biology, Japan), NOCE, Toshiaki (Mitsubishi Kagaku Institute of Life Sciences, Japan), OKABE, Masaru (Osaka University, Japan), SAWADA, Hitoshi (Nagoya University, Japan), SUNANAGA, Takeshi (Kochi University, Japan), TACHIBANA, Kazunori (Tokyo Institute of Technology, Japan), TAKAHASHI, Yoshiko (Center for Developmental Biology, RIKEN, Japan), YAMAMOTO, Masayuki (University of Tokyo, Japan)

53rd NIBB Conference
Dynamic Organelles in Plants

Organizing Chair: Mikio Nishimura
June 14 (Wed) -17 (Sat), 2006

Because they spread their roots in the ground, plants must survive in a given environment. In order to adapt, they utilize environmental changes in the life cycle as important signals that are necessary for their survival. Recent studies have shown that plant cells can induce, degenerate and differentiate their organelles to adapt to environmental changes.

This conference provided an excellent opportunity to review recent advances in the field of plant organelle studies with special emphasis on their dynamics. Thirty-three lectures were presented in five sessions, namely 1) Differentiation and degradation, 2) Biogenesis and protein transport, 3) Post-genome approach, 4) Metabolic regulation and signal transduction, and 5) Integrated functions. Over 200 researchers, including 20 researchers from overseas, participated in the conference, which also included one plenary lecture and 89 poster presentations.

The participants were inspired to develop their own research on dynamic organelles. The conference was well-timed to provide an excellent opportunity to clarify the molecular mechanisms underling organelle dynamics in plants.

The conference was supported by JSPS (Japanese Society of Promotion of Science), Grant-in Aid for Scientific Research of Priority Areas on ìO rganelle Differentiationî, National Institute for Basic Biology, Japan Plant Science Foundation and the Daiko Foundation.

Scientific topics:

Differentiation and Degradation

Biogenesis and protein Transport

Post-Genome Approach

Metabolic Regulation and Signal Transduction

Integrated Functions

Speakers

BAKER, Alison (University of Leeds, UK), BRODSKY, Jeffrey (University of Pittsburgh, USA), CHRISTELLER, John (Horticulture and Food Research Institute of NZ, New Zealand), DENECKE, Jurgen (University of Leeds, UK), DUPREE, Paul (University of Cambridge, UK), EHRHARDT, David (Carnegie Institution, USA), GREENBERG, Jean (The University of Chicago, USA), HUANG, Anthony (University of California, USA), INOUE, Kentaro (University of California at Davis, USA), KOROLEVA, Olga (John Innes Centre, UK), LEE, Youngsook (POSTECH, South Korea), MITTLER, Ron (University of Nevada, USA), THIEL, Gerhard (Darmstadt University of Technology, Germany)

ASADA, Kozi (Fukuyama University, Japan) HARA-NISHIMURA, Ikuko (Kyoto University, Japan), ISHIGURO, Sumie (Nagoya University, Japan), MIMURA, Tetsuro (Kobe University, Japan), MORITA, Miyo (Nara Institute of Science and Technology, Japan), NAKANO, Akihiko (The University of Tokyo, Japan), NISHIKAWA, Shuh-ichi (Nagoya University, Japan), NISHIMURA, Mikio (National Institute for Basic Biology, Japan), NISHITANI, Kazuhiko (Tohoku University, Japan), NISHIZAWA, Naoko K. (The University of Tokyo, Japan), OHSUMI, Yoshinori (National Institute for Basic Biology, Japan), SAITO, Kazuki (Chiba University/ RIKEN, Japan), SAKAMOTO, Wataru (Okayama University, Japan), SHIBATA, Daisuke (Kazusa DNA Research Institute, Japan), SHIKANAI, Toshiharu (Kyushu University, Japan), SHIMAZAKI, Ken-ichiro (Kyushu University, Japan), SHIRASU, Ken (RIKEN, Japan), TAKANO, Hiroyoshi (Kumamoto University, Japan), TANAKA, Kan (The University of Tokyo, Japan), UCHIMIYA, Hirofumi (The University of Tokyo, Japan), YAMAYA, Tomoyuki (Tohoku University, Japan)

The Second NIBB-EMBL Symposium
Frontiers in Bioimaging

Organizing Chair: Naoto Ueno
Jan Ellenberg (EMBL Heidelberg, Germany)
March 22 (Wed) ñ 23 (Thu), 2006

Bioimaging is one of the chief subjects of the collaboration between NIBB and EMBL. This meeting was held with the aim of exchanging information and discussing emerging and innovative technologies including chemical probes, microscopes with new concepts, and image data processing.

Dr. Stelzer (EMBL) reported on the Selective Plane Illumination Microscopy (SPIM), which is a recently developed microscope that allows scientists to observe large-sized (up to a few millimeters) and even living specimens. Dr. Sedat (UCSF) talked about the advanced microscopic system ìO MXî, the first practical implementation of structured illumination (SI) in which the grid is superimposed onto the sample to gain sharper images. Dr. Ellenberg talked about the genome-wide screening of mitosis-regulating genes called ìM itoCheckî based on the mass image analysis of cultured cells combined with the disruption of genes with RNA interference.

Because of the high quality of the presentations and the lively discussions among the participants, this meeting was of significant importance for the field of bioimaging.

Scientific topics:

Imaging Diffusion & Activity

Emerging Technologies

Bioluminescence

Networks & Screening

3D Imaging

Mitosis/Trafficking

Organism Model

Speakers

BASTIAENS, Philippe I. (European Molecular Biology Laboratory (EMBL), Germany), BRUNNER, Damian (European Molecular Biology Laboratory (EMBL), Germany), DENK, Winfried (Max-Planck Institute for Medical Research, Germany), ELLENBERG, Jan (European Molecular Biology Laboratory (EMBL), Germany), ISSAD, Tarik (CNRS, France), JOHNSSON, Kai (Ecole Polytechnique Federale de Lausanne, Switzerland), KERPPOLA, Tom K. (University of Michigan and Howard Hughes Medical Institute, USA), LIPPINCOTT-SCHWARTZ, Jennifer A. (National Institutes of Health, USA), MEYER, Tobias (Stanford University School of Medicine, USA), SEDAT, John W. (University of California, San Francisco, USA), SINGER, Robert H. (Albert Einstein College of Medicine, USA), SO, Peter T. C. (Massachusetts Institute of Technology, USA), STELZER, Ernst H. K. (EMBL Heidelberg, Germany), SWEDLOW, Jason (University of Dundee, UK), WEIJER, Cornelis J. (University of Dundee, UK), WITTBRODT, Joachim (European Molecular Biology Laboratory (EMBL), Germany)

HAMADA, Hiroshi (Osaka University, Japan), HIRAOKA, Yasushi (Kansai Advanced Research Center, NICT, Japan), ITO, Kei (The University of Tokyo, Japan), KASAI, Haruo (The University of Tokyo, Japan), KINJO, Masataka (Hokkaido University, Japan), KODAMA, Ryuji (National Institute for Basic Biology, Japan) KUSUMI, Akihiro (Kyoto University, Japan), MIYAWAKI, Atsushi (Brain Science Institute, RIKEN, Japan), NAGAYAMA, Kuniaki (National Institute for Physiological Sciences, Japan), OHSUMI, Yoshinori (National Institute for Basic Biology, Japan), OZAWA, Takeaki (Institute for Molecular Science, Japan), TABATA, Tetsuya (The University of Tokyo, Japan), TANAKA, Minoru (National Institute for Basic Biology, Japan), TERAKAWA, Susumu (Hamamatsu University School of Medicine, Japan), UENO, Naoto (National Institute for Basic Biology, Japan), WADA, Masamitsu (National Institute for Basic Biology, Japan)

The Fourth NIBB-EMBL Symposium
Biology of Protein Conjugation: Structure and Function

Organizing Chair: Yoshinori Ohsumi
Winfried Weissenhorn (EMBL Grenoble, France)
December 3 (Sun) ñ 5 (Tue), 2006

The Fourth NIBB-EMBL Symposium was held gathering distinguished researchers in the field of the biology of protein conjugation from Europe, USA and Japan. There were 27 oral presentations, of which 12 were by foreign invitees, and 18 poster presentations. The symposium was a successful one as shown in the following comment given by a participant.

The NIBB-EMBL conference in Okazaki, Japan this past December was an outstanding international conference that included scientist from Japan, Europe and the US. It was an exciting meeting with considerable discussion both during the meeting sessions and the social activities. The program included innovative technologies in structural biology, live cell imaging and molecular biology. Each speaker presented new unpublished data and discussed the key questions that need to be addressed in their field. New insights into the roles for ubiquitin and ubiquitin-related molecules in cell signaling, membrane trafficking and protein turn-over were presented. It was the best meeting in 2006 that I attended covering these key topics in cell and molecular biology. (Scott D. Emr, UCSD, USA)

Scientific topics:

SUMO

Membrane Biology

Ubiquitin

Mechanism of UBL Conjugation

Modification by Lipid and Sugar

Speakers

DIKIC, Ivan (Johann Wolfgang Goethe University Hospital, Germany), EMR, Scott D. (UCSD School of Medicine, USA), GOODY, Roger S. (Max-Planck-Institute of Molecular Physiology, Germany), KLEVIT, Rachel E. (University of Washington, USA), LIMA, Christopher D. (Sloan-Kettering Institute, USA), POLO, Simona (IFOM, The FIRC Institute for Molecular Oncology, Italy), RORTH, Pernille (European Molecular Biology Laboratory, Germany), SCHULMAN, Brenda A. (St. Jude Childrenís Research Hospital, USA), SOMMER, Thomas (Max-Delbruck-Center for Molecular Medicine, Germany), ULRICH, Helle D. (Cancer Research UK, UK), WALTERS, Kylie J. (University of Minnesota, USA), WEISSENHORN, Winfried (European Molecular Biology Laboratory, France)

INAGAKI, Fuyuhiko (Hokkaido University, Japan), IWAI, Kazuhiro (Osaka City University, Japan), KAMURA, Takumi (Nagoya University, Japan), KATO, Shigeaki (The University of Tokyo, Japan), KATO, Koichi (Nagoya City University, Japan), KAWAHARA, Hiroyuki (Hokkaido University, Japan), KIHARA, Akio (Hokkaido University, Japan), KONDO, Takao (Nagoya University, Japan), MOROHASHI, Ken-ichirou (National Institute for Basic Biology, Japan), OHNO, Ayako (RIKEN, Japan), OHSUMI, Yoshinori (National Institute for Basic Biology, Japan), SAITOH, Hisato (Kumamoto University, Japan), SHIRAKAWA, Masahiro (Kyoto University, Japan), SUZUKI, Tadashi (Osaka Univeristy, Japan), TANAKA, Keiji (The Tokyo Metropolitan Institute of Medical Science, Japan), TENNO, Takeshi (Kyoto University, Japan), WAKATSUKI, Soichi (KEK (High Energy Accelerator Research Organization), Japan), YOSHIDA, Minoru (RIKEN, Japan)

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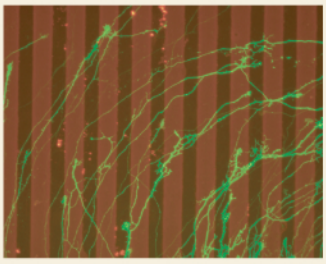

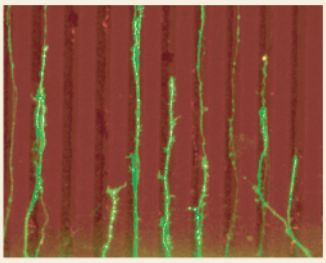

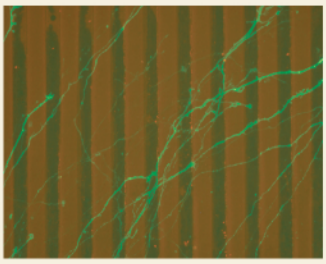

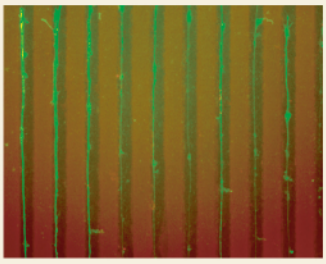

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