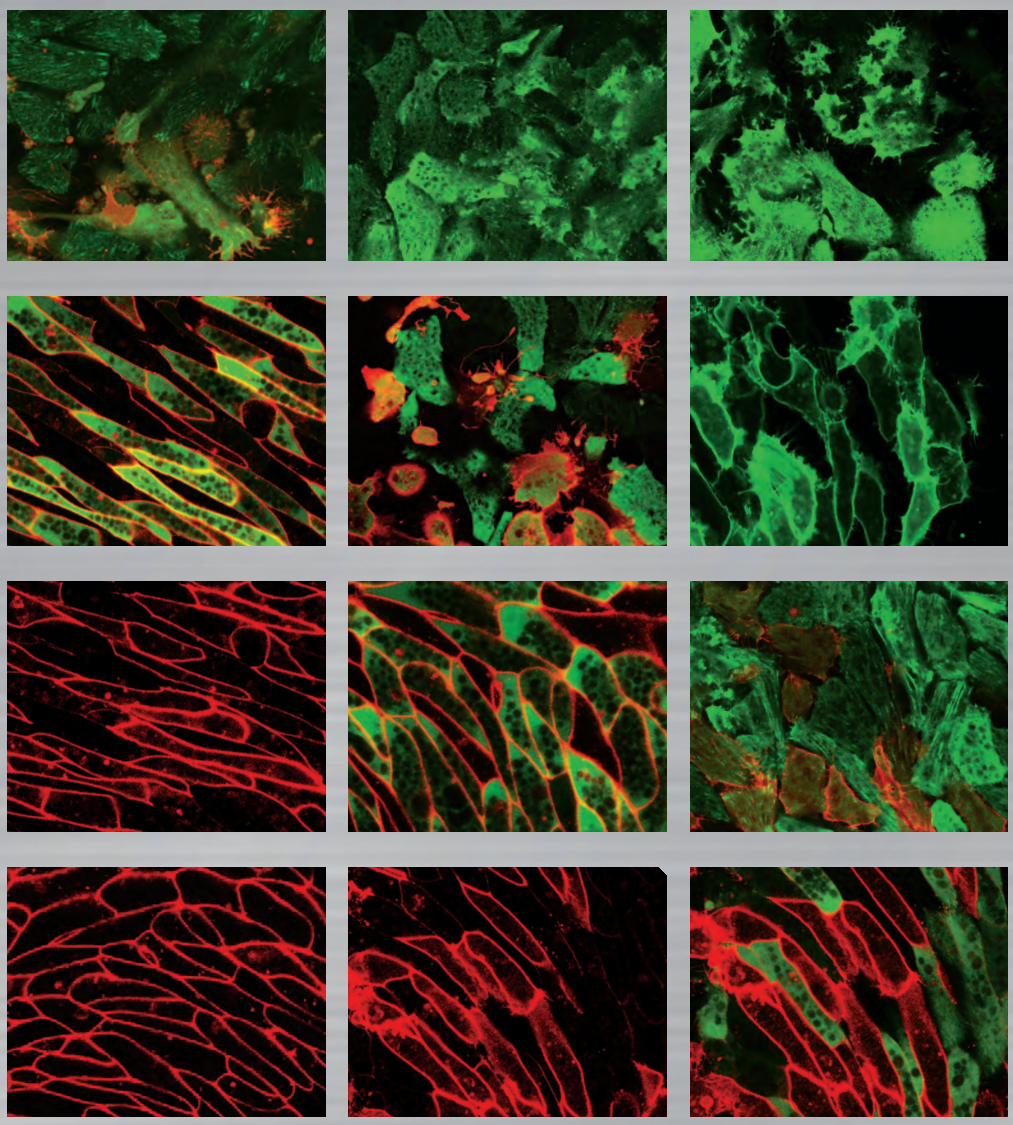


National Intitute for Basic Biology

2007 ANNUAL REPORT

CONTENTS

The cover photographs are excerpts from the study of the role of paxillin in the movement of mesodermal cells of the *Xenopus* embryos published in Nature Cell Biology. The dynamics of cytoskeletal and membrane proteins during gastulation were analyzed using laser scanning confocal microscopy. See page 21 for the details.

INTRODUCTION

Kiyotaka Okada

Our institute, the National Institute for Basic Biology, was established in 1977, and we proudly celebrated our 30th anniversary on June 1, 2007. We hosted a ceremony of celebration with our sister institute, the National Institute for Physiological Sciences (NIPS), with nearly 300 friends, supporters, former researchers and officers attending. Since its foundation, the NIBB has developed as a center of excellence in research, education, and inter-university collaboration in the various fields of basic biology. In 2004, the NIBB and four national research institutes established a new organization, the National Institutes of Natural Sciences (NINS), one of four Inter-University Research Institute Corporations. We now cover a wide variety of fields including cell biology, developmental biology, neurobiology, evolutionary biology and biodiversity, environmental biology, theoretical biology and imaging science, and are studying the basic action of genes and cells common to all living organisms, as well as the mechanisms involved in producing the diversified structures and activities of life adapting to an ever-changing environment. Research in these basic biological fields is maintained at a high level while we continue to search for new and challenging fields of study. We are also involved in ongoing collaboration with a number of researchers from Japanese and foreign universities and institutes. In addition to research, the education of the next generation of researchers is another important aim. As a member of the Graduate University for Advanced Studies, we are involved in training graduate students. We had five new doctors in March of 2008.

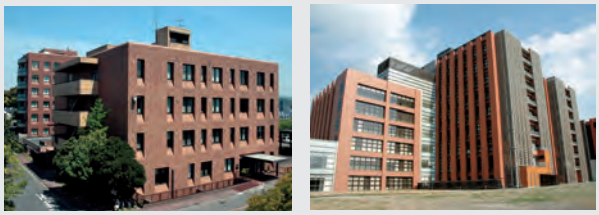
NIBB's activities during 2007, as well as our goals for the future, are summarized in this booklet. We are always open to questions, comments and suggestions.

Kiyotaka OKADA, D. Sci.
Director-general, NIBB

ORGANIZATION OF THE NATIONAL INSTITUTE FOR BASIC BIOLOGY

The National Institute for Basic Biology (NIBB) was founded in 1977 on a hill overlooking the old town of Okazaki in Aichi prefecture as one of the Inter-University Research Institutes to promote and stimulate studies of biology. NIBB conducts first-rate research on site as well as in cooperation with national, public and private universities and research organizations. NIBB attempts to elucidate the general and fundamental mechanisms underlying various biological phenomena throughout a wide variety of biological fields, such as cell biology, developmental biology, neurobiology, evolutionary biology, environmental biology, and theoretical biology.

NIBB and four other national institutes, the National Astronomical Observatory (NAO), the National Institute for Fusion Science (NIFS), the National Institute for Physiological Sciences (NIPS), and the Institute for Molecular Science (IMS), constitute the National Institutes of Natural Sciences, which is one of the four Inter-University Research Institute Corporations. NIBB's activities are supported by the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan. The Okazaki Institute for Integrative Bioscience was established as a common facility for the three institutes (NIBB, NIPS, and IMS) of the Okazaki campus.



Buildings in the Myodaiji area (left) and Yamate area (right).

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 Advisory Committee makes recommendations on the appointments of new Director-Generals, faculty appointments, the NIBB's annual budget and future prospects.

The Strategic Planning Department is a central office for public relations and the management of conferences and other extramural activities. The department also assists the director-general with NIBB's evaluation procedures and in planning a long-range strategy for the institute.

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

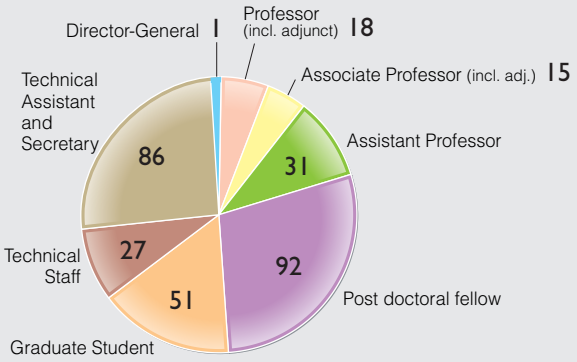
Research and Its Support

The NIBB conducts its research programs through thirty-one research units and three research support units. Each research unit is called either a division, led by a professor, or a laboratory, led by an associate professor or an assistant professor. Each research unit forms an independent project team. Some of the research units are adjunct and led by professors who hold joint appointments with other universities. Adjunct divisions have a resident assistant professor. This arrangement facilitates exchange in research activities in Japan. The Technical Division manages the activities of the technical staff and helps to promote the research activities of each research unit and to maintain the common research resources of both the research support units of the NIBB and some of the research facilities of the Okazaki campus.

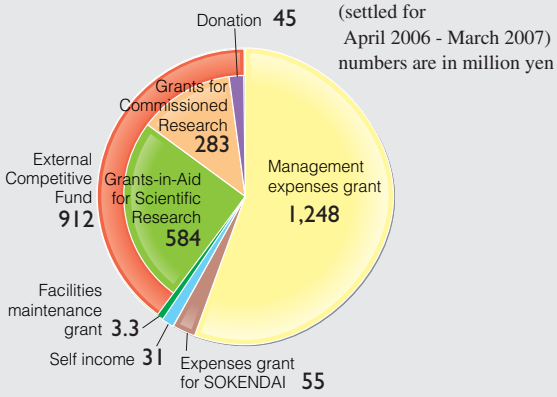
The Okazaki Institute for Integrative Bioscience was founded to conduct interdisciplinary molecular research on various biological phenomena and is shared by all three institutes in Okazaki. Some of the divisions function also as NIBB's divisions.

Members in the NIBB

(as of 1st April, 2007)

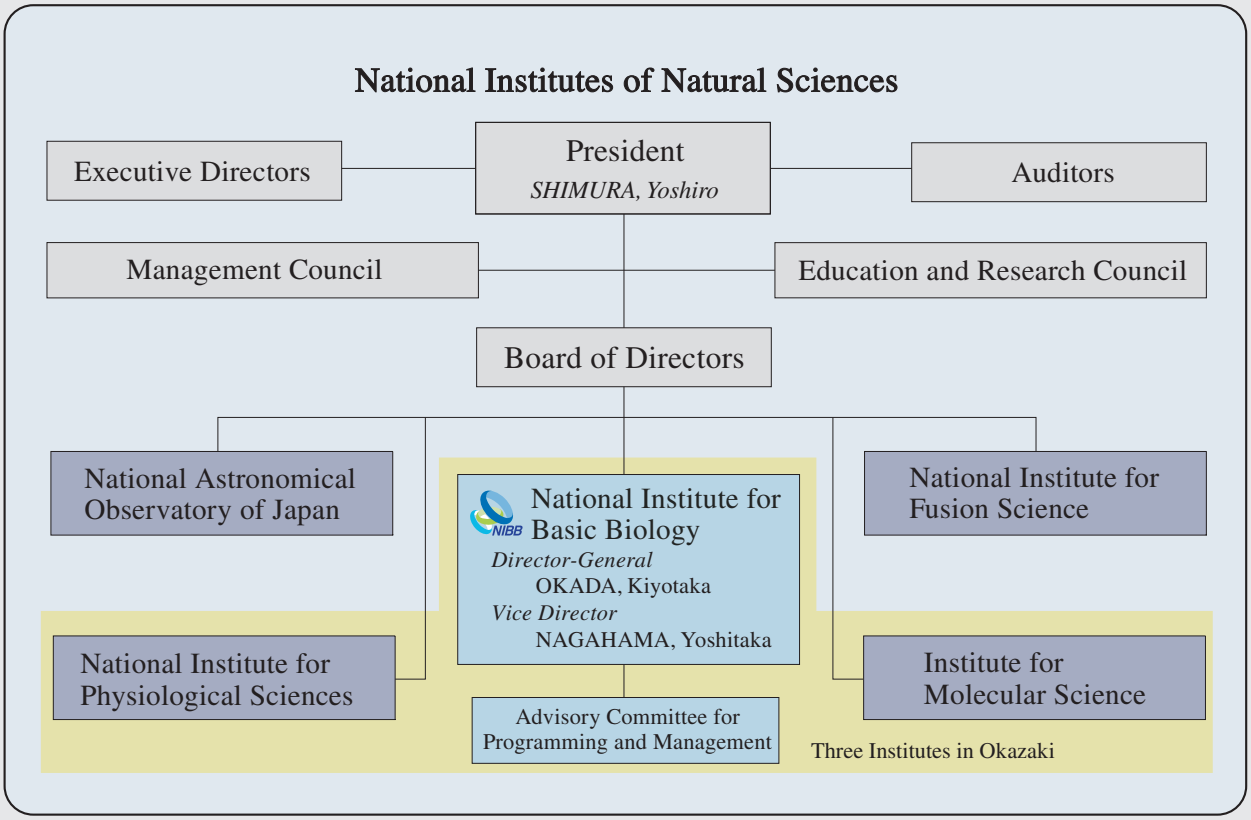


Financial Configuration of the NIBB



The NIBB acquires numerous competitive funds in an effort made by individual researchers, including Grants-in-Aid for Scientific Research and Grants for Commissioned Research, in addition to national subsidies (Management Expenses Grants and Expenses Grants for SOKENDAI).

Organization



Members of the Advisory Committee for Programming and Management

Chairperson	OHSUMI, Yoshinori	Professor, National Institute for Basic Biology
Vice-Chair	IWASA, Yoh	Professor, Kyushu University
Extra NIBB member	HASEGAWA, Mariko	Professor, The Graduate University for Advanced Studies (SOKENDAI)
	ISHINO, Fumitoshi	Professor, Tokyo Medical and Dental University
	KATO, Kazuto	Associate Professor, Kyoto University
	MATSUOKA, Makoto	Professor, Nagoya University
	OSUMI, Noriko	Professor, Tohoku University
	SAKANO, Hitoshi	Professor, The University of Tokyo
	SATOH, Noriyuki	Professor, Kyoto University
	TABATA, Satoshi	Deputy Director, Kazusa DNA Research Institute
NIBB member	HASEBE, Mitsuyasu	Professor, National Institute for Basic Biology
	HORIUCHI, Takashi	Professor, National Institute for Basic Biology
	IGUCHI, Taisen	Professor, Okazaki Institute for Integrative Bioscience
	IIDA, Shigeru	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
	TAKADA, Shinji	Professor, Okazaki Institute for Integrative Bioscience
	UENO, Naoto	Professor, National Institute for Basic Biology
	YAMAMORI, Tetsuo	Professor, National Institute for Basic Biology

National Institute for Basic Biology

Research Units

- **Cell Biology**
  - Division of Cell Mechanism
  - Division of Molecular Cell Biology
  - Division of Cell Proliferation (adjunct)
  - Laboratory of Cell Structure
  - Laboratory of Cell Sociology
- **Developmental Biology**
  - Division of Reproductive Biology
  - Division for Sex Differentiation
  - Division for Morphogenesis
  - Division of Developmental Genetics †
  - Division of Molecular and Developmental Biology ††
  - Laboratory of Molecular Genetics for Reproduction
  - Laboratory of Plant Organ Development
- **Neurobiology**
  - Division of Molecular Neurobiology
  - Division of Brain Biology
  - Division of Behavioral Biology (adjunct)
  - Laboratory of Neurophysiology
  - Laboratory of Neurochemistry
- **Evolutionary Biology and Biodiversity**
  - Division of Molecular Genetics
  - Division of Genome Dynamics
  - Division of Evolutionary Biology
  - Division of Speciation Mechanism (adjunct)
  - Laboratory of Morphodiversity
  - Laboratory of Bioresource
- **Environmental Biology**
  - Division of Molecular Environmental Endocrinology †††
  - Division of Plant Developmental Genetics (adjunct)
  - Division of Photobiology (adjunct)
  - Laboratory of Photoenvironmental Biology
- **Theoretical Biology**
  - Division of Theoretical Biology
  - Laboratory of Genome Informatics
- **Imaging Science**
  - Division of Developmental Dynamics (adjunct)
  - Laboratory for Spatiotemporal Regulations

Research Support Units

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

Other Research Support Sections

- **Technical Division**
- **Strategic Planning Department**

Okazaki Research Facilities

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

*-***) These divisions also function as NIBB's divisions †-†††) , respectively.
Other divisions of the OIIB are not shown.

Research Facilities run jointly with National Institute for Physiological Sciences

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

GOALS OF THE NIBB

NIBB has set five goals for its activities in pursuing the progress of biology. We envision contributing to the world-wide community of biologists through our efforts to accomplish these goals. This chapter briefly explains four of the goals. The last goal, the promotion of academic research, is accomplished through our research activities, which are introduced throughout this brochure.

Promotion of Collaborative Research

■ Collaborative Research Projects

Research projects in collaboratation with the NIBB's divisions/laboratories and research activities to be conducted using the NIBB's facilities are solicited from external researchers at other universities and institutes. In addition to conventional "individual collaborative research projects," "collaborative experiments using the Large Spectrograph," and "NIBB workshops," new types of research projects are solicited that will facilitate the strategic organization of collaborative research projects. "Priority collaborative research projects" are carried out in one to three years as group research by internal and external researchers with the purpose of developing pioneering research fields in biology. Three projects have already been carried out, including "molecular mechanisms for controlling the individuals of higher plants." The category of "collaborative research projects for model organism/technology development" was established in 2007 with the aim of developing and establishing new model organisms. Two projects have already been carried out, including "development of the transgenic strain of *Cabombaceae* (primitive angiosperm)." In the belief that the methods of conducting collaborative research projects must be constantly modified according to the demands of the age and the biology community, the NIBB always encourages discussion on such projects.

Collaborative research projects by year

year	2005	2006	2007
Priority collaborative research projects	2	3	1
Collaborative research projects for model organisms/technology development	–	–	2
Individual collaborative research projects	41	37	34
NIBB workshops	4	1	1
Collaborative experiments using the Large Spectrograph	19	18	12
total	64	59	50

■ Enhancement of the Large Spectrograph

The Large Spectrograph Laboratory, a world-leading research facility in photobiology, has succeeded in a large number of collaborative research projects since its foundation in 1980. To ensure further high-level achievements, the Laboratory has promoted the enhancement of its laboratory equipment, including advanced control systems, the use of laser sources, and sophisticated analysis equipment. To supplement the currently operating fixed (partly tunable) wavelength laser sources, the introduction of tunable laser sources covering a wide range of wavelengths from UV to IR is in preparation.

International Cooperation

■ Collaborative research projects with the EMBL

The European Molecular Biology Laboratory (EMBL), established in 1974, is a research institute funded by 18 European. It conducts comprehensive, high-level basic research programs and leads the world in the field of molecular biology. The NIBB takes the leading role in collaborative research programs between the EMBL and the National Institutes of Natural Sciences (NINS), which were launched in 2005, and promotes personal and technological exchange through symposia, exchange between researchers and graduate students, and the introduction of experimental equipment (see page 83 for details of the EMBL meeting held in 2007).



Seminar (left) and discussions (right) in EMBL, Heidelberg

■ NIBB Conference

The NIBB Conference is an international conference organized by the NIBB's professors once or twice a year with the participation of a guest lecturer from abroad. Since the first conference in 1977 (the year of the NIBB's foundation), the NIBB Conference has provided researchers in basic biology with valuable opportunities for international exchange.

■ International Practical Course

With the cooperation of researchers from Japan and abroad, the NIBB international practical course, a practical training course, is given at the laboratory specifically prepared for the course at the NIBB. The first course, titled "Developmental Genetics of Zebrafish and Medaka," was held in 2007. Graduate students from various East Asian nations and areas, including China, Hong Kong, Taiwan, and India, were provided with training in state-of-the-art techniques for small fish research.

■ Bio Resources

The National BioResource Project (NBRP) is a national project for the systematic accumulation, storage, and supply system of nationally recognized bio resources (experimental animals and plants, cells, DNA, and other genetic resources), which are widely used as materials in life science research. To promote this national project, the NIBB has been appointed as a research center for research on "Medaka (*Oryzia latipes*)" whose usefulness as a vertebrate model first developed in Japan. The usability of Medaka as a research material in biology has drawn increasing attention since its full genome sequence recently became available. The NIBB also works as a sub-center for this national project for research on Japanese morning glory and Zebrafish. In addition, the NIBB provides databases containing research data on the moss *Physcomitrella patens, Daphnia, Xenopus laevis,* plant cell organelles, and bacterial genomes.

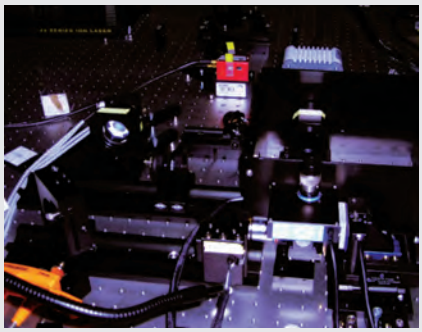
Top: Medaka strain for which the full genome sequence was determined.
Middle: Transgenic Medaka with red fluorescence
Bottom: A strain with transparent body

Development of New Fields of Biology

■ Bioimaging

Recently, the capability of optical microscopes has greatly improved, and biophotonics probes have also been developed. The combination of these technologies allows us to use living samples and observe biological phenomena in real time, which, in the past, could only be estimated based on fragmentary information from fixed samples. The NIBB aims to maximize the application of these techniques for visualizing biological phenomena (bioimaging) in biological research and to develop new imaging techniques.

1) *Imaging Science Laboratories* The NIBB aims to be a center for developing microscopes and biophotonics probes.
2) *Advisory Committee on Bioimaging* Regular meetings are held with several leading researchers in the bioimaging field in Japan to formulate advice on imaging research.
3) *Bioimaging Forum* This provides an opportunity for researchers in the NIBB, members of the Advisory Committee, and company engineers to frankly discuss practical difficulties and needs regarding imaging.
4) *Introduction of DSLM (Digital Scanned laser Light sheet Microscopy)* As part of collaborative work with the EMBL, the NIBB has introduced DSLM, which is effective for the three-dimensional observation of living samples, and is the first of its kind in Japan.
5) *Bioimaging Symposium* This provides an opportunity for academic exchanges with overseas cutting-edge researchers in the imaging field, mainly from the EMBL.



Optical path of the DSLM

■ Okazaki Biology Conferences

The NIBB holds Okazaki Biology Conferences (OBC) that, with the endorsement of the Union of Japanese Societies for Biological Science, support the formation of international communities in future biological research fields with the goal of identifying new research issues in biology. Dozens of top-level researchers from Japan and abroad spend about one week together in exhaustive discussions seeking strategies for addressing future critical issues in biology. Past Conferences have promoted the formation of international researcher communities. (see page 81 for OBCs held in 2007)

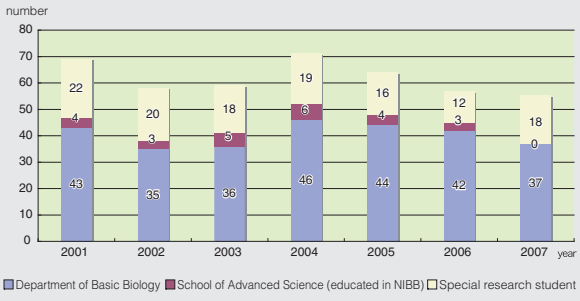
Cultivation of Future Researchers

The NIBB sponsors two education programs for graduate students.

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 (SOKENDAI). The department provides a five-year course for university graduates and a three-year doctoral course for graduate students with a master's degrees.
2. Graduate Student Training Program
   Graduate students enrolled in other universities and institutions can apply to be special research students eligible to conduct research for fixed periods of time under the supervision of NIBB professors.

   In both cases above, graduate students can live an academic life and receive financial support from the NIBB based on the research assistant (RA) system.

Graduate students educated by NIBB



Personnel changes in 2007*

Newly assigned

Name	Position	Research Unit	Date
OKADA, Kiyotaka	Director-General		April 1
NARUSE, Kiyoshi	Associate Professor	Laboratory of Bioresource	April 1
WATANABE, Taka-aki	Assistant Professor	Division of Genome Dynamics	April 1
OKUBO, Tadashi	Assistant Professor	Division of Molecular and Developmental Biology	August 1

Newly affiliated

Name	New Affiliation	Position	Date
KATSUKI, Motoya	National Institutes of Natural Sciences	Executive Director (Part-time)	March 31
SHIGENOBU, Shuji	Japan Science and Technology Agency	PRESTO Researcher	March 31
MUKAI, Masanori	Konan University	Lecturer	March 31
MOROHASHI, Ken-ichirou	Kyushu University	Professor	April 1
YOSHIKUNI, Michiyasu	Kyushu University	Professor	April 1
KOSHIDA, Sumito	The University of Tokyo	Associate Professor	August 1
FUKUI, Yuko	National Institute for Longevity Sciences	Laboratory Head	August 1

* Changes in Director-General, professors, ascociate and assistant professors are shown. All the research associates were reaffiliated as assistant professors on April 1.

Awardees in 2007

Name	Position	Award
SHINTANI, Takafumi	Assistant Professor	Japanese Association for Protein Phosphatase Research Award
WADA, Masamitsu	Professor	The Fellow of American Society of Plant Biologists Award
OHSUMI, Yoshinori	Professor	The Academic Award of the Botanical Society of Japan
HORIUCHI, Takashi	Professor	The Kihara Award of the Genetics Society of Japan
YAMAGUCHI, Takahiro	Assistant Professor	The Best Poster Award of the Botanical Society of Japan
OKADA, Tokindo S.	Professor Emeritus	Order of Culture
TSUKAYA, Hirokazu	Professor (adjunct)	Japan Society for the Promotion of Science Prize

Note on the member list from P. 8 to P. 80: All the members who belonged to the unit during 2007 are listed irrespective of the length of the period. Those appearing twice in the same list under different titles are those whose title was changed during 2007.

DIVISION OF CELL MECHANISM

Professor
NISHIMURA, Mikio

Associate Professor
HAYASHI, Makoto

Assistant Professors	MANO, Shoji
	YAMADA, Kenji
Technical Staff	KONDO, Maki
Postdoctoral Fellows	ARAI, Yuko
	KAMIGAKI, Akane
	OIKAWA, Kazusato
	SINGH, Tanuja
Graduate Students	OGASAWARA, Kimi
	GOTO, Shino
	KANAI, Masatake
	CUI, Songkui
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 "Organelle Differentiation as the Strategy for Environmental Adaptation in Plants" was started to clarify the molecular mechanisms underlying organelle differentiation.

Ⅰ. Reversible transformation of plant peroxisomes

Dramatic metabolic changes that underlie the shift from heterotrophic to autotrophic growth occur in the greening of seed germination. Accompanying these metabolic changes are the functional transformations of many constitutive organelles. 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. We also 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, called *PEX* genes, for peroxisomal biogenesis factors. 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 (Figure 1). 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 *PEX* genes regulating for peroxisomal protein import. These analyses revealed that PEX5, a receptor for PTS1, is involved in both lipid metabolism and photorespiration by regulating the import of

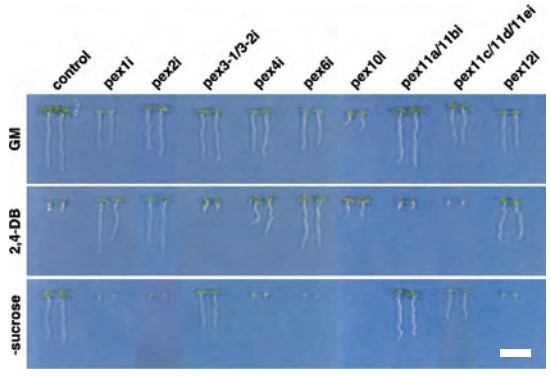


Figure 1. Effects of 2,4-DB and sucrose on growth of knockdown mutants. The name of each transgenic plant at the top of the panels indicates a silenced *PEX* gene or a combination of silenced *PEX* genes. Homozygous T3 progenies of these transgenic plants and parental plants (control) were grown for 7 days on growth medium (GM) containing 0.25 μg/mℓ of 2,4-DB (2,4-DB) or growth medium without sucrose (-sucrose) under constant illumination. Bar = 10 mm.

both PTS1- and PTS2-containing proteins. In contrast, PEX7, a receptor for PTS2, is involved only in lipid metabolism by regulating the 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 sizes and numbers can be visualized with GFP.

Of these *apm* mutants, *apm1* mutants contain slightly larger peroxisomes with long string-like tails. Interestingly, the division of mitochondria is also disturbed in *apm1* mutants. We revealed that *APM1* encodes dynamin-related protein 3A (DRP3A), one of a member of the dynamin family, and that DRP3A has a role in the division of both peroxisome and mitochondria on each membrane. Two other *apm* mutants, *apm2* and *apm4*, showed GFP fluorescence in the cytosol as well as in peroxisomes, indicating the decrease of efficiency of PTS1-dependent protein transport to peroxisomes. Interestingly, both mutants are defective in PTS2-dependent protein transport as well. *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, 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. Other *apm* mutants are under investigation so that we can identify the 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. *NAI1* encodes a transcription factor that has a basic-helix-loop-helix (bHLH) domain. Transient expression of *NAI1* induced ER bodies in the *nai1* mutant. These results provide direct evidence that NAI1 plays a role in the formation of ER bodies. We are trying to isolate additional components that are involved in ER body formation.

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

The vacuolar processing enzyme (VPE) belongs to the cysteine protease family found in higher plants and animals. VPE exhibits substrate specificity toward asparagine and aspartic acid residues, and is responsible for the maturation of various type of vacuolar proteins. Plant VPE homologues are separated into three subfamilies: seed type, vegetative type, and seed-coat type. We revealed a novel function of VPE in various programmed cell death (PCD) in plants. 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* δ*VPE* expresses specifically and transiently in two cell layers of the seed coat that causes PCD accompanying cell shrinkage. In a δ*vpe* mutant, shrinkage of these cell layers was delayed. 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 began to characterize HSP90s, using a specific inhibitor of HSP90 or transgenic plants expressing mutated *Arabidopsis* HSP90. HSP90 inhibitor induced heat-inducible genes and heat acclimation in *Arabidopsis* seedlings (Figure 2). HSP90 inhibitor induced the genes with heat shock response element (HSE) motifs in their promoters, suggesting that heat shock transcription factor (HSF) is involved in the response. *Arabidopsis* HSFs (AtHsfA1d, AtHsfA7a and AtHsfB1) interacted with *Arabidopsis* HSP90.2. Heat shock reduced cytosolic HSP90 activity in vivo. Thus, it appears that in the absence of heat shock, HSP90 actively suppresses HSF function. Upon heat shock, HSP90 is transiently inactivated, which leads to HSF activation. Plant survival requires the ability to acclimate to heat. This data indicates that HSP90 regulates correct gene expression for heat acclimatization in plants. We also observed that HSP90 is involved in hormone responses in *Arabidopsis*. The evolutional and functional characterization is now under experiment.

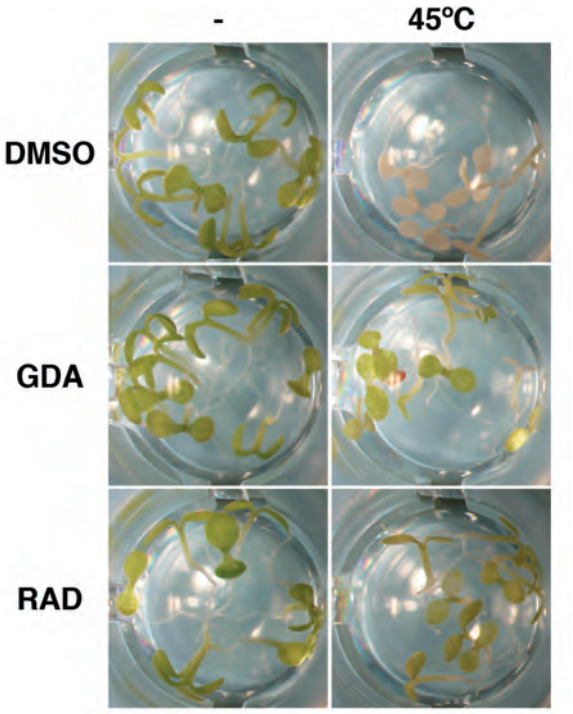


Figure 2. Pretreatment with HSP90 inhibitors induces the heat shock response and high temperature tolerance. Seedlings of *Arabidopsis* were treated with 50 μM of geldanamycin (GDA) or radicicol (RAD), HSP90 inhibitors, for 6 h, and they were washed three times with fresh medium. After plants were allowed to recover at 22 °C for 2 h, they were incubated at 22 °C (-; left) or 45 °C (right) for 1 h. As a negative control, plants were treated with dimethyl sulfoxide (DMSO), chemical solvent. The panels show the 2 days after the treatment.

Ⅶ. The Plant Organelles Database (PODB) – Databases of plant organelles visualized with fluorescent and nonfluorescent probes, and protocols for functional analysis

The Plant Organelles Database (PODB) was built to promote a comprehensive understanding of organelle dynamics and is maintained by the Scientific Research of Priority Areas on "Organelle Differentiation as the Strategy for Environmental Adaptation in Plants." This database consists of 3 individual parts: the organellome database, the functional analysis database, and external links to other databases and Web pages (Figure 3). The organellome database provides images of various plant organelles that were visualized with fluorescent and nonfluorescent probes in various tissues of several plant species at different developmental stages. The functional analysis database is a collection of protocols for plant organelle research. This public database is open to all researchers. We expect that this database will be a useful tool to help researchers gain greater knowledge of plant organelles.

Figure 3. The graphical user interfaces of PODB (http://podb.nibb.ac.jp/Orgenellome).

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- Yamada, K., Fukazawa, M., Hayashi, M., Suzuki, I., and Nishimura, M. (2007). Cytosolic HSP90 regulates the heat shock response that is responsible for heat acclimation in Arabidopsis thaliana. J. Biol. Chem. *282*, 37794-37804.

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- Kuroyanagi, M., Hatsugai, N., Nishimura, M., and Hara-Nishimura, I. (2007). Vacuolar processing enzyme, a key molecule in both pathogen-induced and phytotoxin-induced cell death in higher plants. Mol. Plant Microbe Interact. 5, 208-214.

DIVISION OF MOLECULAR CELL BIOLOGY

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All cellular activity is maintained by the balance between the synthesis and degradation of related proteins. Degradation processes, therefore, play important roles in many physiological aspects as well as the regulation of gene expression. Autophagy is a bulk degradation system for cytosolic proteins and organelles in lysosomes/vacuoles that is highly conserved in eukaryotic cells. This division aims to understand the physiological roles and molecular mechanisms of autophagy in yeast and higher eukaryotes.

Ⅰ. Background

Upon nutrient starvation, the autophagic process starts as a building up of membrane structures called 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, the budding yeast *Saccharomyces cerevisiae*, and morphologically and genetically defined the whole process.

Ⅱ. Identification and characterization of autophagy-specific proteins, Atg17, Atg29, and Atg31

In S. *cerevisiae*, autophagy is induced under nutrient starvation. In growing conditions another autophagy-related transport, the Cvt pathway, is observed. While most of the Atg proteins are involved in both pathways, only a few Atg proteins, such as Atg17 and Atg29, are uniquely required for starvation-induced autophagy. Identification of novel autophagy-specific Atg protein(s) and their characterization are urgent issues for the elucidation of the induction mechanism of autophagy.

We identified Cis1/Atg31 as a third autophagy-unique protein. *ATG31* null mutant cells were defective in autophagy and lost viability under nitrogen-depleted conditions.

Atg29 and Atg31 localize to the pre-autophagosomal structure (PAS; a putative generation site of the autophagosome) in an *ATG17*-dependent manner (Figure 1), and Atg17-Atg29 and Atg17-Atg31 physical interactions are detected, suggesting that these three proteins make a ternary complex. We further observed that this autophagy-unique protein complex binds to Atg1, and this association is regulated by nutrient conditions. When the Cvt pathway is disrupted by deletion of *ATG11*, encoding a Cvt-specific protein, recruitment of the Atg17 complex to the PAS is stimulated by starvation. In addition, in *atg11Δ* cells, the trimeric complex is required for the PAS recruitment of other Atg proteins under starvation conditions. These findings indicate that the Atg17 complex plays an important role in the assembly of other Atg proteins to the autophagy-specific PAS to generate the autophagosome.

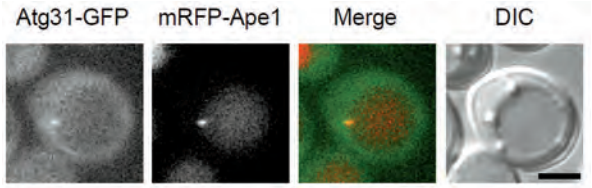

Figure 1. Atg31 localizes to the PAS. Yeast cells expressing Atg31-GFP and mRFP-Ape1 were incubated with rapamycin for 3 h. Fluorescence and DIC images are shown. Ape1 is used as a marker for the PAS. Bar: 2 μm.

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). We have reported on the functions of these conjugates using in vitro reconstitution systems consisting of purified protein components and PE-containing liposomes.

First, we showed that Atg8 mediates the tethering and hemifusion of membranes (liposomes), which are evoked by lipidation of the protein and reversibly modulated by Atg4 that catalyzes the deconjugation of Atg8-PE (Figure 2A). In addition, mutational analyses suggested that these functions of Atg8 observed *in vitro* are required for autophagosome formation *in vivo*; membrane tethering and hemifusion represent bona fide functions of Atg8. These results provide key insights into the unique membrane dynamics of autophagy, which cannot be explained by the mechanisms that have been elucidated in conventional vesicular trafficking systems.

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 purified the Atg12-Atg5 conjugate formed in *E. coli* and found that it dramatically accelerates the Atg8-PE conjugation reaction *in vitro*, in which transfer of Atg8 from the E2 enzyme Atg3 to PE is thought to be activated through the direct interaction between Atg12-Atg5 and Atg3 (Figure 2B). These results indicate that Atg12-Atg5 serves as a novel E3 enzyme in the ubiquitin-like conjugation reaction of Atg8. Further studies will shed light on understanding how these ubiquitin-like systems cooperate in autophagosome formation in the cell.

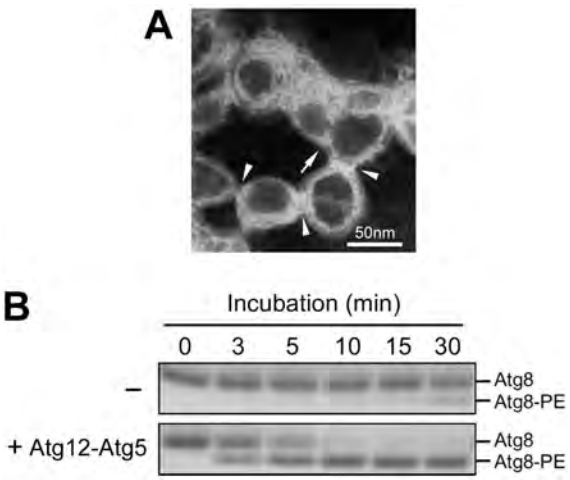

Figure 2. Functions of two ubiquitin-like protein conjugates. (A) Liposomes clustered upon in vitro production of Atg8-PE were stained with phosphotungsticacid and analyzed by electron microscopy. Arrowheads and an arrow, respectively, show junctions between liposomes and a structure suggested to represent hemifusion of liposomes. (B) In vitro conjugation reaction of Atg8-PE was performed in the presence or absence of the Atg12-Atg5 conjugate.

Ⅳ. Identification of another type of autophagy (microautophagy) under a stress condition

Our efforts to elucidate the molecular mechanism of autophagy have led to the accumulation of enormous amounts of knowledge regarding macroautophagy (macroautophagy is here simply described as autophagy). In contrast, little is known at present about the physiological functions and molecular machinery of another type of autophagic pathway, termed microautophagy (MIA). In MIA cargo components are sequestered directly by the vacuolar (lysosomal) membrane and transported into the vacuolar lumen after an inward pinch-off of the sequestering membrane (Figure 3A). In the yeast S. *cerevisiae*, MIA has been observed under some nutrient-starved conditions, but its biological significance remains unclear.

After phenotypic analyses of 18 *ATG*-gene disruptants, we found that disruptions of genes encoding a subset of phosphatidylinositol (PI) 3'-kinase complex led to severe viability defects when the cells were cultured on glycerol as a solo carbon source at 37ºC and eventually exposed to extensive oxidative stress. The frequency of the cells possessing vacuole invaginations increased under the culture condition, which was dependent on the PI 3'-kinase components necessary for the cell viability. Moreover, the product of the PI 3'-kinase, phosphatidylinositol 3'-monophosphate (PI3P), was found to be enriched at the site of vacuole invagination (Figure 3B). Subsequent morphological studies indicated that the vacuole invaginations are followed by a transport of lipid bodies into vacuole, thereby showing that MIA toward lipid bodies is induced under the stress condition.

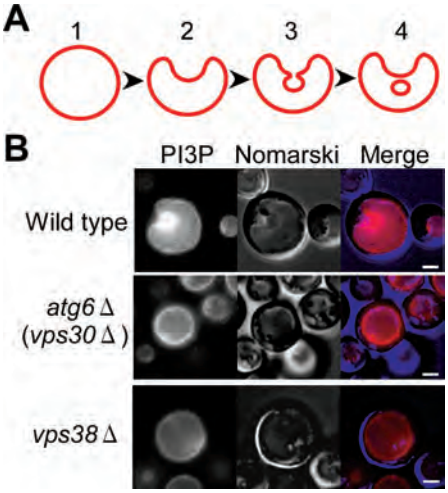

Figure 3. Membrane dynamics of MIA. (A) Schematic drawing of vacuolar membrane morphology undergoing MIA during glycerol culture at 37ºC. A part of a round vacuole (1) forms an invagination (2) dependent on some components of the PI 3'-kinase complex. Within the invagination a smaller vesicle structure emerges (3) and buds off into vacuolar lumen (4). (B) Localization of PI3P at the site of vacuole invagination. A tandem repeat of FYVE domain known to specifically bind to PI3P is expressed in fusion to monomeric RFP (mRFP). Fluorescence microscopy of the mRFP-(2x)FYVE was done for the denoted strains. Along with the mRFP signal (shown as PI3P), Nomarski images (Nomarski) and their superimposed images (Merge) are also shown. Bar, 2 μm.

Ⅴ. Lap3p is a novel cargo of selective autophagy during glycerol culture

Under growing conditions, a constitutive autophagy-like pathway, termed the Cvt (cytoplasm-to-vacuole-targeting) pathway, utilizes similar molecular machinery and vesicle structures to those of autophagy for a biosynthetic transport of aminopeptidase I (Ape1) into the vacuole. Recently, we discovered that in the cells grown on glycerol as a sole carbon source, the Cvt pathway was enhanced. Under this condition, we found that Leucyl aminopeptidase Ⅲ (Lap3), a neutral cysteine protease, was the selective cargo protein of the Cvt pathway (Figure 4A). Lap3 co-localized with Ape1 (Figure 4B), and Atg19 was involved in delivering Lap3 to the vacuole as a receptor protein. Further studies showed that a portion of Lap3 was degraded inside vacuoles, and thus suggested that the Cvt pathway might function as a degradative pathway and that Lap3 might be a cargo of the pathway.

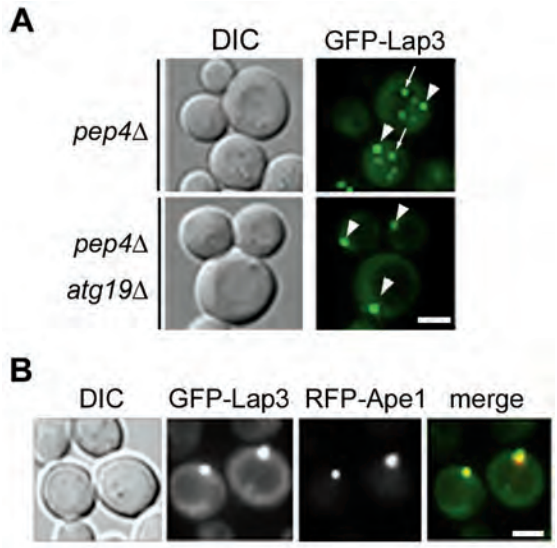

Figure 4. (A) A proteinase A deficient (*pep4*Δ) and *pep4*Δ *atg19*Δ cells expressing GFP-Lap3 were grown to logarithmic phase in the glycerol medium and examined by differential interference contrast (DIC) and fluorescence microscopy. Lap3 was selectively delivered to vacuole by the Cvt pathway. Arrows indicate the intravacuolar structures including GFP-Lap3 positive structures. Arrowheads indicate the punctate structures near vacuole. (B) Cells co-expressing GFP-Lap3 and RFP-Ape1 were examined by microscopy as described in (A). Lap3 co-localized with Ape1 near the vacuole. Bar, 4 μm.

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- Hanada, T., Noda, N.N., Satomi, Y., Ichimura, Y., Fujioka, Y., Takao, T., Inagaki, F., and Ohsumi, Y. (2007). The Atg12-Atg5 conjugate has a novel E3-like activity for protein lipidation in autophagy. J. Biol. Chem. *282*, 37298-37302.
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〔Review article〕

- Suzuki, K., and Ohsumi, Y. (2007). Molecular machinery of autophagosome formation in yeast, *Saccharomyces cerevisiae*. FEBS Lett. *581*, 2156-2161.

LABORATORY OF CELL STRUCTURE

Associate Professor
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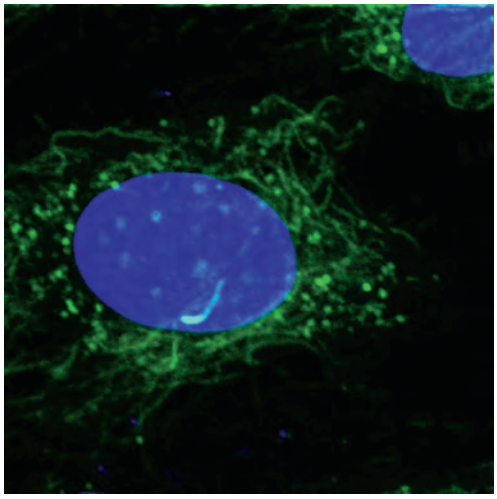
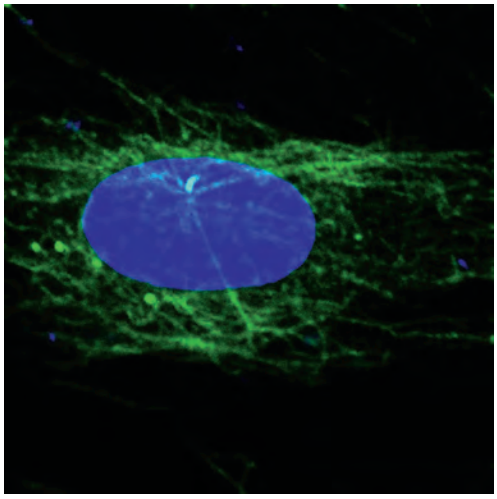
Numerous proteins localized in the cilia are linked to human diseases such as PCD (primary ciliary dyskinesia) and PCKD (polycystic kidney disease). Primary cilia are defined as single cilia that grow out of one of the centrioles during interphase in otherwise unciliated animal cells. They show a 9+0 pattern losing a central pair of microtubule, contrasting with motile cilia with a well-known 9+2 pattern.

Such cilia can been seen in cultured cells such as 3T3, 3T6, BHK21, NRK, MDCK. They are quite common centriolar specializations in vivo and in vitro. The incidence of primary cilia within a cell culture is related with the degree of confluency. Examination of confluent cell monolayers shows that the primary cilia within a single preparation of a given cell line varied considerably in length. In most cases, cell lines previously used were not cloned and the results remained obscure.

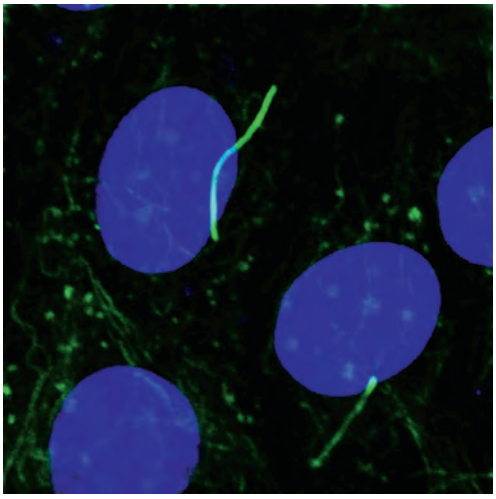
In this year, four cell lines originating from adult mouse kidney were established to study the proteomics of the primary cilia. They were named nibb-K1, K4, K5, and K8. The primary cilia of cells were observed by indirect immunofluorescence microscopy (Figure 1). In the cloned cell, each cell has a distinct length of cilium while K5 cell has the longest one among them (up to 10 μm).



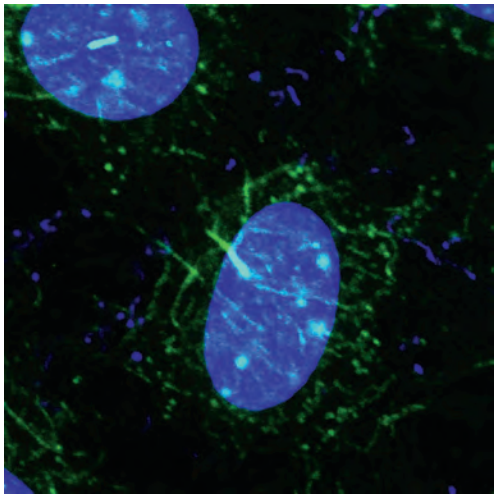
nibb-K1



nibb-K4



nibb-K5



nibb-K8

Figure 1. Typical primary cilia of four cell lines. Cells cultured on the cover slips were reacted with anti-acetylated tubulin antibody, followed by FITC-labeled secondary antibody. DNA was stained with DAPI.

LABORATORY OF CELL SOCIOLOGY

Assistant Professor
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 endeavouring to discover the cellular and molecular mechanisms at work during 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 the cells of the chorion as well as the ectoplacental cone; these 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 take 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 a normal invasion of angiogenic allantoic mesenchyme followed by premature formation of fetal blood vessels in the mutant placentas as early as E9.0. However, the specification of trophoblast subtypes appeared not to be drastically disturbed. Thus, in the developing mouse placenta, *Notch2* is likely not involved in cell fate decision, but rather participates in a formation of circulatory systems in the labyrinth layer where the expression of *Notch2* was detected (Figure 1). Although inadequate formation of maternal vascular beds was partially restored by aggregating mutant diploid embryos with wild type tetraploid embryos (Figure 2), networks of maternal vascular beds appeared still compromised in 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.

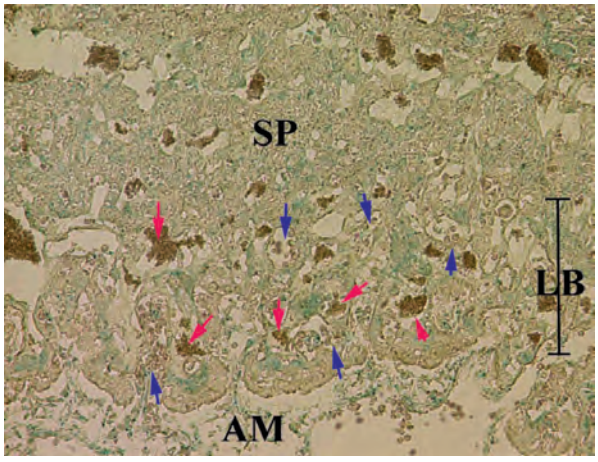


Figure 1. Expression of *Notch2* gene in developing mouse placenta. A cryo-section of *Notch2*$^{+/LacZ}$ placenta at E10.0 was incubated in X-gal staining solution. Trophoblast in maternal vascular bed side expresses the gene, but not in allantoic mesenchyme (AM) side in labyrinth layer (LB). The expression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

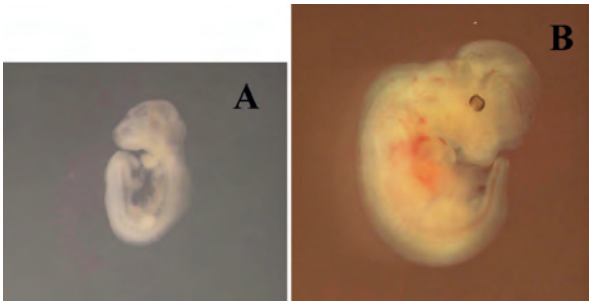


Figure 2. Survival of *Notch2* mutant embryo in a chimera with tetraploid wild type embryo. The mutant obtained in the intercrosses by the heterozygotic mice shows developmental retardation at E10.5 (A) and die before E11.5. The mutant is able to survive in a chimera with wild type tetraploid embryo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

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DIVISION OF REPRODUCTIVE BIOLOGY

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	SHIBATA, Emiko
	TAKAKI, Chikako
Secretary	SHIMADA, Yu

Fish exhibit a range of gonadal forms from gonochorism to several types of hermaphroditism, thus providing an excellent animal model to study the molecular mechanisms of sex determination/differentiation and gametogenesis in vertebrates. Our research using several teleost fishes 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

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. We identified *DMY* as the sex-determining gene of medaka. *DMY* encodes a protein of 267 amino acids including a DNA-binding motif, the DM domain, found in other genes involved in sexual development. A genomic DNA fragment carrying *DMY* was sufficient to induce testis differentiation and subsequent male development, producing fertile sperm (Figure 1). It is important to note that *DMY* transgenic XX medaka are fully functional and fertile males, whereas *Sry* transgenic mice are sterile (Koopman *et al*., 1991). Thus, medaka is the first

Figure 1. *DMY* transgenic adult medaka (XX) with white body color (A) having testis (B)

transgenic vertebrate shown to undergo complete sex reversal. Interestingly, *DMY* is a homolog of *DMRT1*, another DM domain gene that is involved in male development, and appears to be derived from a duplicated copy of autosomal *DMRT1*. *DMY* is also found in *O. curvinotus*, which is most closely related to medaka, but is not found in other *Oryzias* species or other fishes. These findings clearly illustrate the vast diversity of sex-determining genes in fish.

In tilapia, all genetic female (XX) or male (XY) broods are available. Through cDNA subtraction between XX and XY gonads during sex differentiation and microarray hybridization followed by gene expression analyses by RT-PCR and *in situ* hybridization, we have concluded that in tilapia, *Foxl2/Cyp19a1* plays a crucial role in ovarian differentiation and *DMRT1* in testicular differentiation. The transcripts of Foxl2 and aromatase (there are two forms of aromatase in teleost fishes: the ovarian form (Cyp19a1) and brain form (Cyp19a2)) were expressed only in XX gonads at 5 days after hatching (dah), with a marked elevation in expression during the next two days. The critical role of Foxl2 in ovarian differentiation was confirmed by male sex reversal of XX transgenic tilapia carrying a dominant-negative mutant of *Foxl2*. In XY 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. It is of great interest to note that some of the sex reversed XX tilapia produced sperm with extremely high motility (Wang *et al*., unpublished).

We also investigated the expression pattern of a novel type of P450c17 (P450c17-Ⅱ) lacking the lyase activity (see below) in gonads during sex differentiation. The results on tilapia and medaka (for example, its first appearance at 10-20 dah in XX tilapia and 70 dah in XY tilapia) suggest that P450c17-Ⅱ might be involved in the initiation of meiosis during early sex differentiation in these fishes. Further studies on these lines are expected to reveal whether 17α,20 β-DP or another C-21 steroid is involved in the initiation of meiosis and, consequently, sex differentiation.

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. We examined the involvement of gonadotropins in sex change 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. The expression of the *GtHRs* was found

to be confined to the active gonad of the corresponding sexual phase. When the sex change was occurring from female to male, the ovary initially had high levels of *FSHR* and *LHR*, which eventually went up in the testicular tissue once the fish had realized the fact that it was bigger than the other fish in the aquarium. The opposite of this scenario was observed if another fish bigger than the newly sex-changed male was introduced into the aquarium. Swapping of the gonads started with switching of the *GtHR* expression that was discernible within 8-24 hrs of the visual cue. Further *in vitro* culture of the transitional gonads with a supply of exogenous gonadotropin (hCG) revealed that the to-be-active gonad acquired the ability to produce the corresponding sex hormone within one day of the activation of GtHR. Conversely, the to-be-regressed gonad did not respond to the exogenous gonadotropin, demonstrating the absence of *GtHR* expression. A successive sex changing fish like *T. okinawae* is an excellent animal model to elucidate the mysterious role of the brain in bringing out the sexuality of an individual and also for the depiction of sexual plasticity at the organismal level (Figure 2).

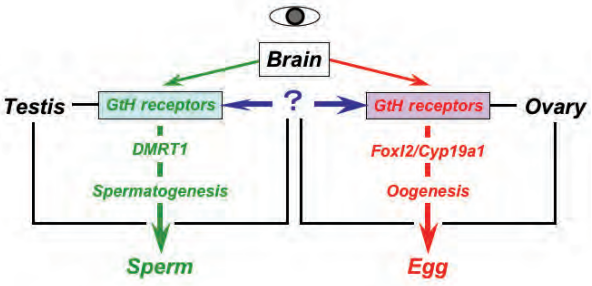

Figure 2. Gonadal sex change in *T. okinawae* may be triggered by visual/brain-stimulated switching of gonadotropin (GtH) receptor gene expression in gonads.

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

Appropriate development of GnRH neurons is essential for reproductive competence. The mechanisms underlying this process are, however, poorly defined because of the unavailability of an *in vivo* animal model. We have generated transgenic medaka that express GFP under the control of the GnRH gene promoters and shown that they could provide a useful model for the study of the GnRH neuronal development, including human disorders of GnRH deficiency. It has recently been shown that mice deficient for p73, a newly described homolog of the tumor suppressor gene p53, are not interested in mating, although its mechanism is unknown. Using the transgenic medaka, we here examined a possible role for p73 in the development of GnRH neurons. Antisense-mediated ablation of p73 led to the fusion of the bilateral GnRH neuronal clusters in the terminal nerve ganglion and an inappropriate increase in the number of these neurons. This result identified p73 as a novel factor involving the development of GnRH neurons and would account for the reproductive and behavioral defects in p73-deficient mice.

Ⅳ. Endocrine regulation of oocyte maturation and ovulation

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. Oocyte maturation has been studied in a variety of vertebrates and invertebrates including mammals, amphibians, fishes, and starfishes, but the endocrine regulation of oocyte maturation has been investigated most extensively in fishes. 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 (Figure 3). In both species, three major mediators have been shown to be involved (*Three step model*): a gonad-stimulating substance (GSS), 1-methyladenine (maturation-inducing hormone, MIH), and a 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 complete 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).

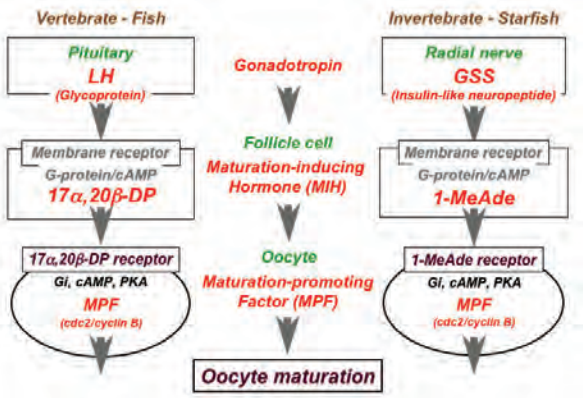

Figure 3. Hormonal control of oocyte maturation in fish and starfish – *Three step model*

In fish, LH acts on ovarian follicle cells to produce fish MIH (17α,20β-DP) immediately prior to oocyte maturation. There is a distinct shift in follicular steroidogenesis from estradiol-17β (E2) during oocyte growth (vitellogenesis) to 17α,20β-DP during oocyte maturation. This occurs in two stages, the first being the shift in the synthesis of precursor steroids in thecal cells, while the other is the shift in the final steroidal mediator production in granulosa cells. We have already shown that there is a distinct shift in the steroidogenic enzyme genes from ovarian aromatase (*Cyp19a1*) to 20β-hydroxysteroid dehydrogenase (*20β-HSD*), occurring in the granulosa cells of ovarian follicles prior to oocyte maturation. The triggering of the steroidogenic shift by GtHs in granulosa cells can be achieved by the differential actions of two transcription

factors, Ad4BP/SF-1 (aromatase) and CREB (20β-HSD).

The major remaining question was the differential availability of precursor steroid, 17α-hydroxyprogesterone. This was essentially because, until now, a single enzyme P450c17, possessing 17α-hydroxylase and 17, 20 lyase activities to mediate the production of estrogen and 17α,20β-DP, has been described among the vertebrates in general. Recently, we discovered a novel type of P450c17 (P450c17-Ⅱ) lacking the lyase activity in several teleost species, and showed that P450c17-II, but not P450c17-I, is responsible for the shift in precursor steroid from testosterone to 17α-hydroxyprogtesterone in both medaka and tilapia. Thus, our studies have resolved a long-standing question in the field of steroidogenesis with respect to oocyte maturation. As the novel type of P450c17 is found to have only the hydroxylase activity, we investigated whether this is the gene which is responsible for the cortisol production by analyzing its expression pattern in the head kidney of tilapia during different developmental stages. Interestingly, only P450c17-Ⅱ is found to be expressed in the interregnal cells of the head kidney from very early stages (5 dah) to adulthood (8 month old) (Figure 4). Since one of the most important physiological functions of the interrenal cell is to produce cortisol, our data suggests that only P450c17-Ⅱ is responsible for the cortisol production in the interrenal cells

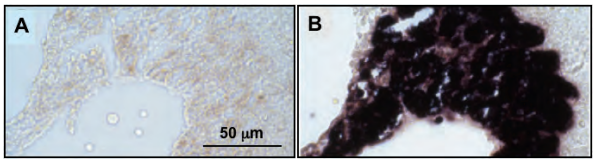

Figure 4. Expression of tilapia *P450c17-I* (A) and –Ⅱ (B) in the interrenal cells of the head kidney

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.

Ovulation is a precisely timed process by which a mature oocyte is released from an ovarian follicle. This process is initiated by the pituitary surge of LH and is temporally associated with the transcriptional regulation of several genes. The molecular mechanisms that control the complex process of ovulation are not well understood in vertebrates. Our recent studies (Shibata *et al*., unpublished) on medaka have demonstrated that 17α,20β-DP can induce ovulation (follicle rupture) in the mature follicles *in vitro*. It is particularly important to note that this action of 17α,20β-DP is mediated through its nuclear progestin receptors (nPRs) expressed in the granulosa cells. We were able to find that nPR mRNA expression is induced by gonadotropin prior to ovulation. Thus, 17α,20β-DP is the key hormone for the induction of not only maturation (through its membrane receptors), but also ovulation (through its nuclear receptors).

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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 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 division's research has focused primarily on the transcriptional control of the genes implicated in gonad sex differentiation.

Ⅰ. Function of Forkhead transcription factor Fkhl18 during testicular vasculature development

There is a general agreement that gonad sex determination in mammals is a process initiated by *Sry* gene. Downstream of *Sry*, *Sox9* specifies Sertoli cell lineages, organization of the testicular cord, and production of male hormones. In addition, the vasculature system develops differentially between the testis and ovary. Before the actions of *Sry* are evoked at around embryonic day 11.0 (E11.0), the structure of the primitive vasculature in the genital ridge is similar irrespective of sex. During the early phase of gonad sex differentiation, however, the mesonephric cells migrate vigorously into the developing testis to form a vasculature structure characteristic of the testis. In contrast, no such active cell migration is observed in the developing fetal ovary. This difference gives rise to sexually dimorphic vascular patterns in the gonads. Especially, in the testis, a large artery is formed at the coelomic surface at around E12.5. This male-specific vascular system that develops during fetal life is thought to be required for the export of testosterone from the testis to the rest of the fetus to ensure masculinization.

Vasculogenesis starts during fetal development. Precursor cells for blood vessel endothelia, which share their origins with hematopoietic progenitors, assemble into a primitive vascular network of small capillaries. Subsequently, the vascular plexus progressively expands by sprouting and matures into stable blood vessels. During this phase of angiogenesis and arteriogenesis, nascent endothelial cells become covered by periendothelial cells (pericytes and smooth muscle cells) and association with these cells is required to regulate proliferation, survival, migration, differentiation, vascular branching, blood flow and vascular permeability.

Forkhead transcription factors are characterized by a winged helix DNA binding domain, and the members of this family are classified into 20 subclasses by phylogenetic analyses. *Fkhl18* is structurally unique, and is classified in the FoxS subfamily. We found *Fkhl18* expression in periendothelial cells of the developing mouse fetal testis. In an attempt to clarify its function, we generated mice with *Fkhl18* gene disruption. Although KO mice developed normally and were fertile in both sexes, we frequently noticed unusual blood accumulation in the fetal testis. Electron microscopic analysis demonstrated frequent gaps, measuring 100-400 nm, between the endothelial cells of blood vessels. To visualize the entire structure of the testicular vasculature system, we injected carbon ink into the umbilical vein of E14.5 fetuses. Although the whole view of branches of the vasculature system was indistinguishable

between the wild-type and *Fkhl18* KO testes (Figure 1a, b), the area around the vasculature looked dark and blurred in the *Fkhl18* KO testes. The gonads were subsequently sectioned, and we found leaking of the injected carbon ink from the testicular vessels and coelomic vessel. The leakage of the carbon ink suggested a defect in the sealing structure of the vasculature in the *Fkhl18* KO mice.

These gaps probably represented ectopic apoptosis of testicular periendothelial cells, identified by caspase-3 expression, in KO fetuses. No apoptosis of endothelial cells was noted. *Fkhl18* suppressed the transcriptional activity of *FoxO3a* and *FoxO4*. Considering that *Fas ligand* gene expression is activated by Foxs, the elevated activity of *FoxOs* in the absence of Fkhl18 probably explains the marked apoptosis of periendothelial cells in *Fkhl18*-KO mice.

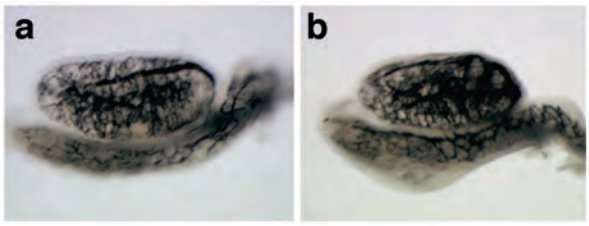

Figure 1. Visualization of the vasculature structure using carbon ink. Carbon ink was injected into the testes through the umbilical vein. The whole views of the wild-type (a) and *Fkhl18* KO testes (b) are shown.

Ⅱ. Function of Dioxin Receptor (AhR) in the Male Reproductive Tract

The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor belonging to the basic helix-loop-helix (bHLH)-PAS super-gene family. Since AhR can bind with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD, dioxin), the molecular properties of AhR as a transcription factor have been extensively studied. Recently, the intrinsic functions of AhR have been investigated with regards to animal reproduction. Indeed, our recent study in *AhR*(-/-) mice demonstrated that AhR is involved in female reproduction by regulating estradiol synthesizing *Cyp19* (*P450 aromatase*) gene expression. Based on the essential functions of estradiol in the female reproductive process such as folliculogenesis, ovulation, and implantation, it was concluded that AhR plays an indispensable function in female reproduction. Its function in male reproduction, however, remained unknown.

The accessory internal reproductive systems, derived from the Wolffian duct for males and from the Mullerian duct for females, are clearly different between the two sexes. The male internal reproductive system consists of multiple tissues such as the epididymis, the deferens duct, the seminal vesicle, the coagulating gland, and the ejaculatory duct. Developmentally, all these tissues are known to be regulated by androgen signaling. The mature seminal vesicle consists of numerous outpouchings of alveolar glands that empty into the ejaculatory duct. Although semen mostly contains materials secreted from the seminal vesicle, a definite functional relationship linking the seminal vesicle to male fertility has yet to be elucidated. The coagulating gland secretes a substance that, when mixed with the secretions from the seminal vesicle, forms a vaginal plug, and it has been thought that the vaginal plug is required for efficient pregnancy after insemination.

We showed age-dependent regression of the seminal vesicles, probably together with the coagulating gland, in *AhR*(-/-) male mice. The KO mice had abnormal vaginal plugs, low sperm counts in the epididymis, and low fertility. Moreover, serum testosterone concentrations and expression of steroidogenic 3ß hydroxysteroiddehydrogenase (3ßHsd) and steroidogenic acute regulatory protein (StAR) in testicular Leydig cells were decreased in *AhR*(-/-) males. Taken together, our results suggest that impaired testosterone synthesis in aged mice induces regression of seminal vesicles and the coagulating glands. Such tissue disappearance likely resulted in abnormal vaginal plug formation, and eventually in low fertility. Together with previous findings demonstrating AhR function in female reproduction, AhR has essential functions in animal reproduction in both sexes.

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The complex morphogenesis of organisms is achieved by consecutive cell-to-cell interactions during development. Recent studies suggest that growth factors play crucial roles in controlling such intercellular communications in a variety of organisms. In addition to secretory factors that trigger intracellular signaling, transcription factors that act in the nucleus to regulate gene expression are thought to be essential for the determination of cell fates. Our main interest is to know how pattern formation and morphogenesis during development are 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. Establishment of cell polarity during vertebrate embryogenesis

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 the molecular level. During *Xenopus* gastrulation, mesodermal cells migrate to the inside of the embryo and move on the blastocoel roof. One of the important mechanisms for this process is the cell movement called “convergent extension (CE)”. As convergent extension begins, cells are polarized and aligned mediolaterally, followed by the mutual intercalation of the cells that acquired planar cell polarity (PCP). In the regulation of convergent extension, several growth factor signaling pathways including Wnt/PCP pathway are implicated.

To understand how PCP is established within the cells, we have recently introduced a new method to visualize microtubule (MT) dynamics based on the assumption that cytoskeletal reorganization is one of the earliest events of the cell polarity formation. Using a GFP-fusion protein of EB3 (end-binding protein) which preferentially binds to the plus-end of growing MTs, we have been able to demonstrate that MT dynamics in non-polarized and polarized cells differ in that in polarized cells MT growth is more restricted towards the two ends of the cells even before the cells change their morphology to spindle shape. We also found that the cell polarity revealed by the MT dynamics is influenced by extracellular environments such as the notochord-somite tissue boundary and that physical contact of chordamesodermal explants with heterogenous tissues has significant impacts on the cell polarity, possibly by establishing a new tissue boundary between the two tissues. We are currently investigating what actually triggers the cell polarity formation during gastrulation.

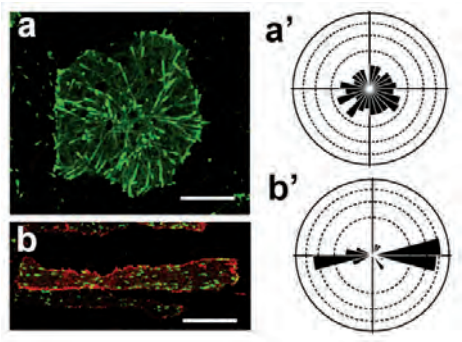

Figure 1. Tracking of EB3-GFP movements captures microtubule growth in *Xenopus* explants. In an animal cap cell (a), EB3-GFP shows radially symmetrical movements toward the rim of the cell in the rose diagram (a’). In chordamesoderm cell (b), the movements are mostly bidirectional toward both ends of the cell (b’).

Ⅱ. The involvement of protein ubiquitination systems in the noncanonical Wnt signaling pathway during gastrulation.

The noncanonical Wnt signaling pathway has been shown to play an essential role in the regulation of gastrulation movements. However, the molecular mechanisms of how Wnt signals intracellularly and how it regulates the tissue movements remain elusive. In order to clarify the Wnt signal transduction mechanism, we searched for the proteins essential for this signaling pathway, and identified a novel ubiquitin ligase complex, consisting of Rab40 GTPase and Cullin. This complex is localized in the Golgi apparatus and is essential for the regulation of the localization of Dishevelled, which plays the pivotal role in the Wnt signaling pathway. 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 another ubiquitination system essential for gastrulation, which ubiquitinates and destabilizes Paxillin, one of the focal adhesion components.

We found that the focal adhesion plays an important role in convergent extension. Paxillin stability is regulated at the focal adhesions, and the destabilization of Paxillin promotes the dynamics of focal adhesions and cell movements (Figure 2). 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 Paxillin. These findings implicated two previously-unidentified ubiquitin systems in the regulation of gastrulation movements in *Xenopus* embryos.

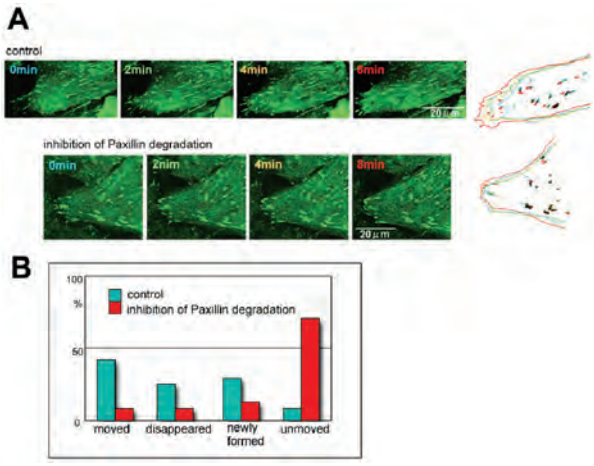

Figure 2. Paxillin degradation promotes focal adhesion dynamics and cell movement. (A) EGFP-Paxillin was expressed in the cells undergoing convergent extension. Inhibition of Paxillin degradation by antisense morpholino against XRNF185, which plays a crucial role in Paxillin degradation, decreased focal adhesion dynamics and cell movement. (B) Statistical data of focal adhesion dynamics.

Ⅲ. *Brachyury*-downstream notochord genes and convergent extension in *Ciona intestinalis* embryos

Formation of the chordate body is accomplished by a complex set of morphogenetic movements including the convergent extension of notochord cells. In the ascidian *Ciona intestinalis, Brachyury* plays a key role in the formation of the notochord, and more than thirty *Bra*-downstream notochord genes have been identified. In the present study, we examined the effects of functional suppression of nine *Bra*-downstream notochord genes, which include *Ci-PTP*, *Ci-ACL*, *Ci-prickle*, *Ci-netrin*, *Ci-trop*, *Ci-Noto3*, *Ci-ASAK*, *Ci-ERM* and *Ci-pellino*. When the function of the first two genes (*Ci-PTP* and *Ci-ACL*) was suppressed with specific morpholinos, the notochord cells failed to converge, while functional suppression of *Ci-prickle* resulted in a failure of intercalation, and therefore the cells in these three types of embryo remained in the mid-dorsal region of the embryo (Fig. 3). Functional suppression of the next four genes (*Ci-netrin*, *Ci-trop*, *Ci-Noto3* and *Ci-ASAK*) resulted in the partial defect of intercalation, and the notochord did not align in a single row. In addition, when the function of the last two genes (*Ci-ERM* and *Ci-pellino*) was suppressed, notochord cells failed to elongate in the embryo, even though convergence/extension took place normally. These results indicate that many *Bra*-downstream notochord genes are involved in convergence/extension of the embryo.

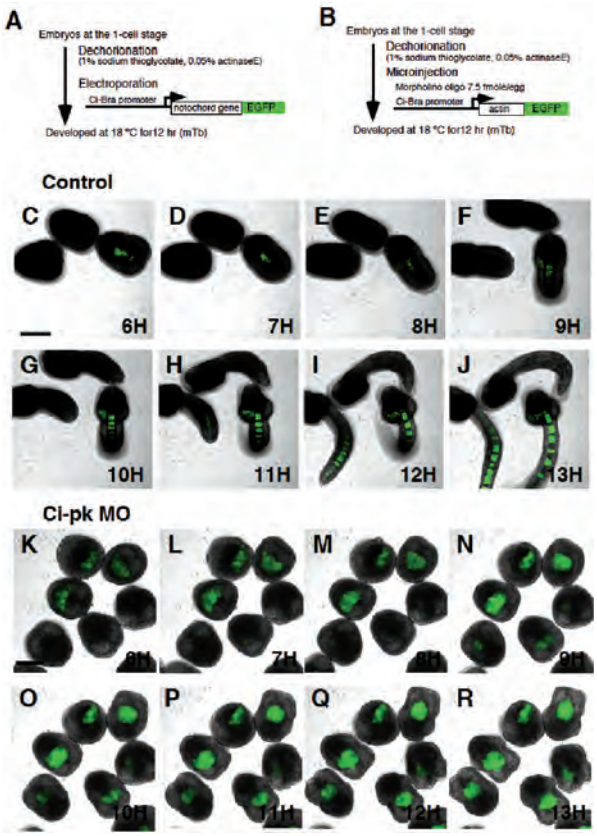

Figure 3. Examination of intracellular localization and function of proteins encoded by *Ci-Bra*-downstream notochord genes. (A) Experimental procedure. Fertilized eggs were dechorionated and electroporated with *Ci-Bra(promoter)/Bra*-downstream notochord gene:EGFP constructs. Manipulated embryos were allowed to develop at 18 ℃ for 12 hrs (to the mid tailbud stage). (B) Experimental procedure. Fertilized eggs were dechorionated and microinjected with specific morpholino oligos against *Ci-Bra* downstream genes together with the *Ci-Bra(promoter)*/actin:EGFP construct. Manipulated embryos were allowed to develop at 18 ℃ for 12 hrs (to the mid tailbud stage). (C-J) Control embryos at (C) 6, (D) 7, (E) 8, (F) 9, (G) 10, (H) 11, (I) 12, and (J) 13 hrs after fertilization. Notochord cells with actin:EGFP expression show convergence (C, D), intercalation (D-F), extension (F-H) and elongation (H-J). Scale bar, 100 μm. (K-R) Functional suppression of *Ci-pk* results in the failure of intercalation and extension of notochord cells, which are evident with actin:EGFP expression. Embryos at (K) 6, (L) 7, (M) 8, (N) 9, (O) 10, (P) 11, (Q) 12, and (R) 13 hrs after fertilization are shown to compare with normal embryos (C -J). Scale bar, 100 μm.

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

Translational regulation takes a major role in *Drosophila* body patterning. One of the eIF4G family proteins NAT1/p97/DAP5 has been identified and analyzed mainly in vertebrate culture cells. NAT1 has a structural similarity to 2/3 of eIF4G-C-terminal region. Since NAT1 deletes a binding motif to eIF4E, it has been hypothesized that this

protein participates in CAP-independent translation or plays an antagonistic role in translation. To elucidate *in vivo* function of *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 allele corresponding to the *dNAT1* locus. One of the P-element insertion lines $dNAT1^{GS1}$ shows severe embryonic lethality with abnormal germband extension defect. The lethality and morphological phenotype were completely rescued by introduction of the 12 kb *dNAT1* genomic DNA fragment by germ line transformation. We also found some similarity in the phenotype between *dNAT1* hypomorphic mutant and mutants in ecdysone signaling cascade. Both mutants show lethality at late 3rd instar larva to pupal stage with various metamorphosis defects. We found that one of the ecdysone target genes E74A is not properly translated in the *dNAT1* mutant animals. E74A is one of the rare examples of the genes whose translation is started from non-AUG start codon. E74A is translated from CUG start condon. Our analysis strongly suggested that dNAT1 participates in translation initiation from CUG start condon. We also speculate that dNAT1 also regulates other target(s) translation in early embryogenesis, since *dNAT1* null mutant shows early lethality. We would like to elucidate the general importance of NAT1 mediated non-AUG translation initiation in *Drosophila* development as well as vertebrate development.

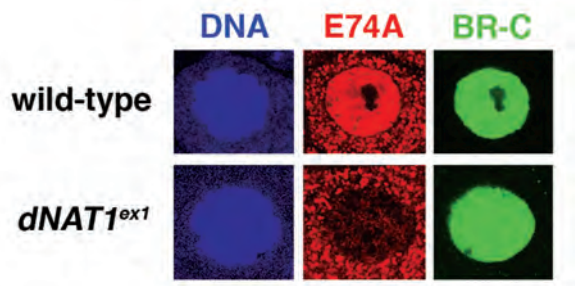

Figure 4. Salivary gland cells of wild-type and *dNAT1* mutant were stained with antibodies against E74A (red) BR-C (green) as well as DNA-staining dye (blue). In a *dNAT1* mutant cell, nuclear signal of E74A is completely missing while another nuclear marker BR-C is expressed normally.

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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. Maternal Nanos protein is required in pole cells to repress their apoptosis

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.

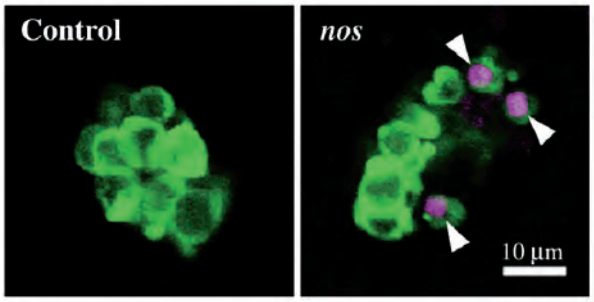

Figure 1. Nanos prevents apoptosis in pole cells. Confocal images of the pole cells in control (left) and *nos* (right) embryos at stage 13, stained with TUNEL labeling (magenta) and an antibody against Vas (green). Arrowheads show TUNEL-positive pole cells.

Several components of germ plasm have been identified. One of these components is maternal *nanos* (*nos)* RNA, which is enriched in germ plasm during oogenesis and translated *in situ* to produce Nos protein after fertilization. Nos 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.

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 the 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 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.

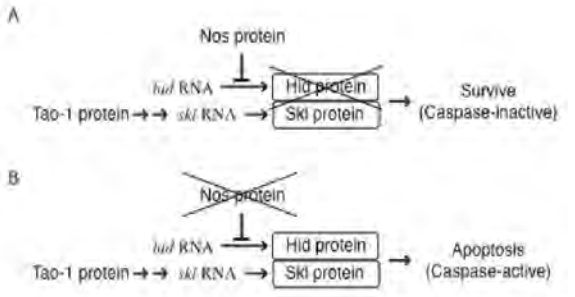

Figure 2. A model for the regulation of apoptosis in pole cells by the maternal factors, Nos and Tao-1. (A) In normal pole cells, maternal Tao-1 is inherited by pole cells and induces *skl* expression. Although *skl* alone does not induce apoptosis, it sensitizes pole cells to induction of apoptosis by *hid*. *Hid* mRNA is expressed in pole cells, but translation is repressed by maternal Nos. (B) Once pole cells lack Nos, hid mRNA is translated to produce its protein product that in turn acts together with *skl* to induce apoptosis.

We demonstrated 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 (Figure 2). These findings provide the first evidence that the germline is maintained through the regulated expression of RHG genes.

Ⅱ. Maternal Mamo protein is required in pole cells for their differentiation as the functional germ cells

Meiosis is a specialized cell division that produces haploid gametes from diploid progenitors. This is accomplished through two chromosomal segregation events without an intervening DNA replication. To accommodate the specialized processes, meiotic chromosomes undergo specific morphological changes (Figure 3). In *Drosophila* oocytes, upon entry into meiosis, a synaptonemal complex (SC) is formed between homologous chromosomes to stabilize their pairing, and the meiotic recombination occurs. Following disassembly of the SC, the meiotic chromosomes condense to form specialized prophase I chromosome structures, “karyosomes”, and these structures are maintained during later oogenesis. After ovulation, the oocyte initiates meiotic division and produces both a single female pronucleus and three polar-body nuclei which later fuse to form a rosette structure. These dynamic changes by the meiotic chromosomes are critical for meiosis. Genetic screens have identified many genes involved in the regulation of the meiotic chromosomes. The mechanism by which the germline acquires the potential to execute meiosis, however, remains elusive.

We demonstrated that a novel maternal factor, *mamo* (maternal gene required for meiosis), is autonomously required in pole cells to produce functional gametes. Mamo protein which contains both a BTB/POZ (Broad Complex, Tramtrack, Bric-a-brac/ Pox virus and Zinc finger) domain and C_2H_2 zinc finger motifs is enriched in PGCs during embryogenesis. The PGCs with reduced maternal Mamo activity are able to undergo oogenesis, but fail to execute meiosis properly (Figure 3). In the resulting oocytes, meiosis-specific chromosomal configurations are impaired (Figure 3). We additionally show that the decondensation of fertilized sperm nuclei is also affected in the eggs. We propose that maternal Mamo activates downstream genes to promote specialized morphological changes within both the female meiotic chromosomes and the sperm nucleus, which are critical in zygote formation.

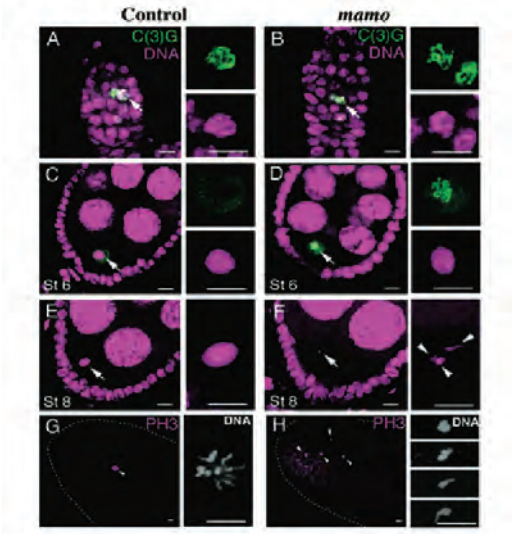

Figure 3. Maternal *mamo* mutation affects the structure of the SC, karyosome, and polar body. (A-F) Germarium regions (A and B), Stage-6 (C and D) and stage-8 (E and F) egg chambers were stained for C(3)G (green), a marker of the SC, and DNA (propidium iodide, magenta). (Insets) higher-magnification images of the oocyte nuclei are shown. (A and B) In the germarium region, C(3)G protein localized to the DNA in both the control and the *mamo* oocytes (arrows). (C and D) C(3)G was not associated with the DNA, and was diffusely distributed throughout the nuclear matrix (arrow) in a control oocyte at stage 6 (C). C(3)G failed to dissociate from the DNA (arrow) in a *mamo* oocyte (D). (E and F) The karyosome was compact and spherical in a control oocyte at stage 8 (arrows) (E), while the karyosome was fragmented (arrow) in a *mamo* oocyte at stage 8 (F). (G, H) Chromosomes were stained with an anti-PH3 antibody and TOTO-3 in control (G) and *mamo* eggs (H) at 0-1 hr AEL. (G) In a control egg, the rosette structure derived from polar bodies was observed (arrow). (H) In a *mamo* egg, rosette structure was indiscernible. The chromosomes were scattered, and their number was significantly reduced. (Insets) Higher-magnification images of the chromosomes shown in (G) and (H). Dotted lines outline the eggs. Scale bars: 10 μm.

Ⅲ. Signaling from pole cell to the gonadal soma is required for proper formation of the germline-stem-cell niche

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 collectively 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*). How the formation of hub progenitors is restricted in the anterior of embryonic gonads, however, remains elusive. We demonstrate that a receptor tyrosine kinase, Sevenless (Sev), provides a cue to ensure that the niche

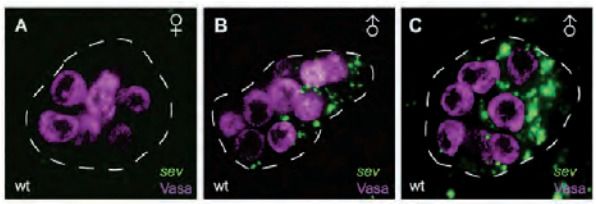

Figure 4. Expression of *sev* within the embryonic gonads
The gonads in a female embryo at stage 16 (A) and in male embryos at stage 13 (B) and 16 (C). Embryos were double-stained for *sev* mRNA (green) and a marker for the germline, Vasa (magenta). In all panels, anterior is to the left, and the embryonic gonads are outlined by white lines.

develops in the anterior region of the male embryonic gonads. Sev is expressed by somatic cells within the posterior region of the gonads (Figure 4), 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.

Ⅳ. Studies on short ORF-containing transcripts in *Drosophila* (Kageyama group)

Transcriptome analyses of eukaryotes, including mice and humans, have identified poly(A)-containing transcripts that only contains short ORFs (sORFs; less than 100 aa). These sORF transcripts are believed to most likely function as non-coding RNAs (ncRNAs), but their translational capacities and biological activities have not been characterized in detail.

To elucidate the biological roles of sORF genes, we use *Drosophila* as an excellent model system. By computational filtration and expression analysis, we have identified 33 putative poly(A)-containing ncRNAs, referred as to MREs (mRNA-like ncRNAs in embryos). In this year, we have been focusing on the biological roles of two MRE genes, MRE32 and MRE29. MRE32 is specifically expressed in the central nervous system and detailed transcript mapping strongly suggests that it really functions as ncRNA. Mutational analysis revealed that the *MRE32* gene is required for the eclosion behavior of the flies. Eclosion timing is sexually dimorphic in *Drosophila melanogaster*, in which females eclosed one day earlier than males. In *MRE32* mutant flies, female eclosion was specifically delayed, resulting in similar eclosion profiles in both sexes. In addition, microarray analysis of female and male adult heads demonstrated that a lack of *MRE32* severely altered expression of many genes in females. Thus, MRE32 is one of the crucial regulators of the Drosophila nervous system that acts on developmental timing. Another MRE, MRE29, is in fact transcribed into a polycistronic mRNA that contains evolutionarily conserved four ORFs that encode tiny peptides (11 and 32 aa). *MRE29* is expressed in epithelial tissues during embryogenesis and a lack of the *MRE29* gene eliminated apical cuticular structures, including the epidermal denticles and tracheal taenidia (Figure 5). Considering these phenotypes, we renamed the gene as *polished rice* (*pri*). Cytological analysis demonstrate that *pri* is essential for the formation of specific F-actin bundles that prefigure the formation of the denticles and taenidium (Figure 5), indicating that *pri* plays essential roles in epithelial morphogenesis by regulating F-actin organization.

These results demonstrate that sORF genes play important roles in *Drosophila* and further analysis on other MRE members should elucidate unexplored functions of the *Drosophila* genome.

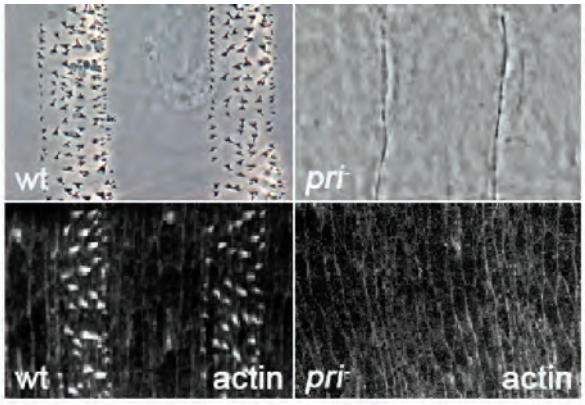

Figure 5. Epidermal phenotype of *polished rice*. In contrast to wild-type embryos (left column), pri mutant embryos exhibit complete loss of denticles (right, upper panel) and actin bundle formation beneath denticles (right, lower panel).

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- Kitadate, Y., Shigenobu, S., Arita, K., and Kobayashi, S. (2007). Boss/Sev signaling from germline to soma restricts germline-stem-cell-niche formation in the anterior region of *Drosophila* male gonad. Dev. Cell *13*, 151-159.
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- Mukai, M., Hayashi, Y., Kitadate, Y., Shigenobu, S., Arita, K., and Kobayashi, S. (2007). MAMO, a maternal BTB/POZ-Zn-finger protein enriched in germline progenitors is required for the production of functional eggs in *Drosophila*. Mech. Dev. *124*, 570-583.
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The body and tissues of the developing embryo are repeatedly divided into sub-regions specified by characteristic gene expression and morphology. The process that gives rise to these sub-regions along some defined pattern is called “pattern formation” or “patterning.” The most popular model to explain the patterning process is the “morphogen gradient and threshold” theory. Actually, many genetic results indicate that secreted signal proteins such as Wnt, BMP, and Hedgehog function as morphogens in many aspects of patterning processes. However, in spite of the accumulation of genetic evidence, the biochemical characteristics, including modification and higher order structure, of morphogens remain to be elucidated. Thus, one of our major goals is to reveal *the real image* of morphogens and the molecular mechanism underlying the formation of morphogen gradients, including the secretion and extracellular transport of these morphogens.

In contrast, the segmental sub-regions of the paraxial mesoderm, or somites, appear not to be simply directed by the morphogen gradient and threshold, but by a unique mechanism proceeding periodically. Somites are sequentially generated in an anterior-to-posterior order by converting oscillatory gene expression into periodical structures. The molecular mechanism underlying this conversion and morphological segmentation, however, has not yet been fully understood. Thus, another goal of our current studies is to reveal the molecular mechanism of *this other and unique modes of patterning* that underlies the periodical and sequential sub-division in somite formation.

Ⅰ. Molecular mechanism for secretion of Wnt proteins

The Wnt family of secreted signal proteins plays a key role in numerous aspects of embryogenesis. Most Wnt proteins transmit their signals locally, presumably since their secretion and transport are under tight control. One important step regulating the extracellular transport of various secreted signal proteins involves post-translational modification with lipid moieties. In the case of Wnt, Nusse and co-workers reported that murine Wnt-3a is S-palmitoylated at a conserved cysteine residue, and proposed that palmitoylation of this cysteine residue may be required to produce an increased local concentration of Wnt on the plasma membrane. In contrast, there is strong evidence to suggest that lipid modification is involved in the processing and intracellular trafficking of Wnt prior to secretion.

To resolve inconsistencies between the previous studies and to better understand the biological significance and molecular mechanism of lipid modification of Wnt, we carefully examined its modification. Unexpectedly, we found that murine Wnt-3a is modified with a mono-unsaturated fatty acid, palmitoleic acid, at a conserved Ser residue. Wnt-3a defective in this modification 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 this Ser-dependent modification, as well as for Wnt-3a transport from the ER for secretion. These results strongly suggest that Wnt protein requires a particular lipid modification for proper intracellular transport during the secretory process (Figure 1). We expect that the discovery of this unexpected lipid modification might provide a clue regarding the higher-order structure of secreted Wnt proteins and their gradient formation.

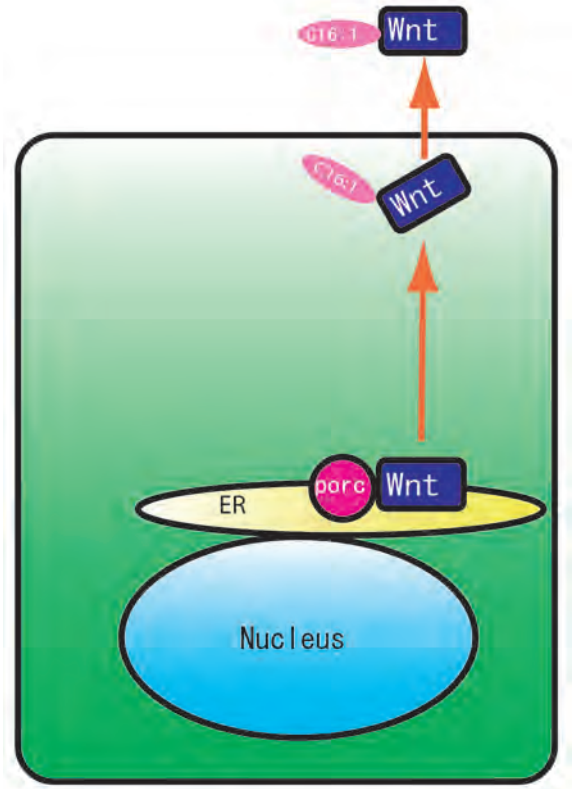

Figure 1. The function of palmitoleic lipid modification of Wnt protein. Wnt proteins are modified with palmitoleic acid (C16:1) by acytransferase, Porcupine (porc), in the ER. This modification is required for trafficking of Wnt proteins from the ER.

Ⅱ. Identification and characterization of genes required for somite development

2-1 Paf1 complex homologs are required for Notch-regulated transcription during somite segmentation

The molecular mechanism underlying the periodical formation of somites is coupled to an internal oscillator, referred to as the "segmentation clock," which has been evidenced by the cyclic expression of genes in the presomitic mesoderm (PSM). For instance, *hairy/Enhancer of split (Espl)*-related bHLH genes, including *her1* and *her7* in zebrafish, are expressed in a dynamic pattern of stripes across the PSM in a posterior to anterior direction. The oscillating and anteriorly propagating wave of gene expression, which is maintained in the posterior PSM, becomes fixed to cause segmentation in the anterior PSM. Interestingly, many genes that exhibit cyclic expression are regulated by Notch signaling in the PSM. Notch signaling is also involved in other aspects of segmentation, synchronization of the oscillating phase between cells in the PSM, and establishment of the rostral-caudal compartment within a somite.

In previous studies, we identified numerous ethylnitrosourea (ENU)-mutagenized zebrafish mutants with altered somite morphogenesis. For instance, we found that *integrinα5* and *fibronectin* were mutated in embryos showing defective boundary formation in their anterior somites. Detailed analysis of these mutantas indicated that Integrina5-directed assembly of Fibronectin appears critical for the epithelialization and boundary maintenance of somites. This result indicates that our strategies are effective for the identification of the genes involved in the somite segmentation process.

We are also searching for other genes involved in this process by both the expression screening and the mutagenesis screening methods. This year, we characterized one mutant obtained in our screens, *kt641*, which exhibited reduced but still striped expression of the Notch target genes in the PSM (Figure 2). In contrast to previously identified Notch signaling mutants, in which the striped expression of Notch target genes is perturbed to form salt-and-pepper patterns, *kt641* exhibited reduced but still striped expression of the Notch target genes in the PSM. We found that the gene responsible for this phenotype is a zebrafish homolog of yeast *rtf1*.

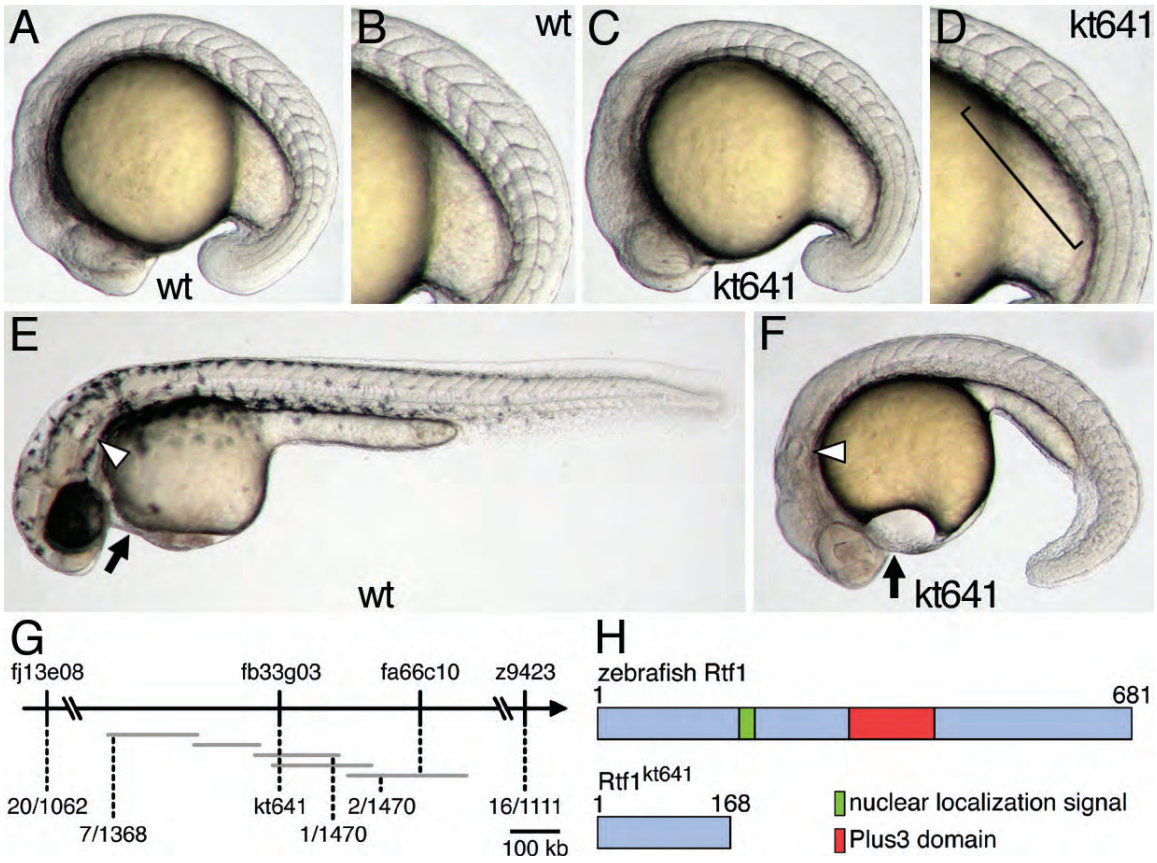

Figure 2. Phenotype of kt641 mutant and the structure of Rtf1. (A-F) Somite boundaries are disrupted in the posterior trunk of the *kt641* mutant (bracket in D). At later stages, the *kt641* mutation causes reduced pigmentation, limited tail growth, and abnormal heart (arrow) and ear (arrowhead) development (F). (G) Meiotic and physical mapping of *kt641* mutation. Horizontal gray bars represent contigs deposited in LG 13. (H) Schematic diagrams of zebrafish Rtf1 proteins encoded by wild-type and *kt641* alleles.

Rtf1 is a member of the Paf1 complex, which has been implicated in various processes, such as transcription initiation and elongation, histone modification, phosphorylation of pol II, and RNA processing and export. Members of Paf1 complex, which is composed of at least five components (Paf1, Rtf1, Cdc73, Leo1, and Ctr9), are conserved from yeast to human. Although these proteins have been implicated in RNA polymerase II-mediated transcription, their roles in vertebrate development have not been elucidated. We showed that a zebrafish mutant exhibiting a somite segmentation defect is deficient in *rtf1*. In addition, embryos deficient in *rtf1* or *ctr9* display abnormal development of the heart, ears, and neural crest cells. *rtf1* is required for proper RNA levels of the Notch-regulated genes *her1*, *her7*, and *deltaC* as well as for Notch-induced *her1* expression in the presomitic mesoderm. Furthermore, the phenotype observed in *rtf1*-deficient mutants is enhanced by an additional deficiency in mind bomb, which encodes an effector of Notch signaling. Thus, zebrafish homologs of the yeast Paf1 complex appear to preferentially affect a subset of genes, including Notch-regulated genes, during embryogenesis (Figure 3).

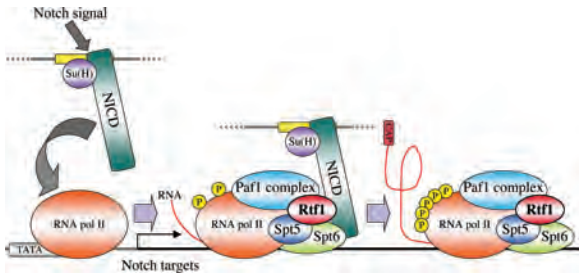

Figure 3. A model to explain Rtf1 function in the transcription of a Notch target gene. Our results suggest that the Paf1 complex may regulate the transcription of Notch targets in association with Spt5 and Spt6.

2-2 Transcriptional repression by Groucho-associated transcriptional mediator Ripply1 in somite segmentation

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 a 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.

In *ripply1*-deficient embryos, the expression of *mesp-b*, a key regulator in somite segmentation, is upregulated in a cell-autonomous manner, whereas, in embryos injected with *ripply*1mRNA, the expression of *mesp-b* is highly suppressed in the anterior PSM. These results suggest that Ripply1 regulates the proper expression of *mesp-b* in the anterior PSM. Taking into account that the expression of *mesp-b* could be induced by T-box transcription factor (Tbx), we can speculate that Ripply1 may antagonize the function of Tbx in the transcription of *mesp* genes. Therefore, we examined precisely the relationship between Tbx24 and Ripply1 in the transcription of *mesp-b*. We showed that Ripply1 is coprecipitated with Tbx24 and converts it from an activator to a repressor in culture cells. The other members of the Ripply family, Ripply2 and Ripply3, can also antagonize the transcriptional activation mediated by Tbx24. On the other hand, Ripply1 also antagonizes the transcriptional activation of another T-box protein, No tail (Ntl), both *in vitro* and *in vivo*. These results indicate that the intrinsic transcriptional property of T-box proteins is controlled by Ripply family proteins, which act as specific adaptors that recruit the global corepressor Groucho/TLE to T-box proteins.

Publication List

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- Takada, I., Mihara, M., Suzawa, M., Ohtake, F., Kobayashi, S., Igarashi, M., Youn, M.-Y., Takeyama, K., Nakamura, T., Mezaki, Y., Takezawa, S., Yogiashi, Y., Kitagawa, H., Yamada, G., Takada, S., Minami, Y., Shibuya, H., Matsumoto, K., and Kato, S. (2007). A histone lysine methyltransferase activated by non-canonical Wnt signalling suppresses PPAR-gamma transactivation. Nat. Cell Biol. *9*, 1273 - 1285.

LABORATORY OF MOLECULAR GENETICS FOR REPRODUCTION

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	YONEMITSU, Masako

Laboratory Scope

Our laboratory aims to reveal the molecular mechanisms of the 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 are available, which allows us to investigate biological phenomena by the means of molecular genetics. Furthermore, 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 differentiation common to many organisms.

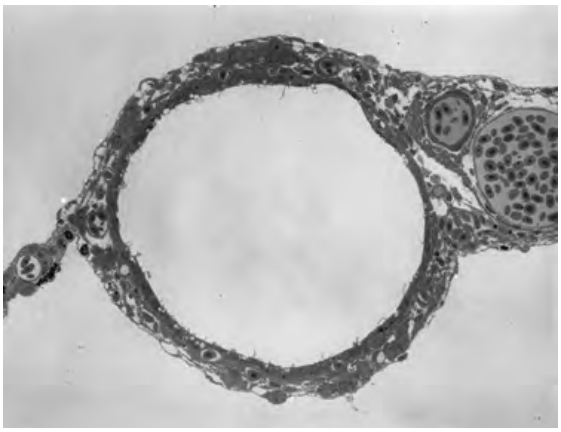

Figure 1. The germ cell-deficient gonad exhibits a single fundamental structure common to both ovary and testis. A large lumen is located in the middle and a single layer of supporting-like cells encloses the lumen, which is separated by a basement membrane from an outer stromal region.

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

We generated medaka that completely lack germ cells in the gonad by impairment of PGC migration. The morphology of the gonad in the germ cell-less medaka exhibits a tube-like structure which is composed of a lumen in the middle surrounded by a single layer of inner cells separated by a basement membrane from an outer stromal layer (Figure 1). This structure is common to the unit that constitutes ovarian follicles and testicular tubules, indicating that germ cells are essential for organizing sex-specific structures in the gonads.

This medaka also exhibits another interesting phenotype, male secondary sex characteristics irrespective of the genetic sex. Novel *aromatase*-expressing theca cells that we identified in ovary did not develop properly in the germ cell-deficient gonads and female supporting cells that control gametogenesis began to express male-specific genes as development of the gonad proceeded in the absence of germ cells, suggesting trandifferentiation from female to male supporting cells. The cells producing male sex steroid hormone persisted in the germ cell-deficient medaka. All lines of the data indicated that the gonadal somatic cells are predisposed to adopting male development and the production of male steroid hormone is responsible for male secondary sex characteristics. Thus, we demonstrated that, contrary to accepted thinking, germ cells are essential for sexually dimorphic gonads (Kurokawa *et al*., 2007, PNAS *104*, 16958-16963).

Ⅱ. Successful identification of a mutated gene that causes male to female sex reversal (*hotei* mutant)

In collaboration with the 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, *hotei*, is of particular interest because of the excessive number of germ cells that are arrested in the 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 successfully identified a candidate gene as the gene for a type Ⅱ receptor of anti-Müllerian hormone (*amhrⅡ*). This result reveals that amhr Ⅱ, together with its ligand, anti-Müllerian hormone (amh), regulates the proliferation of germ cells. Both *amh* and *amhrⅡ* are expressed in the gonadal somatic cells. It is interesting to note that *amhrⅡ* is not detected in the absence

of germ cells, suggesting that a reciprocal cross talk between germ cells and gonadal somatic cells is present during the term of sex differentiation. These results also support our contention that the proliferation of germ cells is closely related to sex differentiation of the gonads. (Morinaga *et al*., 2007, PNAS *104*, 9691-9696).

Another mutant, *zenzai*, is a good contrast with the *hotei* mutant in that germ cells are not maintained in the gonad (Figure 2). 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*.

Figure 2. Blue staining shows PGCs in the gonad. Left: *zenzai* mutant that cannot maintain gerrn cells. Middle: wild type. Right: *hotei* mutant that shows overproliferation of germ cells.

Ⅲ. Modes of Germ Cell Proliferation are important components of gonadal sex differentiation

In order to investigate the mode of germ cell proliferation, a small number of germ cells are labeled by fluorescence and monitored in the developing gonads of living medaka embryos. These observations reveal that two modes of proliferation are taking place, one type (type Ⅰ) with intermittent division found in both male and female developing gonads, and another type (type Ⅱ) with successive and synchronous division, leading to cystic germ cells and subsequent meiosis and oogenesis, which is only detected in the female (Figure 3).

Since type Ⅰ-proliferation is impaired in *zenzai* mutants, resulting in the depletion of germ cells, type Ⅰ is responsible for the maintenance of germ cells and type Ⅱ is the mode of germ cell proliferation which commits to gametogenesis. Our results also demonstrate that transition from type Ⅰ to type Ⅱ is regulated in a sex-dependent manner (Saito *et al*., 2007, Dev. Biol. *310*, 280-290).

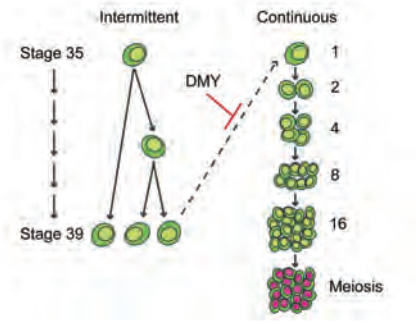

Figure 3. Two types of germ cell proliferation are important components of sexual dimorphism of medaka developing gonads.

Ⅳ. 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 with different colors 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 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 EMBL's Jochen Wittbrodt Lab.

Publication List

〔Original papers〕

- Hano, T., Oshima, Y., Kinoshita, M., Tanaka, M., Mishima, N., Ohyama, T., Yanagawa, T., Wakamatsu, Y., Ozato, K., and Honjo, T. (2007). Quantitative bioimaging analysis of gonads in olvas-GFP/ST-II YI medaka (transgenic *Oryzias latipes*) exposed to ethinylestradiol. Environ. Sci. Tech. *41*, 1473-1479.
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- Morinaga, C., Saito, D., Nakamura, S., Sasaki, T., Asakawa, S., Shimizu, N., Mitani, H., Furutani-Seiki, M., Tanaka, M (Corresponding Author), and Kondoh, H. (2007). The *hotei* mutation of medaka in the anti-Mullerian hormone receptor causes the dysregulation of germ cell and sexual development. Proc. Natl. Acad. Sci. USA *104*, 9691-9696.
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- Takamatsu, N., Kurosawa, G., Takahashi, M., Inokuma, R., Tanaka, M., Kanamori, A., and Hori, H. (2007). Duplicated Abd-B class genes in medaka hoxAa and hoxAb clusters exhibit different expression patterns in pectoral fin buds. Dev. Genes Evol. *217*, 263-273.

LABORATORY OF PLANT ORGAN DEVELOPMENT

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Plant organs - leaves, flowers, and roots - show impressive, symmetrical shapes, based on an ordered arrangement of differentiated cells. The organs are formed from a group of undifferentiated cells located at the tip of the stem or the root. In the case of leaves, the process of organogenesis starts with the formation of a leaf primordium in the peripheral zone of the shoot apical meristem at the fixed position, following an order called phyllotaxis. Cells in the primordium then proliferate and differentiate according to three spatially fixed axes: the apical-basal axis, the lateral axis (central-marginal axis), and the adaxial-abaxial (foreside-backside) axis. In the course of proliferation and differentiation, the plant cells are believed to exchange information with neighboring or separated cells in order to regulate the organ architecture. We are trying to understand the mechanism of the information exchange between plant cells during the development of lateral organs, such as leaves, sepals, petals, stamens and carpels.

Ⅰ. Genetic approach

Recent studies of *Arabidopsis* mutant show a couple of genes are involved in the axes-dependent control of lateral organ development. FILAMENTOUS FLOWER (FIL) and YABBY3, members of the YABBY/FIL gene family encoding a protein with a zinc finger and an HMG-related domains, are among them and control the specification of the abaxial side of lateral organs. FIL gene expression was restricted at the abaxial side of the lateral organ primordia (Figure 1). On the contrary, PHABULOSA (PHB) gene encoding a class III homeobox-ZIP protein, and its homologs, REVOLUTA (REV) and PHAVOLUTA (PHV) are responsible for formation of the adaxial-side tissue. We showed that PHB is expressed in cells of the adaxial side and separated clearly from the abaxial side-specific FIL gene expressing cells, by action of microRNA165/166 which targeted the PHB, REV, and PHV messenger RNAs. A series of section analyses showed that the separation of the adaxial side- and the abaxial side-specific genes was observed in the leaf primordium of stage 0, indicating that the microRNA action is working in the earliest stage of leaf development.

Several lines of evidence showed that the fixing of the adaxial-abaxial boundary in the leaf primordium is a key step of the subsequent expression of the region-specific genes and cell proliferation and differentiation. We then screened mutants with altered adaxial-abaxial boundary position by mutagenizing seeds of transgenic Arabidopsis carrying the abaxial-side specific FIL gene promoter::GFP. One of the mutants, enf1-1, showed a fluctuating boundary position, while another mutant, enf2-1, revealed a shift of the boundary to the adaxial side. Further analysis of the mutants will unveil the molecular mechanism for determining the position of the boundary, an important process of lateral organ development.

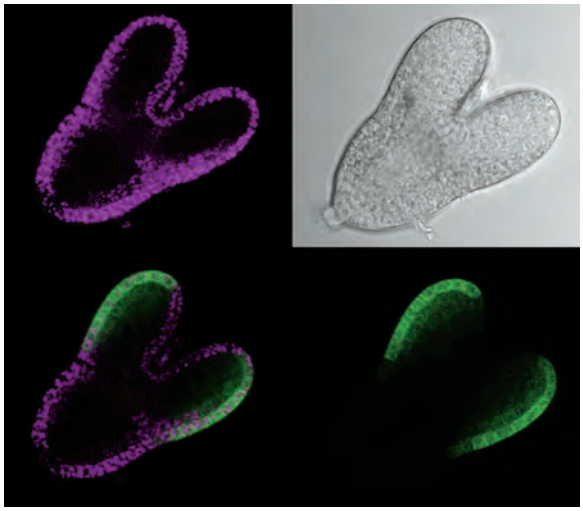

Figure 1. Region-specific expression of the FIL gene at the abaxial side-region of the developing cotyledons of Arabidopsis at the late heart stage embryo. Top right: light-field microscope picture. Top left: Pink signal show auto-fluorescence of chloroplasts. Bottom right: Green signals show the FIL promoter-drived green fluorescence protein (GFP). Bottom left: Merged picture of chloroplast auto-fluorescence and of GFP.

Ⅱ. Biochemcal approach

We are taking another approach to studying the intercellular signaling system by analyzing small peptides as candidates for intercellular signaling ligands, which are present in the apoplastic region of the shoot apical meristem. Small peptides were identified by LC/MS in the apoplastic liquid of edible cauliflower, which is a huge clump of inflorescence and premature flowers, or of *cauliflower* mutant of Arabidopsis, which makes clumped meristem identical to the vegetable. More than 400 peptide species were identified and their functions in lateral organ development are now being investigated.

Publication List

〔Original papers〕

- Ishida, T., Hattori, S., Sano, R., Inoue, K., Shirano, Y., Hayashi, H., Shibata, D., Sato, S., Kato, T., Tabata, S., Okada, K., and Wada, T. (2007). Arabidopsis TRANSPARENT TESTA GLABRA2 is directly regulated by R2R3 MYB transcription factors and is involved in regulation of GLABRA2 transcription in epidermal differentiation. The Plant Cell *19*, 2531-2543.
- Tominaga, R., Okada, K., and Wada, T. (2007). Functional analysis of epidermal-specific Myb genesgenes CAPRICE and WEREWOLF in Arabidopsis. The Plant Cell *19*, 2264-2277.

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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 the 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 developing retina as the basis of the topographic retinotectal projection.

In the past ten years, we have devoted our efforts to searching for molecules with asymmetrical distribution in the embryonic chick retina, and to the characterization of their roles in the topographic retinotectal projection. We performed a large-scale screening using differential hybridization and restriction landmark cDNA scanning (RLCS) on the embryonic day 8 (E8) chick retina, and we successfully identified 33 asymmetric molecules along the nasotemporal (N-T) axis and 20 along the dorsoventral (D-V) axis.

We subsequently conducted misexpression and knockdown experiments on the embryonic chick retina by *in ovo* electroporation using retroviral vectors to elucidate the molecular functions of these asymmetric molecules and the hierarchy among them. We have revealed gene cascades of topographic molecules for the retinal patterning and for the topographic retinotectal projection (see Annual Report 2006).

Although it is known that different neuronal subtypes are organized asymmetrically with respect to the two axes in the retina, the mechanisms of the final differentiation of neuronal subtypes still remain unclear. The region-specific distribution and development of these cells in the retina are also attributable to the retinal patterning which should be determined by morphogens and transcription factors expressed asymmetrically in the developing retina. Among the RLCS clones, we have already identified some molecules that are expressed in a specific subtype of the retinal ganglion cells (Figure 1). Studies to explore functional roles of these genes are currently underway.

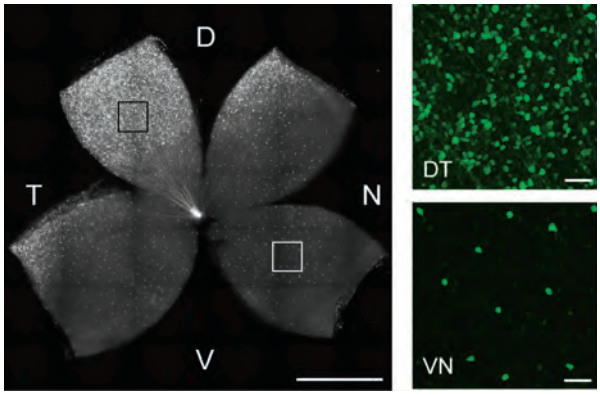

Figure 1. SPIG1 expression in the mouse retina at P5. SPIG1-positive retinal ganglion cells are densely distributed in the dorsotemporal retina. In the remaining region of the retina, only a subtype of the retinal ganglion cell appears to express SPIG1, showing a mosaic distribution. Enlargements of the boxed regions are shown on the right. N, T, D, and V indicate nasal, temporal, dorsal, and ventral, respectively. Scale bars: 1 mm (left panel), 50 μm (right panels).

Ⅱ. Mechanisms for the topographic retinotectal projection

At the onset of the retinotectal projection at E12, the axons of retinal ganglion cells exhibit a crude topographic order both anteroposteriorly and mediolaterally on the tectum/superior colliculus. Next, axonal sprouting begins predominantly in the vicinity and towards the site of the normal terminal zone (TZ). Further axon branching and arborization lead to the formation of mature TZs, while aberrant axon segments and branches are eliminated in an activity-dependent manner. These processes are completed at around E16 in chick (Figure 2) and P8 in mouse, respectively.

General attention is now devoted to the molecular mechanisms for the axon branching and arborization and their selective elimination. Among the region-specific

molecules in the developing retina, we have already identified several molecules which induce abnormal branching and arborization when their expression was experimentally altered *in vivo*. We expect that our research will shed light on these issues.

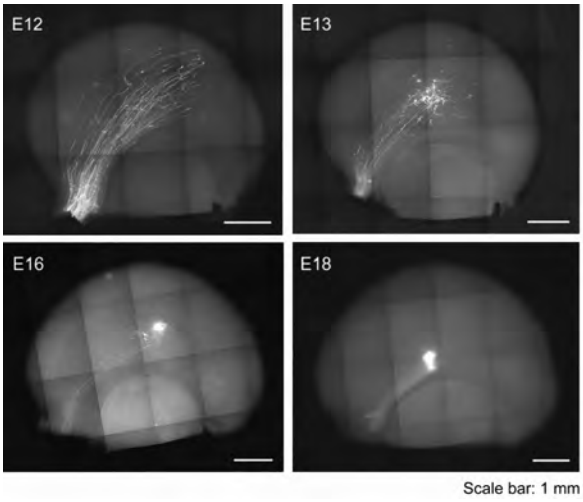

Figure 2. Development of retinotectal projection in chicks. Dorsal axons were labeled with DiI and the feature of axon terminals on the tectum was developmentally explored.

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. Among the twenty-one RPTPs expressed in mammals, only the R5 subfamily, Ptprz and Ptprg (PTPγ), have a canonical PDZ-binding motif (-S-L-V) at their carboxyl-termini. ErbB4 is a member of the ErbB-family tyrosine kinases known as a neuregulin (NRG) receptor. Ptprz and ErbB4 are reported to bind to postsynaptic density-95 (PSD95) on the second and the first/second PDZ (PSD95/Disc large/zona occludens1) domains, respectively, through the PDZ-binding motif of their carboxyl termini. We found a functional interaction between Ptprz and ErbB4.

An intracellular carboxyl-terminal region of Ptprz pulled-down PSD95 and ErbB4 from an adult rat synaptosomal preparation. ErbB4 and Ptprz showed co-localization in cell bodies and apical dendrites of neurons in the prefrontal cortex. *In vitro* experiments using the whole intracellular region (ICR) of ErbB4 also showed that PSD95 stimulates the autophosphorylation of ErbB4, and that the ICR of Ptprz dephosphorylates ErbB4 independent of the presence of PSD95 (Figure 3A, B). In HEK293T cells, phosphorylation of ErbB4 was raised by co-expression of PSD95, which was repressed by additional expression of Ptprz (Figure 3C). Taken together with the finding that the tyrosine phosphorylation level of ErbB4 was increased in *Ptprz*-knockout mice, these results suggest that Ptprz has a role in suppressing the autoactivation of ErbB4 by PSD95 at the postsynaptic density in the adult brain.

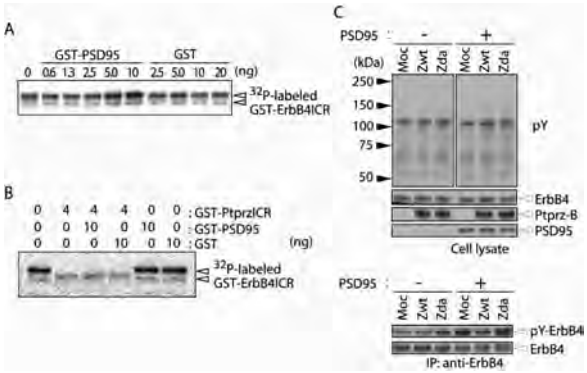

Figure 3. Tyrosine phosphorylation of ErbB4 is enhanced by PSD95 and repressed by Ptprz. (A) *In vitro* phosphorylation assay of ErbB4. GST-ErbB4ICR pre-incubated with indicated amounts of GST-PSD95 or GST was subjected to autophosphorylation with [γ-^{32}P]ATP, and then analyzed by SDS–PAGE followed by autoradiography. (B) *In vitro* dephosphorylation assay of ErbB4. After indicated amounts of GST-PtprzICR, GST-PSD95 and GST were mixed and preincubated, the ^{32}P-labelled GST-ErbB4ICR was added and incubated, and then analyzed as above. (C) Tyrosine phosphorylation of ErbB4 and its dephosphorylation by Ptprz in HEK293T cells. The tyrosine phosphorylation of cellular proteins and ErbB4 was analyzed by Western blotting, along with the protein expression.

Ⅳ. Mechanisms of Na-level sensing in the brain for the body-fluid homeostasis

Dehydration causes an increase in the sodium (Na) concentration and osmolarity of body fluids. For Na homeostasis of the body, controls of Na and water intake and excretion are of prime importance. Although it was suggested that the circumventricular organs (CVOs) are involved in body-fluid homeostasis, the system for sensing Na levels within the brain, which is responsible for the control of Na- and water-intake behavior, has long been an enigma. Na_x is an atypical sodium channel that is assumed to be a descendant of the voltage-gated sodium channel family. Our studies on the *Na$_x$*-knockout mice revealed that Na_x channels are localized to the CVOs and serve as a sodium-level sensor of body fluids. *Na$_x$*-knockout mice do not stop ingesting salt when dehydrated, while wild-type mice avoid salt.

As the first step toward understanding the cellular mechanism by which the information sensed by Na_x channels is reflected in the activity of the organs, we dissected the subcellular distribution of Na_x. Double-immunostaining and immuno-electron microscopic analyses revealed that Na_x is exclusively localized to perineuronal lamellate processes extending from ependymal cells and astrocytes in the organs. In addition, glial cells isolated from the subfornical organ (SFO), a member of the CVOs, were sensitive to an increase in the extracellular sodium level, as analyzed by an ion-imaging method. These results suggest that glial cells bearing Na_x channels are the first to sense a physiological increase in the level of sodium in body fluids, and regulate the neural activity of the CVOs by enveloping neurons. Thus, close communication between inexcitable glial cells and excitable neural cells is supposedly the basis

of the central control of salt homeostasis.

This year, we revealed direct interaction between Na_x channels and α subunits of Na^+/K^+-ATPase, which brings about Na-dependent activation of the metabolic state of the glial cells. The metabolic enhancement leading to extensive lactate production was observed in the SFO of wild-type mice, but not of the *Na$_x$*-knockout mice. Furthermore, lactate, as well as Na, stimulated the activity of GABAergic neurons in the SFO (Figure 4). These results suggest that the information on a physiological increase of the Na level in body fluids sensed by Na_x in glial cells is transmitted to neurons by lactate as a mediator to regulate neural activities of the SFO. It is likely that this leads to the control of salt-intake behavior (Figure 5).

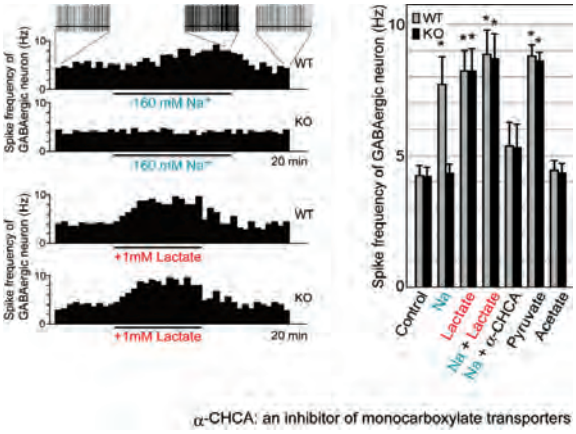

Figure 4. Properties of GABAergic neurons in the SFO of wild-type and *Na$_x$*-KO mice. There exist GABAergic neurons spontaneously firing in the SFO. Lactate, as well as Na, stimulated the activity of GABAergic neurons in the SFO. This activation by lactate is suppressed by α-CHCA, an inhibitor of lactate transporter.

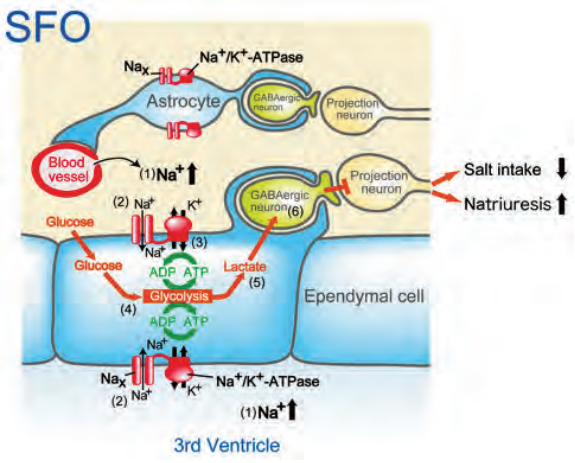

Figure 5. Schematic drawing of the Na-level sensing mechanism and Na-dependent regulation of neural activity in the SFO. When animals are dehydrated, Na concentration in plasma and cerebrospinal fluid increases above the usual level of ~145 mM (1). When the extracellular Na concentration exceeds ~150 mM, Na_x channels open, and the intracellular Na concentration in these glial cells is increased. This leads to activation of Na^+/K^+-ATPase in these cells (2). Activated Na^+/K^+-ATPase consumes ATP larger than the usual level to pump out Na ions (3). To fuel Na^+/K^+-ATPase with ATP, the glial cells enhance the glucose uptake to stimulate the anaerobic glycolysis (4). Lactate, the end product of the anaerobic glycolysis, is released from the glial cells and supplied to neurons, including GABAergic neurons, through the processes enveloping them (5). Lactate stimulates the activity of the GABAergic neurons through production of ATP, which presumably regulate hypothetic neurons involved in the control of salt-intake behavior (6).

Publication List

〔Original papers〕

- Fujikawa, A., Chow. J.P.H, Shimizu. H., Fukada. M., Suzuki. R., and Noda, M. (2007). Tyrosine phosphorylation of ErbB4 is enhanced by PSD95 and repressed by protein tyrosine phosphatase receptor type Z. J. Biochem. *142*, 343-350.
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- Yamamoto, H., Kamegaya, E., Hagino, Y., Imai, K., Fujikawa, A., Tamura, K., Enokita, T., Yamamoto, T., Takeshima, T., Koga, H., Uhl, G.R., Ikeda, K., and Sora, I. (2007). Genetic deletion of vesicular monoamine transpoter-2 (VMAT2) reduces dopamine transporter activity in mesencephalic neurons in primary culture. Neurochem. Int. *51*, 237-244.

〔Review article〕

- Noda, M. (2007). Hydromineral neuroendocrinology: Mechanism of sensing sodium levels in the brain. Exp. Physiol. *92*, 513-522.

DIVISION OF BRAIN BIOLOGY

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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 mammalian evolution, the neocortex was markedly expanded while the total number of genes in the mammal was little changed. In order to answer 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 Figure 1.

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. The question remained, however, whether or not there are any genes that show marked neocortical area difference.

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

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

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

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

iv) 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).

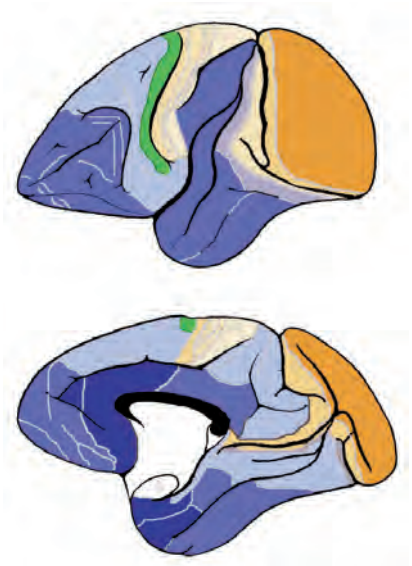

Figure 1. The expression of *occ1* (orange color) m *Rbp* (blue) and *gdf7* (green) are schematicaly illustrated in Brodmann's area figure in the guenon monkey. Top and bottom views are medial and lateral surfaces, respectively. (The figure is cited from Yamamori & Rockland, Neurosci. Res., *55*, 11-27, 2006).

v) *occ1* is strongly expressed in the mouse LGN. We then monocularly deprived activity 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 (Figure 2; Takahata *et al*., J. Chem Neuroanat., in press).

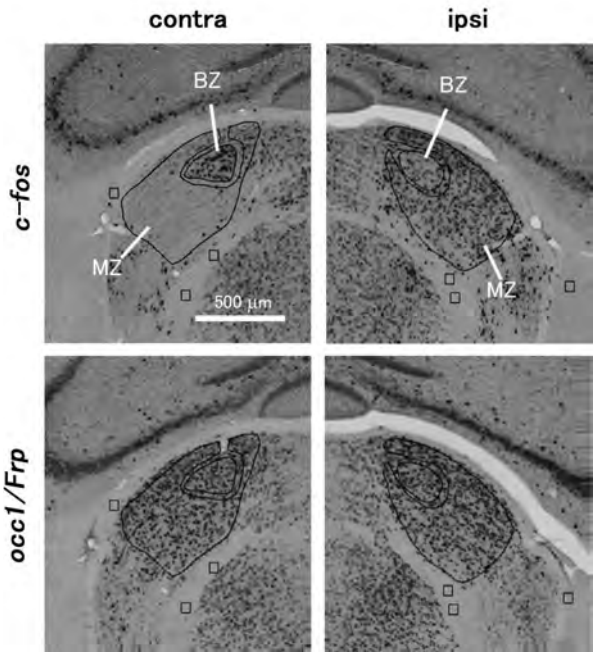

Figure 2. The expression of *c-fos* (top) and *occ1/Frp* (bottom) in mouse LGN are shown. In the monocular zone (MZ) of the contra LGN to the deprived eye, *c-fos* mRNA expression was dramatically decreased, whereas the *occ1/Frp* mRNA expression is not affected. (The original figure is shown in Takahata *et al*., J. Chem Neuroanat., 2007 Sep 16; [Epub ahead of print]).

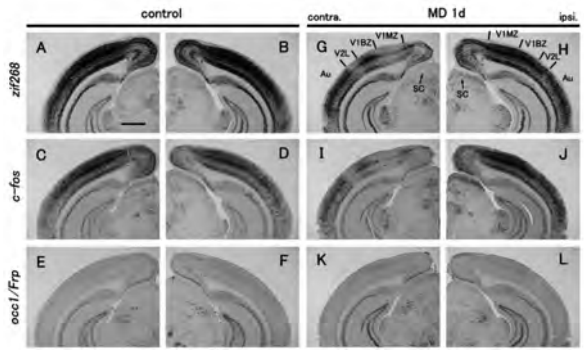

Figure 3. The expression of *zif268* (top), *c-fos* (middle) and *occ1/Frp* (bottom) in mouse visual cortex are shown. In the contra visual cortex (V1MZ: V1 monocular zone) and V2L (lateral secondary visual cortex), the expression of *zif268* and *c-fos* to the deprived eye (MD, 1 day after monocular deprivation), *c-fos* mRNA expression was dramatically decreased, whereas the *occ1/Frp* mRNA expression is not affected. (The original figure is shown in Takahata *et al*., J. Chem Neuroanat., 2007 Sep 16; [Epub ahead of print]).

vi) In the visual cortex of the mouse, it is known that monocular deprivation reduces the expression of immediate early genes such as *c-fos* and *zif268*. However, the expression of *occ1/Frp* in the LGN and visual cortex was not affected by monocular deprivation experiments (Figure 3; Takahata *et al*., J. Chem. Neuroanat., in press).

These results suggest that activity dependency of *occ1* has been acquired during evolution of the primate brain (Takahata *et al*., Society for Neuroscience in North America, 2006, J. Chem Neuroanat., in press).

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 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 standardize 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).

Traditionally, multisensory integration was thought to occurr in higher neocortical areas by merging different modalities of primary sensory information. Our results suggest that the multisensory integration may in fact occur at a relatively "early sensory" area such as V2. Previous elecrophysiological studies also show that there exist multisensory areas in the secondary visual area and the boundary areas between two modal areas (Toldi *et al*., 1986; Barth *et al*., 1995; Wallace *et al*., 2004). This observation is consistent with our findings through the experiments using the newly developed behavioral system and c-Fos analyzing system. Therefore, we have demonstrated evidence for a role for the lateral secondary visual area (V2L) in multisensory informational processing under behavior conditions.

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. Their roles in behavioral tasks, however, have remained obscure. We are currently examining the altered behavior that appears under the pharmacological treatments that affect the metabolism of the interneurons in the striatum.

Ⅲ. Topological relationships between brain and social networks

Network theory has recently revealed that many networks (gene transcription, protein-protein interaction, The Internet, and sociological networks) share global and local properties (Albert and Barabasi, 2002; Barabasi, 2002; Barabasi and Albert, 1999; Milo *et al*., 2002, 2004; Watts and Strogatz, 1998). To understand the global and local design principles of mammalian cerebral cortical networks, we applied network-theoretical approaches to connectivity data from macaque and cat cortical networks. Firstly, we confirmed the "small world" property of these cortical networks. Secondly, we then compared them, based on the significance profile (SP) of thirteen possible network motifs in the real network compared to randomized networks. SPs of different mammalian cortical networks are highly conserved and robust. Our results thus suggest that there are constraints of neocortical development and evolution (Sakata and Yamamori, Neurosci. Res., 51, 309-315, 2005).

This year, we reported on the topological similarities between brain and social networks. The statistical relevance of specific tied structures differs between social "friendship" and "disliking" networks. This suggests a relation-type-specific topology of social networks. Overrepresented connected structures in brain networks are more similar to those in the friendship networks than to those in other networks (Figure 4). We found that, unlike what could be predicted by simply counting mutual connections, balanced and imbalanced reciprocal connections between nodes are significantly abundant and rare, respectively. We interpret these results as evidence of positive selection of balanced mutuality between nodes. These results also imply that there exist common underlying principles between the organization of the brain and social networks.

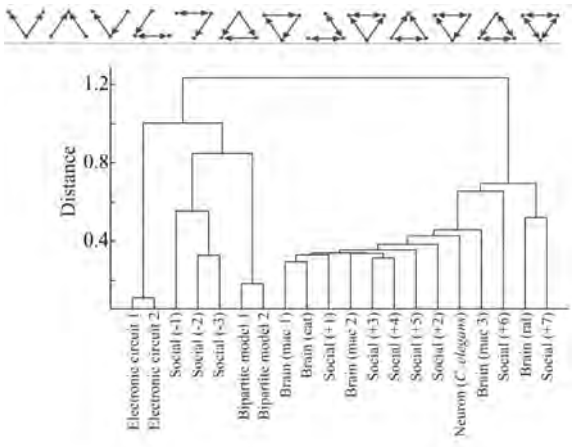

Figure 4. Hierarchical clustering of Triad Significance Profile (TSPs) (see thirteen possible motifs on the top panel) of complex networks. Note that brain and friendship networks fell into the same cluster. Abbreviations: mac. Macaque. The figures are cited from Sakata and Yamamori, Neural Networks., *20*, 12-21, 2007.

Publication List

〔Original papers〕

- Nakamura, K., Watakabe, A., Hioki, H., Fujiyama, F., Tanaka, Y., Yamamori, T., and Kaneko, T. (2007). Transiently increased colocalization of vesicular glutamate transporters 1 and 2 at single axon terminals during postnatal development of mouse neocortex: a quantitative analysis with correlation coefficient. Eur. J. Neurosci. *26*, 3054-3067.
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- Watakabe, A., Ichinohe, N., Ohsawa, S., Hashikawa, T., Komatsu, Y., Rockland, K.S., and Yamamori, T. (2007). Comparative analysis of layer-specific genes in mammalian neocortex. Cereb. Cortex *17*, 1918-1933.

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 structure and 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 mounting in females. These clearly indicate that sex-dependent patterns of behavior are not directly dependent on "sex-specific" 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*. Furthermore, we have generated *ERαas*- and *ARas*-transgenic (Tg) mice in which *ERαas* and *ARas* fragments are constitutively expressed, respectively. We report herein on the functional alteration of mouse brain by these noncoding RNAs.

1-1 Functional alteration of rodent brain by noncoding RNA for ER α promoter

ERα participates in the cellular, reproductive and behavioral mechanisms. We found novel endogenous antisense transcripts on *ERα* locus, named *ERαas*. In the brain, *ERαas* expression showed tissue-, development-, and sex- dependent pattern. *ERαas*-Tg mice gained weight faster than the wild type during prepubertal stages. *ERαas*-Tg females showed the significant reduction of lordorsis and the larger volume of ventromedial hypothalamus (VMH) which are rather reminiscent of the male-like patterns. The search for molecular targets of *ERαas* revealed that the *ERα* promoter was specifically demethylated, resulting in the *ERα* upregulation. It is to be noted that, of discrete brain areas, only the principal portion of the bed nucleus of the stria terminalis, but not the VMH, showed the sex-dependent DNA methylation pattern in mice and rats. Thus, *ERαas*, acting on the *ERα* promoter as a noncoding RNA, is a component that establishes its epigenetic status for gene expression. Spatiotemporal expression of *ERαas* may mediate the functional differentiation of the female and male brains with or without morphological alterations in rodents.

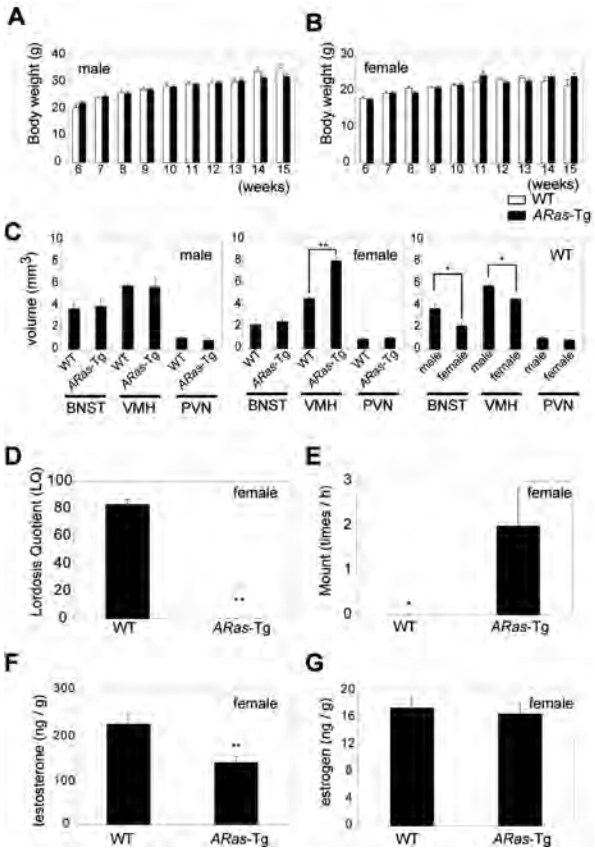

Figure 1. Physiological alterations in *ARas*-Tg mice. (A) Body weights of males. Growth graphs of the 15 weeks old *ARas*-Tg and the wild type. WT denotes wildtype. (B) Body weights of females. Growth graphs of the 15 weeks old *ARas*-Tg and the wild type. (C) Graphs showing the volume of each nucleus of the PVN (paraventriclar nucleus), BNST (principal portion of the bed nucleus of the stria terminalis), and VMH in *ARas*-Tg and wild type mice. (D) lordosis behavior test in *ARas*-Tg and wild type female mice (LQ scores). (E) Mount behavior tests in *ARas*-Tg and wild type female mice. Animals was measured with the times of mount behaviors. (F) Testosterone levels in excrement of *ARas*-Tg and wild type female mice. (G) Estrogen levels in the excrement of *ARas*-Tg and wild type female mice. Bars represent mean ±SE (**$p < .01$, $.01 <$ *$p < .05$; student t-test).

1-2 Functional alteration of rodent brain by noncoding RNA for AR promoter

In addition to the estrogen signaling, AR-mediated androgen signaling participates in the cellular, reproductive and behavioral mechanisms. We found novel endogenous antisense transcripts on *AR* locus, named *ARas*. In the brain, the expression showed tissue-, development-, and sex-dependent pattern. *ARas*-Tg female mice showed the significant reduction of lordorsis, occurrence of mount behavior, and larger volume of VMH which are rather reminiscent of the male-like patterns (Figure 1). The circulating testosterone level was lower in *ARas*-Tg females than in wild type females. The search for molecular targets of *ARas* revealed *AR* promoter to be specifically demethylated, resulting in the *AR* upregulation. Of the discrete brain areas, only VMH showed the sex-dependent DNA methylation pattern in mice and rats. Orchidoectomy and peripheral application of estradiol benzoate at the onset of birth increased and decreased the methylation level at several CG sites in males and females, respectively, suggesting that the sex-specific methylation of *AR* promoter was established/maintained by estrogen that was produced by the aromatization of testosterone derived from the testis. Thus, *ARas*, acting on the *AR* promoter, is a component that establishes its epigenetic status for gene expression in a spatiotemporal manner. It is likely that appropriate control of AR expression by *ARas* mediates the estrogen-induced sexual differentiation of the brain.

Publication List

〔Original papers〕

- Iwata, E., Kikusui, T., Takeuchi, Y., and Mori, Y. (2007). Fostering and environmental enrichment ameliorate anxious behavior induced by early weaning in Balb/c mice. Physiol. Behav. *91*, 318-324.
- Kakuma, Y., Ichimaru, T., Yonezawa, T., Momozawa, Y., Hashizume, C., Iwata, E., Kikusui, T., Takeuchi, Y., Ohkura, S., Okamura, H., and Mori, Y. (2007). Androgen induces production of male effect pheromone in female goats. J. Reprod. Dev. *53*, 829-834.
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- Uematsu, A., Kikusui, T., Kihara, T., Harada, T., Kato, M., Nakano, K., Murakami, O., Koshida, N., Takeuchi, Y., and Mori, Y. (2007). Maternal approaches to pup ultrasonic vocalizations produced by a nanocrystalline silicon thermo-acoustic emitter. Brain. Res. *1163*, 91-99.
- Yamada, S., Uenoyama, Y., Kinoshita, M., Iwata, K., Takase, K., Matsui, H., Adachi, S., Inoue, K., Maeda, K. I., and Tsukamura, H. (2007). Inhibition of metastin (kisspeptin-54)-GPR54 signaling in the arcuate nucleus-median eminence region during lactation in rats. Endocrinology *148*, 2226-2232.

〔Review article〕

- Maeda, K., Adachi, S., Inoue, K., Ohkura, S., and Tsukamura, H. (2007). Metastin/kisspeptin and control of estrous cycle in rats. Rev. Endocr. Metab. Disord. *8*, 21-29.

LABORATORY OF NEUROPHYSIOLOGY

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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. The molecular entities of these receptors, however, were not discovered for a long time. In 2000, by using gene-targeting technology, we first clarified that Na_x sodium channel is a probable candidate for the specific sodium receptor in the brain.

A line of studies using Na_x-gene deficient mice showed that 1) Na_x-deficient mice ingested hypertonic sodium chloride solution in excess in comparison with wild type-mice; 2) Na_x gene is expressed in the circumventricular organs, which are the specialized central organs involved in the sensing of sodium concentration and osmolality in the body fluids; 3) Na_x channel is an extracellular sodium-level sensitive sodium channel (Figure 1); and 4) 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 suggest 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.

Recent immuno-electron-microscopic 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.

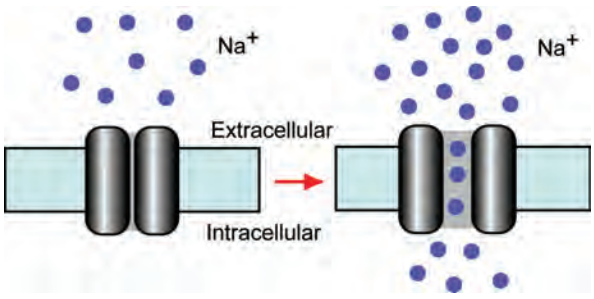

Figure 1. Na_x is a sodium channel sensitive to extracellular sodium level. When the extracellular sodium concentration increases, Na_x channel opens the gate pore and generates the sodium ion influx into the cells. This view was hypothesized by ion-imaging studies.

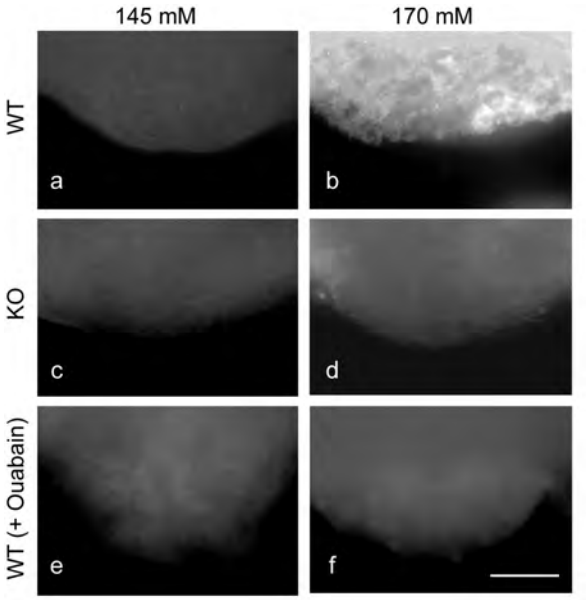

Figure 2. Imaging analysis of the uptake of glucose in the subfornical organ using a fluorescent glucose derivative 2-NBDG. The subfornical organ was isolated from wild-type (WT; a, b, e, and f) and Nax-knockout (KO; c and d) mice, and incubated with 2-NBDG in the 145 mM (a, c, and e) or 170 mM (b, d, and f) sodium solution. In some experiments, the extracellular solutions contained 1 mM ouabain that is a potent blocker of Na^+/K^+-ATPase (e and f). Scale bars: 50 mm. For details see the following paper.

In 2007, we showed direct interaction between Na_x channels and subunits of Na^+/K^+-ATPase, which brings about sodium-dependent activation of the metabolic state of the glial cells (Figure 2). Metabolic enhancement leading to extensive lactate production was observed in the subfornical organ of wild-type mice, but not in the Nax-knockout mice. Furthermore, lactate, as well as Na, stimulated the activity of GABAergic neurons in the subfornical organ. These results suggest that the information ofn a physiological increase of the sodium level in body fluids sensed by Na_x in glial cells is transmitted to neurons by lactate as a mediator to regulate neural activities of the subfornical organ. These findings suggest that the neuron-glia complex plays a key role in sodium sensing in the circumventricular organs.

Publication List

〔Original paper〕

● Shimizu, H., Watanabe, E., Hiyama, T.Y., Nagakura, A., Fujikawa, A., Okado, H., Yanagawa, Y., Obata, K., and Noda, M. (2007). Glial Na_x channels control lactate signaling to neurons for brain [Na^+] sensing. Neuron *54*, 59-72.

LABORATORY OF NEUROCHEMISTRY

Associate Professor
SASAOKA, Toshikuni

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Our major research interest is to understand the physiological role of the dopaminergic system in animal behavior, particularly locomotion and eating behaviors, 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 modulation of locomotor activity, the regulation of several peptide hormones in the pituitary, 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 the dopaminergic system can result in several neurological and psychiatric disorders, such as Parkinson's disease and schizophrenia.

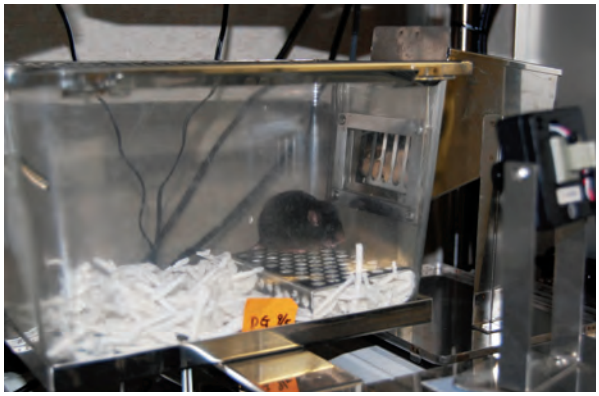

Figure 1. Experimental equipment for measurement of locomotor activity and food/water intake

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

In collaboration with the Laboratory of Dr. Motoya Katsuki, the former 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 severe impairment in locomotion, no initiation of eating, and died by 4 weeks of age. To investigate the molecular mechanism of motor control 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 decreases in locomotion and food/water intake as well as a decrease in the amount of transgene expression after Dox administration. After the withdrawal of Dox administration, the *D1R/D2R* DKO-*D1R* rescued mice exhibited transient hyperactivity and recovered locomotor activity and food/water intake. We are analyzing these results to identify the mechanism of the relationship between the *D1R* expression and altered behavior. In addition, we are also 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 collaboration with Prof. Yo-ichi Nabeshima of Kyoto University, we developed a novel mouse developmental biotechnology of introducing an amino acid substitution into a target gene in a spatially and temporally restricted manner. The goal of the study was 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 (the 595th asparagines, Asp595) of N-methyl-D-aspartate receptor (NMDAR), leading to an aberrant activation of NMDAR. 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.

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). We previously generated the β-SG KO and γ-SG KO mice and found that 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.

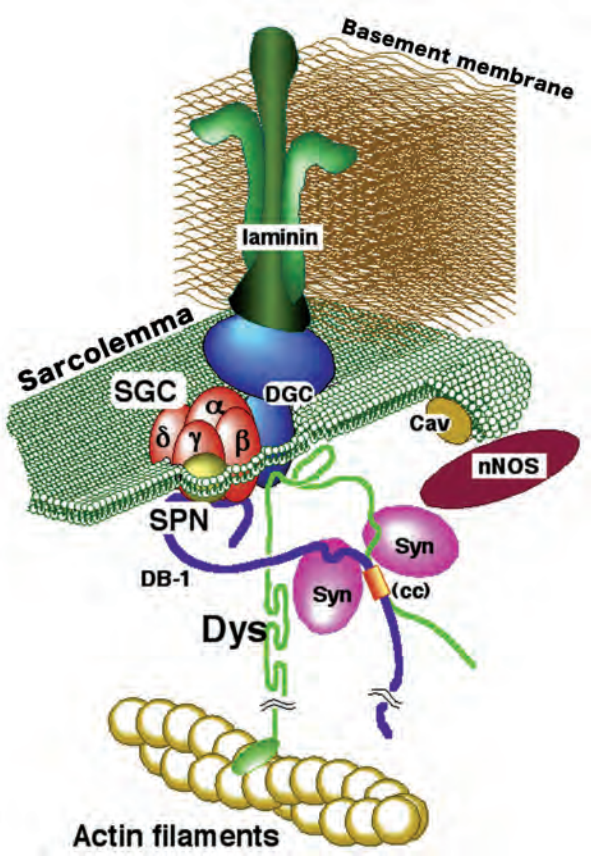

Figure 2. A schematic drawing of the dystrophin complex

Dystroglycan (DG) is a widely expressed transmembrane glycoprotein complex which plays important roles 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 of the precursor to generate α- and β-DG subunits. In collaboration with Dr. Torahiko Tanaka of Nihon University School of Medicine, Tokyo, we performed a series of mutation analyses to determine which amino acid residues and which regions of DG are critical for cleavage in order to clarify the mechanisms involved in DG cleavage and subunit association. We transfected cultured cells with wild-type and various mutant DGs, and confirmed the DG cleavage. We found the following: (i) Disruption of the intramolecular disulfide bridge between Cys669 and Cys713 in β-DG completely abolishes cleavage; (ii) deletions in the loop region (669-713) and in the C-terminal region of α-DG (550-645) abolish the cleavage; (iii) disruption of the disulfide bridge and deletions in the loop region deteriorate the α- and β-DG association; (iv) positions P1' (Ser654) and P6' (Trp659) are critical, particularly at the cleavage site. Thus, the critical role of the Cys669-Cys713 disulfide bridge formation is, most likely, to form a specific tertiary structure, in which the α- and β-DG domains interact and the cleavage site becomes susceptible to proteolytic reactions. The Cys669 and Cys713 pair is broadly conserved in vertebrates and in some invertebrates, suggesting that the disulfide bridge formation was established early in the evolution of DG.

Caveolin-3 is a muscle-specific membrane protein, a component of the dystrophin complex, and serves as a scaffold of various molecules. Its gene mutations cause limb-girdle muscular dystrophy (LGMD1C or caveolinopathy) with mild clinical symptoms. In collaboration with Dr. Yasuko Hagiwara of Musashino University, 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 gene expression profiles of approximately 8,000 genes in the skeletal muscle of Cav3-/- mice using DNA microarray technique. We found that the gene of *osteoponin* (*OPN*), a versatile regulator of inflammation and tissue repair, was significantly down-regulated. This is in contrast to *mdx* mice showing a markedly up-regulated *OPN* gene in their skeletal muscles. Recently, *OPN* has been reported to be important in the pathogenesis of muscular dystrophy. We examined whether up-regulated *OPN* gene expression in *mdx* muscles is altered by the deficiency of caveolin-3. To this end, we developed caveolin-3 and dystrophin double-deficient mice. The levels of *OPN* mRNA and osteopontin in the double-deficient mice clearly decreased compared with those in *mdx* mice. We showed that although the level of *OPN* mRNA expressed in the double-deficient skeletal muscles was lower than that in *mdx* skeletal muscles, macrophage infiltration and muscle regeneration occurred similarly in the double-deficient and *mdx* skeletal muscles. There may still be other factors that are involved in macrophage infiltration and muscle regeneration.

Publication List

〔Original papers〕

- Ohi, Y., Ishii, Y., Haji, A., Noguchi, S., Sasaoka, T., Fujimori, T., Nabeshima, Y., Sasahara, M., and Hattori, Y. (2007). Platelet-derived growth factor (PDGF)-BB inhibits AMPA receptor-mediated synaptic transmission via PDGF receptor-beta in murine nucleus tractus solitarius. Brain Research *1159*, 77-85.
- Watanabe, N., Sasaoka, T., Noguchi, S., Nishino, I., and Tanaka, T. (2007). Cys669-Cys713 disulfide bridge formation is a key to dystroglycan cleavage and subunit association. Genes to Cells *12*, 75-88.

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*, have been domesticated as floricultural plants. Of these, spontaneous mutants of *I. nil* and *I. purpurea* with various flower colors 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. Almost all structural and regulatory

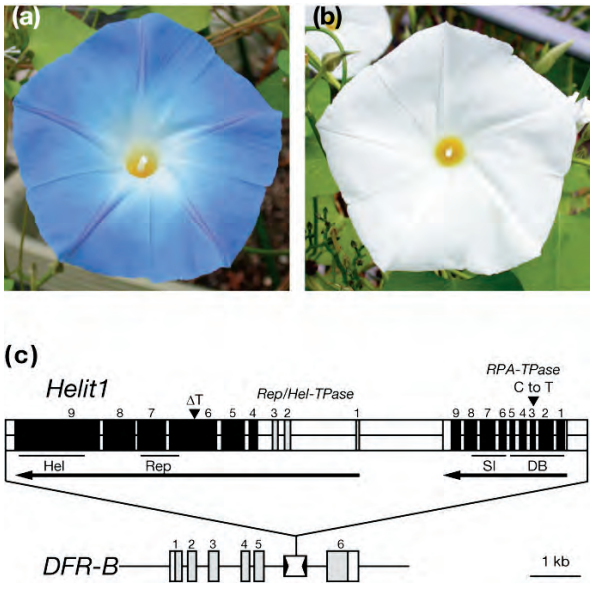

Figure 1. Pigmentation phenotype of the *pearly-s* mutant and structure of the *pearly-s* mutation. (a) Wild-type cultivar Heavenly Blue. (b) A *pearly-s* mutant displays stable white flowers. (c) Structure of the *pearly-s* mutation having the *Hel-It1* transposon integrated into the *DFR-B* gene. The boxes with numerals and the shadowed parts indicate exons and the coding region of the *DFR-B* gene, respectively. The rectangle with the filled arrowheads shows the *MELS2* element. The boxes in *Hel-It1* indicate the predicted exons for the putative wild-type *RPA-TPase* and *Rep/Hel-TPase* genes, respectively. The horizontal lines under the boxes indicate functional domains of these proteins, and the symbols DB, SI, Rep, and Hel represent DNA-binding, subunit interaction, Rep, and Hel domains, respectively. The predicted single-base substitution and deletion are shown by vertical arrowheads with C to T and ΔT, respectively.

genes, which encode enzymes to produce anthocyanidin 3-*O*-sophorosides for their flower pigmentation, and transcriptional regulators, which activate the structural genes for anthocyanin biosynthesis, as well as their spontaneous mutations, have been identified and characterized. The majority of their spontaneous mutations have been shown to be caused by insertions of various DNA transposons belonging to the *hAT*, CACTA, and *MuLE* superfamilies.

Helitrons are newcomers of eukaryotic DNA transposons and were originally identified by computational analysis in the genomes of Arabidopsis, rice, and nematode. They are distinguished from other transposons in their structural features, and their proposed transposition mechanisms are involved in rolling circle replication. Computer-predicted autonomous *Helitrons* with conserved terminal sequences, 5'-TC and CTRR-3', are presumed to encode a putative transposase, *Rep/Hel-TPase*, which contains a characteristic nuclease/ligase domain for the replication initiation protein (Rep) and a DNA helicase domain (Hel). Plant *Helitrons* are thought to carry an additional transposase gene, *RPA-TPase*, which is related to the largest subunit of the replication protein A (RPA70). Although *Helitrons* are found in diverse genomes, neither an autonomous element nor a transposition event has been reported. We found that a spontaneous *pearly-s* mutant of *Ipomoea tricolor*, cultivar Pearly Gates,

exhibiting white flowers and isolated in approximately 1940, has an 11.5-kbp novel *Helitron*, named *Hel-It1*, integrated into the *DFR-B* gene for anthocyanin pigmentation (Figure 1). *Hel-It1* shows the predicted plant *Helitron* structure for an autonomous element with the conserved termini and carries the two putative transposase genes, *Rep/Hel-TPase* and *RPA-TPase*, which contain a nonsense and a frameshift mutation, respectively. *Hel-It1*-related elements are scattered in the *Ipomoea* genome, and only a fraction of the *pearly-s* plants was found to carry *Hel-It1* at another insertion site. The *pearly-s* mutant appears to bear an autonomous element and express the wild-type *RPA-TPase* transcripts, indicating that an *Ipomoea Helitron* also acts as a spontaneous mutagen.

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

Rice (*Oryza sativa* L.), with the sequenced genome of 389-Mb, is an important staple food for more than half of the world's population and is 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 using the *hpt* and *DT-A* genes and have succeeded in modifying the *Waxy* and *Adh2* genes to generate true gene targeting (TGT) repeatedly without the concomitant occurrence of ectopic events, such as one-sided invasion (OSI) and ectopic gene targeting (EGT) (Figure 2). While *Waxy* is a unique gene in the rice genome, the 3 *Adh* genes, *Adh1*, *Adh2*, and *Adh3*, reside on chromosome 11 in the same orientation. The targeting frequencies of *Waxy* and *Adh2* were about 1% and 2% per surviving callus, respectively. To gain information about surviving calli that were not targeted homologous recombinants, a series of PCR analyses was performed to determine the randomly integrated transgene segments present in these calli. All the surviving calli examined contained the selective *hpt* sequence, and none of them carried the intact *DT-A* sequence, indicating that none of the surviving calli was an escapee from the positive-negative selection. The analyses prompt us to speculate the following integration processes of transgenes associated with gene targeting in *Agorbacterium*-mediated transformation (Figure 2).

In *Agrobacterium*-mediated transformation, which has been used by gene targeting in higher plants, T-DNA appears to integrate randomly throughout the plant genome as a single molecule or multiple sequences ligated with each other in various orientations. The majority of the randomly integrated single-copy T-DNA molecules mediated by nonhomologous end-joining are known to contain the entire T-DNA segment with a well-conserved right border, and a left-border sequence that is either conserved or slightly truncated, which we termed as border-associated random integration (BARI) (Figure 2). There appears to be another type of random integration with relatively large deletions at both ends of the T-DNA segment without the border proximal regions, which we termed border-independent random integration (BIRI) (Figure 2). BIRI appears to occur much less frequently than BARI. Since a significant portion of single-stranded T-DNA imported into the plant nucleus can become double-stranded in *Agrobacterium*-mediated transformation, it has been speculated that such BIRI processes must share common recombination mechanisms with random-integration processes by direct DNA delivery methods using double-stranded DNA. Although the targeting frequencies in higher plants have been thought to be low as calculated by TGT events per BARI-mediated transformant, such frequencies may be too low to calculate the proper targeting frequency because *Agrobacterium*-mediated transformation via BARI of T-DNA is a highly efficient process.

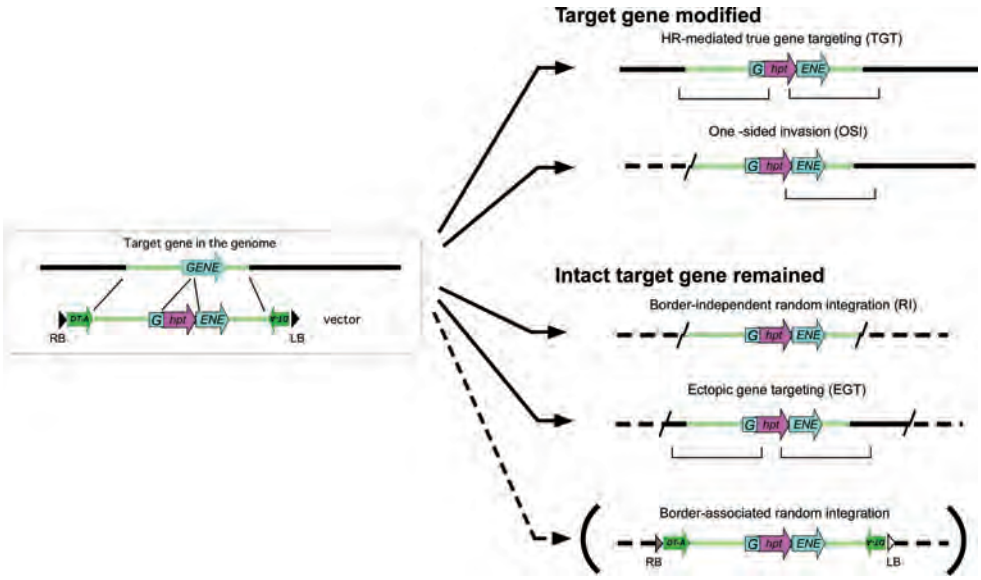

Figure 2. Integration events of a transgene associated with homology-dependent gene targeting with positive-negative selection. The large black arrowheads with RB and LB indicate the right and left borders, respectively. The most efficient BARI events are shown in large parentheses because BARI-mediated calli are killed by the action of TD-A. Zigzag lines represent breakpoints generated by nonhomologous events.

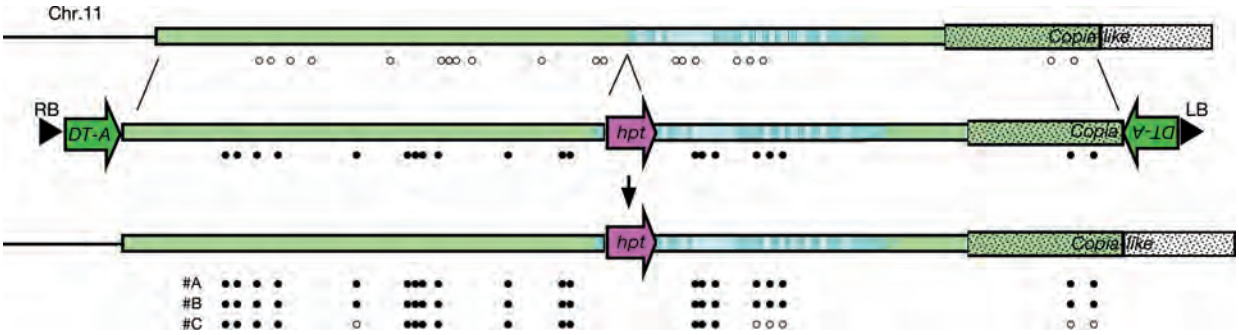

Figure 3. Transfer of base changes from a vector to the rice genome. The rice *Adh2* locus on chromosome 11 bears a *Copia*-like retroelement, and the vector used contains the 6.2-kb *Adh2* promoter sequence including a 0.1-kb 5'-untranslated region, *hpt*, 4.0-kb *Adh2* region, and 2.0-kb 3' part of a *Copia*-like retroelement; the *DT-A* genes were placed at both ends of T-DNA in order to efficiently eliminate BARI. The large black arrowheads with RB and LB indicate the right and left borders of T-DNA, respectively. The blue boxes indicate *Adh2* exons, and *hpt* and *DT-A* are not drawn to scale. The filled and open circles indicate the base changes in the vector and the corresponding genomic sequences, respectively. Homologous crossover sites can be deduced by determining these alternative sequences in the targeted recombinant allele.

To facilitate the molecular analysis of the recombination processes, we began to characterize crossover sites in the targeting of *Adh2* with a vector carrying multiple base changes (Figure 3). Preliminary results indicate that the efficient transfer of base changes (point mutations) occurs from the vector to the rice genome.

Ⅲ. Characterization of mutable *virescent* allele in rice

We have identified an active nonautonomous rice transposon *nDart1* of about 0.6 kb, belonging to the *hAT* superfamily, as a causative transposon of a mutable *virescent* allele *pyl-v* (*pyl-variegated*) conferring pale yellow leaves with dark green sectors in its seedlings. 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*. Mapping data indicated that *aDart* resides within 170-kb region on chromosome 6. The most likely candidate element, *Dart1-27*, was cloned and reintroduced into a stable *virescent* mutant *pyl-stb* (*pyl-stable*) exhibiting pale yellow leaves due to the a deficiency of the *aDart* activity. Clear leaf variegation could be observed in a significant portion of the *pyl-stb* derivatives, in which the demethylated *Dart1-27* element by growing in *Escherichia coli* had been introduced. We can thus conclude that *Dart1-27* is the active autonomous *aDart* element in the mutable *virescent pyl-v* plants. In consistent with this notion, the 5'-terminus of *Dart1-27* in the *pyl-v* lines is less DNA methylated than that in the pyl-stb plants, even though the sequences of the *Dart1-27* elements in *pyl-v* and *pyl-stb* are found to be identical to each other.

Publication List

〔Original papers〕

- Choi, J.D., Hoshino, A., Park, K.I., Park, I.S., and Iida, S. (2007). Spontaneous mutations caused by an active *Helitron* transposon, *Hel-It1*, in morning glory, *Ipomoea tricolor*. Plant J. *49*, 924-934.
- Furukawa, T., Maekawa, M., Oki, T., Suda, I., Iida, S., Shimada, H., Takamure, I., and Kadowaki, K. (2007). The *Rc* and *Rd* genes are involved in proanthocyanidin synthesis in the rice pericarp. Plant J. *49*, 91-102.
- Park, K.I., Ishikawa, N., Morita, Y., Choi, J.D., Hoshino, A., and Iida, S. (2007). A *bHLH* regulatory gene in the common morning glory, *Ipomoea purpurea*, controls anthocyanin biosynthesis in flowers, proanthocyanidin and phytomelanin pigmentation in seeds, and seed trichome formation. Plant J. *49*, 641-659.
- Takagi, K., Ishikawa, N., Maekawa, M., Tsugane, K., and Iida, S. (2007). Transposon display for active DNA transposons in rice. Genes Genet. Syst. *82*, 109-122.
- Terada, R., Johzuka-Hisatomi, Y., Saitoh, M., Asao, H., and Iida, S. (2007). Gene targeting by homologous recombination as a biotechnological tool for rice functional genomics. Plant Physiol. *144*, 846-856.

〔Review article〕

- Iida, S., Johzuka-Hisatomi, Y., and Terada, R. (2007). Gene targeting by homologous recombination for rice functional genomics. In Rice Functional Genomics-Challenges, Progress and Prospects, N.M. Upadhyaya ed., (Springer), pp. 273-289.

DIVISION OF GENOME DYNAMICS

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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 repeated sequence or the mechanisms producing repeated sequences and controlling instability are fully understood. To clarify these aspects of genomes, we are pursuing several themes using *Eschericia coli*, *Saccharomyces cerevisiae* and Chinese Hamster Ovary (CHO) cells. In 2007 we have made advances in understanding a new role for condensin in maintaining a long repeated structure of rDNA in yeast and a relationship between the association of condensin with chromosomes and transcription on those chromosomes. In addition, we also have constructed a new system of gene amplification via DRCR (double rolling circle replication) in yeast by using the Cre-*lox* site-specific recombination. From previous and present results, we concluded that DRCR is an actual amplification mechanism, at least in budding yeast. There exists a possibility that DRCR might work for gene amplification in higher eukaryotes as well.

Ⅰ. Analysis of mechanism maintaining repeated structure of ribosomal RNA genes

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

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 the replication fork barrier (RFB) site, rDNA region-specific recombination and expansion/contraction of rDNA repeats. The latter two activities are likely to be triggered by a double-strand break at the RFB site and a repairing of the break via gene conversion. Thus, this *FOB1*-dependent recombination apparently contributes to the maintenance of the average copy number of rDNA. However, in *Δfob1* cells, the repeats are still maintained without any fluctuation of copy number, thereby suggesting that another, unknown system acts to prevent contraction of the number of repeats.

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

Recently we found that RNA polymerase I (PolI) defective mutation suppresses dramatic reduction of the copy number of rDNA in the condensin and *fob1* double mutant. Because PolI is an rDNA specific transcription enzyme, ΔpolI defective mutant is non-viable. But if the defective cells carry a plasmid, in which rDNA is inserted downstream of Gal-dependent promoter, cells can be viable in the presence of galactose. Using the ΔpolI mutant, we examined the effect of PolI enzyme on condensin association with rDNA. Under *fob1* conditions, PolI enzyme seems to force condensin from transcribing to non-transcribing (IGS) region. This characteristic change of pattern suggests that constant PolI transcription through cell cycle prevents condensin from associating with the transcribing region. Thus, it is expected that, in the triple (*fob1*, condensin and PolI) mutant, the partial defective condensin uniformly associated with rDNA region is responsible for successful separation in anaphase.

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

Ⅱ. Mechanism of condensin recruitment onto RFB site located within the tandem rDNA repeat in budding yeast.

The primary functions of mitotic chromosome condensation are to reduce the length of chromosomes so

that they avoid truncation of genome during cell division and to ensure the proper segregation of sister chromatids. The compaction ratio of mitotic chromosomes relative to double stranded DNA fiber ranges from ~160-fold in budding yeast to ~10000 – 20000-fold in mammalian chromosomes. Condensin is a multi-subunit protein complex that plays a central role in mitotic chromosome condensation and segregation. In vertebrates, condensin has been shown to be distributed in axial part over the whole length of condensed chromosomes, but only at the resolution of light microscopy. The sites where condensin acts in chromatin and the molecular mechanisms of condensin recruitment have largely remained elusive. As described above, we found that condensin localized at the RFB site in Fob1 dependent manner during S-phase. To date, this Fob1-dependent condensin localization is the only example of condensin association with a specific DNA site in a specific protein factor-dependent manner. To understand chromosome condensation in molecular resolution, we are studying mechanisms of condensin localization at the RFB site. Firstly, we haven't obtained any positive data indicating direct interaction between condensin and Fob1 protein from either of two-hybrid or immuno-precipitation experiment, and we therefore excluded the possibility in Figure 1 (1). Secondly, we discovered that condensin could bind to the short DNA fragment containing RFB sequences, even if the sequence was inserted in either orientation (fork blocking or non-blocking orientation) at an ectopic chromosome site, and we therefore excluded the possibility in Figure 1 (2). To gain further information about the specific recruitment of condensin onto the RFB site, we isolated additional factors by genetic approach. So far, we have identified at least three additional factors that were necessary for condensin recruitment to the RFB site (Figure 1 (3)). Protein-protein interaction analysis of these three factors in addition to Fob1 and all subunits of condensin complex suggested that condensin was targeted at the RFB site by ordered interactions among them.

Ⅲ. Construction of a new gene amplification system via DRCR (double rolling circle replication) by using the Cre-*lox* site specific recombination

In addition to rDNA gene amplification in eukaryotes, there is another type of gene amplification, which is involved in various biological phenomena, such as cancer development and drug-resistance. However, the mechanism is largely unknown because of the complexity of the amplification process. Previously, we developed a gene amplification system in *S. cerevisiae* that is based on double rolling-circle replication (DRCR), utilizing break-induced replication (BIR). This system produced two types of amplification products. Type-1 products contain 13 to ~100 copies of the amplification marker, *leu2d* (up to ~730 kb increase) with novel arrangement present as randomly oriented sequences flanked by inverted *leu2d* copies. Type-2

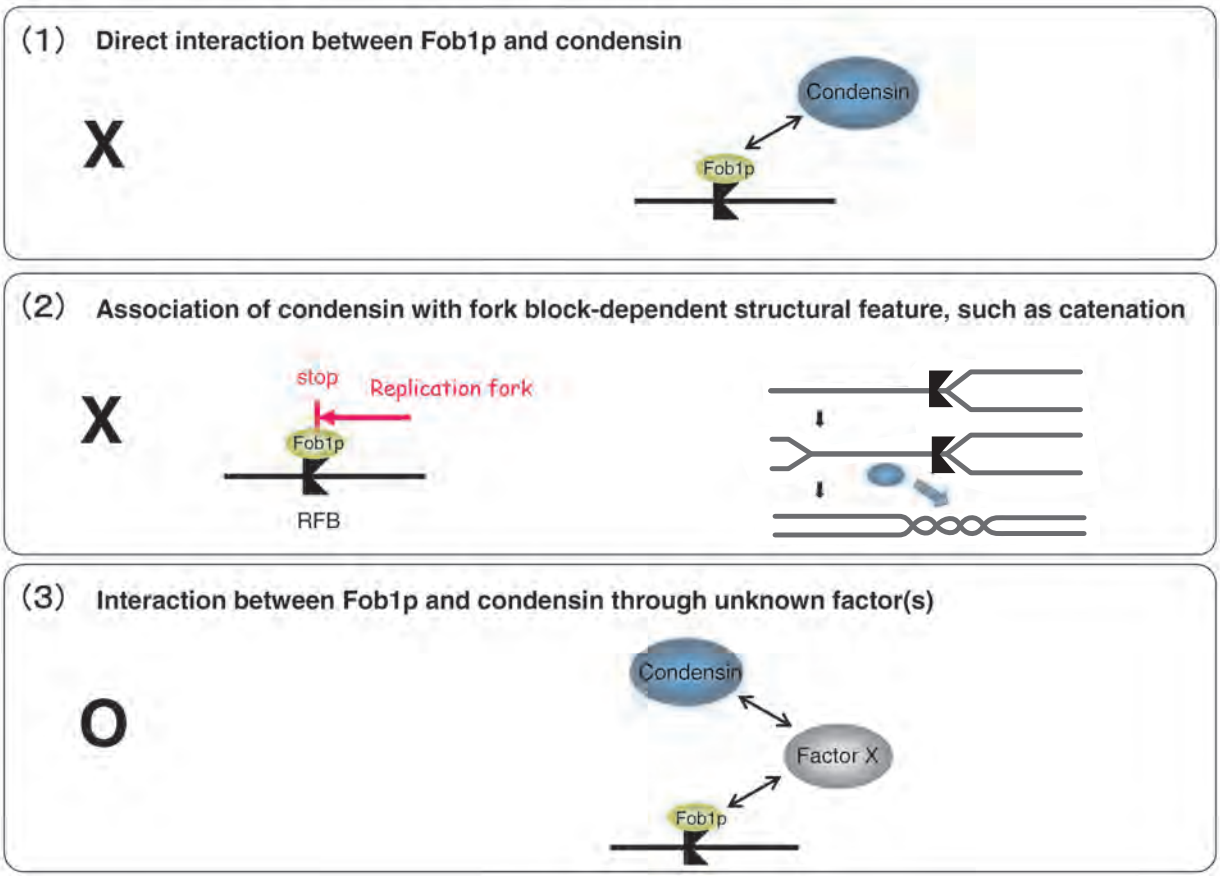

Figure 1. How to associate condensin with the specific RFB site in rDNA repeats

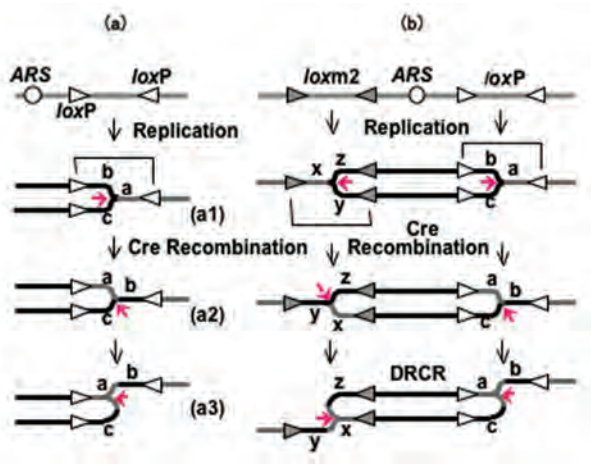

Figure 2. DRCR induced by Cre-*lox* system
(a) Cre-*lox* dependent reversal of replication orientation. (a1) When the replication fork passes through between a pair of *lox* sites, Cre recombination occurs between a pair of *lox* sites, as shown in (a1). Recombination changes replication orientation from un-replicated DNA (parental DNA strand) to replicated DNA (one of sister chromatids) as shown in (a2 and a3), because DNA strand a and b are identical. (b) DRCR is induced by Cre-dependent combinational recombination of two pairs of *lox*P and *lox*m2 as shown in (b). Recombination cannot occur between *lox*P and *lox*m2.

products are acentric multi-copy mini-chromosomes carrying *leu2d*. Structures of type-1 and -2 products resemble those of homogeneously staining region (HSR) and double minutes (DMs) of higher eukaryotes, respectively. Interestingly, products analogous to these were generated at low frequency without deliberate DNA cleavage (*EMBO J* 24, 190-198 (2005)).

If DRCR is an actual gene amplification mechanism in yeast, a quite different initiation reaction, which can induce RDCR, should produce amplification products resembling to HSR and DMs. Thus, we tried to construct a new DRCR amplification system that is induced by another process, Cre-*lox* site-specific recombination. We first predicted that, if Cre recombination occurs between the two *lox* sites, one present on the replicated and the other on the unreplicated regions, as shown in Figure 2(a1), the replication fork should switch the template from the parental (unreplicated) to the sister-chromatid (replicated) DNA strands, as shown in Figure 2(a2) and (a3), and that the Cre recombination system would make this process efficient, as shown in Figure 2(a). Furthermore, a combination of the process, as shown in Figure 2(b), can efficiently induce gene amplification through DRCR. In actuality, this system produced two kinds of products; highly amplified (>100 copies) chromosome products and acentric multi-copy extra-chromosomal products. The structures of these products resemble those of HSR and DMs of higher eukaryotes, respectively. From previous and present results, we concluded that DRCR is an actual amplification mechanism in budding yeast and could be naturally initiated if some structural requirement could be satisfied.

Publication List

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- Cui, T., Moro-oka, N., Ohsumi, K., Kodama, K., Ohshima, T., Ogasawara, N., Mori, H., Wanner, B., Niki, H., and Horiuchi, T. (2007). *E. coli* with a linear genome. EMBO Rep. *8*, 181-187.
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- Johzuka, K., and Horiuchi, T. (2007). RNA polymerase I transcription obstructs condensin association with 35S rRNA coding region and can cause contraction of long repeat in *Saccharomyces cerevisiae*. Genes Cells *12*, 759-771.

DIVISION OF EVOLUTIONARY BIOLOGY

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All living organisms evolved from a common ancestor that lived more than 3.5 billion years ago, and the accumulation of mutations in their genomes has resulted in the present biodiversity. Traces of the evolutionary process are found in the genomes of extant organisms. By comparing the gene networks and their functions of different organisms, we hope to infer the genetic changes that caused the evolution of cellular and developmental processes.

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 based on molecular mechanisms 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. Based on live imaging of microtubules, we have hypothesized that the formation of oblique microtubules, which elongate to the outside of a phragmoplast, drives the centrifugal expansion. We examined the role of γ-tubulin in phragmoplast expansion, because γ-tubulin binds onto existing microtubules and nucleates a new microtubule as a 40 degrees branch in interphase cells. We isolated phragmoplasts from synchronized tobacco BY-2 cells and labeled with anti-γ-tubulin antibody. γ-tubulin was detected at the branched points of microtubules in isolated phragmoplasts (Figure 1). We proposed a hypothesis that cytosolic γ-tubulin complexes are recruited onto existing phragmoplast microtubules and nucleate new microtubules as branches, and the branched microtubules drive phragmoplast expansion. Inhibition of γ-tubulin function during phragmoplast expansion is in progress. T. Murata mainly performed this study.

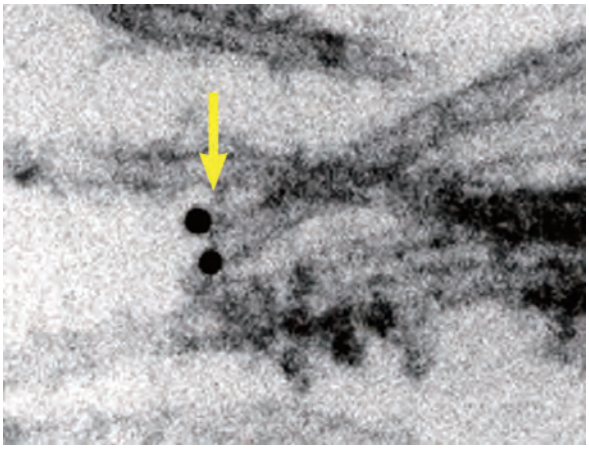

Figure 1. Phragmoplast development in flowering plants. γ-tubulin detected at a microtubule end in an isolated phragmoplast by electron microscopy (yellow arrow).

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 phragmoplast, suggesting that these ubiquitin-like genes likely regulate the stability of phragmoplast microtubules. Further analyses are in progress.

Formation of several types of stem cells to produce different types of differentiated cells is properly regulated during the development of multicellular organisms. However, molecular mechanisms for the stem cell characterization have been largely unknown. We showed that *AINTEGUMENTA/PLETHORA/BABY BOOM* (*APB*) orthologs *PpAPB*s (*PpAPB1*, *2*, *3*, and *4*) are involved in the stem cell characterization in the moss Physcomitrella patens. Gametophore stem cells were induced by exogenous cytokinin in the wild type, while the quadruple disruptants did not form any gametophore stem cells with exogenous cytokinin application. These results suggest that the *PpAPB*s play a critical role in the characterization of a gametophore stem cell. Meanwhile, the expression of *PpAPB*s is regulated by auxin, not cytokinin. This study was mainly performed by Tsuyoshi Aoyama and Yuji Hiwatashi.

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. 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 Evolution of phytohormone regulation

Phytohormones are important regulators for plant development and we investigated their evolution, focusing on polar auxin transport (Fujita et al. in press), cytokinin synthesis (Sakakibara et al. submitted), and gibberellic acid (GA) signal transduction (Hirano et al. 2007).

The shoot is a repeated structure made up of stems and leaves and is the basic body plan in land plants. Vascular plants form a shoot in the diploid generation, whereas non-vascular plants such as mosses form a shoot in the haploid generation. It is not clear whether all land plants use similar molecular mechanisms in shoot development or how the genetic networks for shoot development evolved. The control of auxin distribution, especially by polar auxin transport, is essential for shoot development in flowering plants. We did not detect polar auxin transport in the gametophytic shoots of several mosses, but did detect it in the sporophytes of mosses without shoot structure. Treatment with auxin transport inhibitors resulted in abnormal embryo development, as in flowering plants, but did not cause any morphological changes in the haploid shoots. We fused the soybean auxin-inducible promoter *GH3* with a GUS reporter gene and used it to indirectly detect auxin distribution in the moss *Physcomitrella patens*. An auxin transport inhibitor NPA did not cause any changes in the putative distribution of auxin in the haploid shoot. These results indicate that polar auxin transport is not involved in haploid shoot development in mosses and that shoots in vascular plants and mosses are most likely regulated differently during development (Fujita et al. in press).

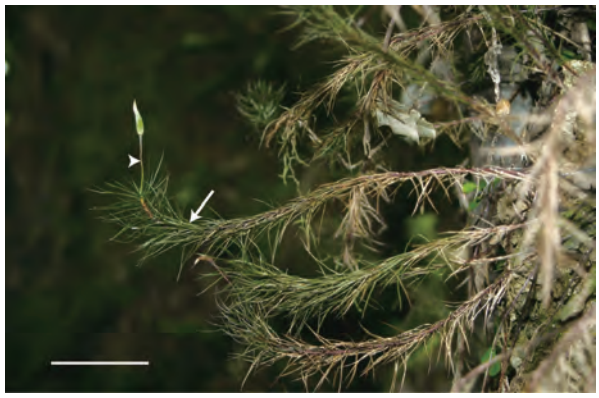

Figure 2. Basipetal polar auxin transport was not detected in a gametophytic shoot (arrow) but in a sporophyte axis (arrow head) of *Dawsonia superba*, which is the biggest moss in the world. Scale bar = 3 cm.

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 international consortium.

We compared the features of *P. patens* genome to those of flowering plants, from which it is separated by more than 400 million years, and unicellular aquatic algae. This reveals genomic changes concomitant with the evolutionary movement to land, including a general increase in gene family complexity, loss of genes associated with aquatic environments, acquisition of genes for tolerating terrestrial stresses, and the development of the auxin and abscisic acid signaling pathways for coordinating multicellular growth and dehydration response. The *P. patens* genome provides a resource for phylogenetic inferences about gene function and for experimental analysis of plant processes through this plant's unique facility for reverse genetics (Rensing et al. in press).

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, (4) 5'-end serial analysis of gene expression (5' SAGE), and (5) a collection of 3' UTR and small RNA sequences as collaborative works with groups associated 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).

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.

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.

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〔Original papers〕

- Hirano, K., Nakajima, M., Asano, K., Nishiyama, T., Sakakibara, H., Kojima, M., Katoh, E., Xiang, H., Tanahashi, T., Hasebe, M., Banks, J. A., Ashikari, M., Kitano, H., Ueguchi-Tanaka, M., and Matsuoka, M. (2007). The GID1-mediated GA perception mechanism is conserved in the lycophyte *Selaginella moellendorffii* but not in the bryophyte *Physcomitrella patens*. Plant Cell *19*, 3058-3079.
- Odahara, M., Inouye, T., Fujita, T., Hasebe, M., and Sekine, Y. (2007). Involvement of mitochondrial-targeted RecA in the repair of mitochondrial DNA in the moss, *Physcomitrella patens*. Genes Genet. Syst. *82*, 43-51.
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- Murata, T., and Hasebe, M. (2007). Microtubule-dependent microtubule nucleation in plant cells. J. Plant Res. *120*, 73-78.
- Murata, T., Tanahashi, T., Nishiyama, T., Yamaguchi, K., and Hasebe, M. (2007). How do plants organize microtubules without a centrosome? J. Integr. Pl. Biol. *49*, 1455-1463.

DIVISION OF SPECIATION MECHANISM (ADJUNCT)

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	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 group's research is to propose and clarify 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 (Figures 1 and 2).

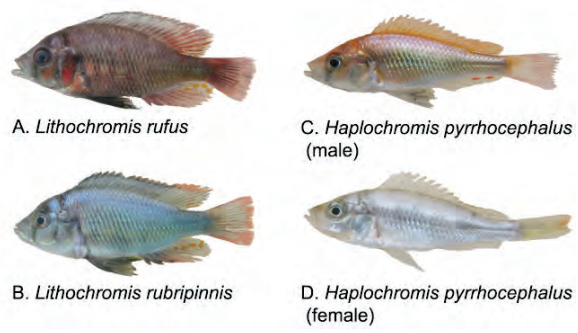

Figure 1. Cichlid fishes in Lake Victoria. These were caught in the field by our group.

Figure 2. Live color of male *Pundamilia nyererei*

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 – Tanganyika, Malawi, and Victoria - and their drainages in East Africa. One thousand or more species of cichlid fishes inhabit these lakes. It is believed that Lake Tanganyika was established 12 million years ago, Lake Malawi 2 million years ago, and Lake Victoria 12,000 years ago. Thus, it is thought that the cichlids evolved in each lake after the lakes had 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 sites 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, which may be a significant factor promoting 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 type. 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. Okada's laboratory (Tokyo Inst. Tech.) have been conducting field expeditions around Mwanza Gulf on the southern shore of Lake Victoria since 2004 (Figure 3). Thousands of fish individuals have been collected so far at various ecological habitats, and the number of species of these cichlids available for our study reached approximately 150 (rough estimation). Pictures were taken of each individual, immediately after collection, to record their live colors (Figure 2). 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

Cichlids' varied body colorations are one of the examples of 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 female's choice. Therefore, it could be considered that the body colors of males play an important role for recognition by the visual system of females during the course of reproduction. In addition, the visual system of cichlids must have been affected by environmental differences in their habitat such as turbidity and depth of the lake water. In a collaborative work with Dr. N. Okada's 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 3, panel B), and we found geographical clines of nuptial coloration on the males. We are carrying out an analysis of the opsin gene family of these species to detect genetic variations of chromatic vision that seem to be related to adaptation for such specific male colors. Another interesting issue concerning the evolution of the opsin gene family can be seen for the species *Haplochromis pyrrhocephalus*, which is broadly distributed in the lake (Figure 1C and D). The light environment for this species is considerably different among populations. To elucidate the mode of adaptation of their vision system to such various light conditions, we are analyzing the six types of their opsin gene family. A more extensive analysis of the molecular evolution of opsin genes in Victorian cichlids is in progress in our division.

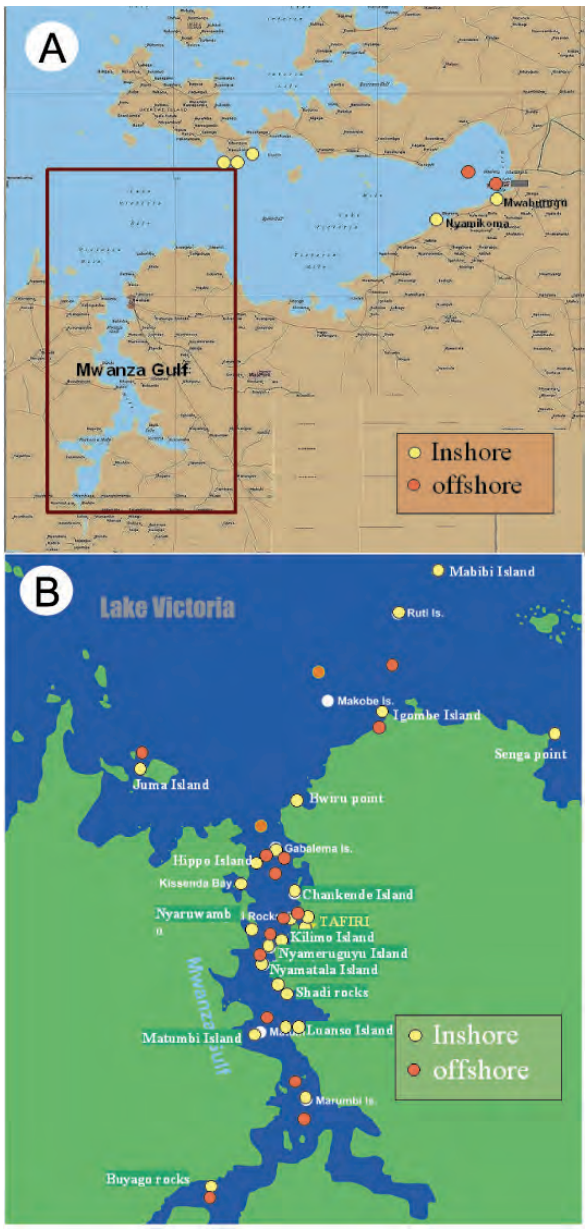

Figure 3. Localities of collection of cichlids in Lake Victoria since 2004. Panel A: Sampling localities in southern shore of Lake Victoria. The area surrounded by a brown rectangle in this panel corresponds to the region shown as a magnified map of panel B. Panel B: Sampling localities in Mwanza gulf. 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〕

- Fujimura, K., and Okada, N. (2007). Development of the embryo, larva and early juvenile of Nile tilapia *Oreochromis niloticus* (Pisces: Cichlidae). Developmental staging system. Develop. Growth Differ. *49*, 301-324.
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LABORATORY OF MORRHODIVERSITY

Associate Professor
KODAMA, Ryuji

The aim of this laboratory is to observe the variety of the morphogenetic processes that occur 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 of the embryogenetic processes of related species is expected to provide an insight into the evolution of morphogenetic processes. This laboratory uses the wings of lepidopteran insects for our morphological studies.

Ⅰ. Wing worphogenesis

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 re-gion have characteristics in common with the apop-totic 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 en-gulfed 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.

Ⅱ. Other research activities

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. Our institute's Division of Sex Differentiation and the Laboratory of Neurophysiology are both involved in these activities.

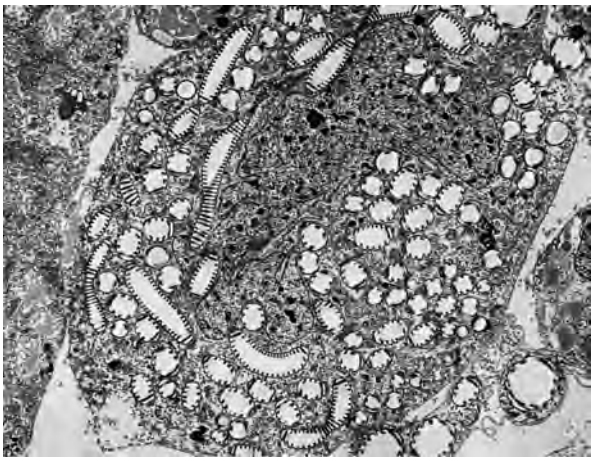

Figure 1. Transmission electron micrograph of the tracheole cell. Many cross-sections of the tracheoles can be observed within the cytoplasm of the tracheole cell. As the cell migrates, the tracheole is laid behind.

LABORATORY OF BIORESOURCES

Associate Professor NARUSE, Kiyoshi

Postdoctoral Fellow SHEN, Xueyan
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TORII, Naoko
AJIOKA, Rie
Secretary SUZUKI, Tokiko

Teleosts comprise about half of all vertebrate species and adapt to a variety of environments, including sea-water, fresh water, the bottom of deep seas, and small creeks and paddy fields. Analysis of their genome structure is important to understand the adaptation and diversification in this interesting group. Medaka is a small egg-laying “secondary” fresh water fish found in brooks and rice paddies in Eastern Asia. This species has a long history as an experimental animal, especially in Japan. Our laboratory has conducted a comparative genomic analysis focusing mainly on fish chromosomes and gene evolution using medaka and other fishes. In addition to these activities, our laboratory is stepping ahead to lead the National BioResource Project Medaka (NBRP Medaka).

Ⅰ. Medaka genome sequence and the vertebrate genome evolution

We have successfully completed the genome sequencing of Hd-rR (Southern inbred strain) at the draft level. Total nucleotide length of the generated scaffblds is 700.4Mbp. Comparative analysis with the genome of the northern inbred line (HNI) revealed 16.4 million single nucleotide polymorphisms (SNPS) which allowed us to develop a high density SNP map that could be successfully used for aligning generated scaffblds. The genome–wide SNP rate between Hd-rR and HNI inbred strains is 3.42 % which is, to our knowledge, the highest SNP rate seen in any vertebrate species. We also identified 20,141 non-redundant genes with 5’-end serial analysis of gene expression (SAGE) tags combining with Genscan. Using this medaka gene model, we conducted four-way comparisons of the synteny of orthologous genes among the human and *Tetraodon* genomes and the zebrafish gene map. Most of the medaka and *Tetraodon* chromosomes have a one-by-one relationship and about half of the medaka and zebrafish chromosomes also correspond to a one-by-one relationship, even though the lineage separation of these two species dates back more than 191 million years. These results showed that the inter-chromosomal arrangements in the teleost lineage were less frequent than those of mammals whose lineage separation dates back to 100 million years ago. We also successfully reconstructed the pre-duplicated proto-chromosomes of the ancestor of all ray-finned fish and described the scenario leading to the present genome structure of medaka, zebrafish and *Tetraodon*.

Ⅱ. Evolution of the sex chromosome and sex determination genes in *Oryzias* fish

The sex-determining gene DMY was identified on the Y chromosome in the medaka, *Oryzias latipes*. However, this gene is absent in most *Oryzias* fishes, suggesting that closely related species have another sex-determining gene. In fact, it has been demonstrated that the Y chromosome in *O. dancena* differs from that in *O. latipes*, whereas both species have an XX/XY sex determination system. Through a progeny test of sex-reversed fish and a linkage analysis of isolated sex-linked DNA markers, we showed that *O. hubbsi*, which is one of the species most closely related to *O. dancena*, has a ZZ/ZW system.

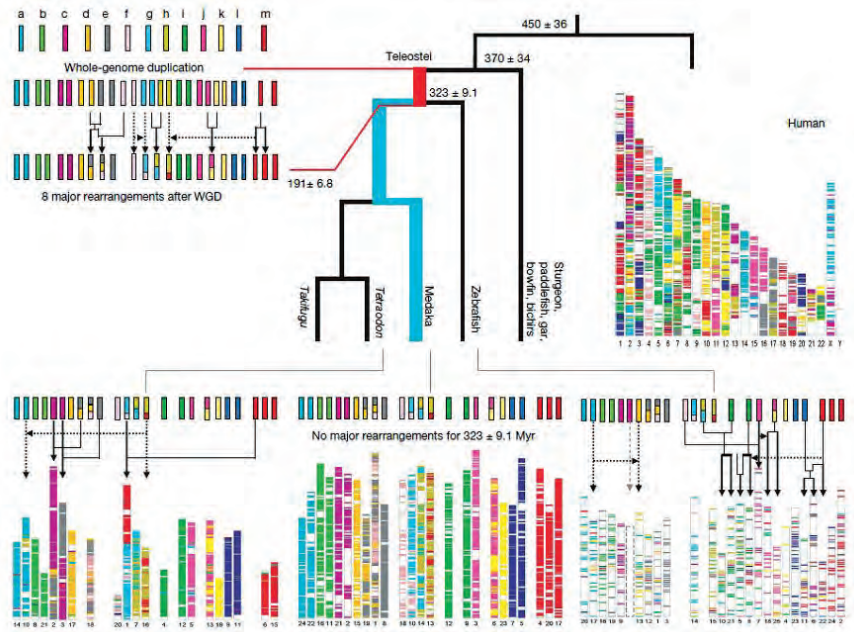

Figure 1. Reconstruction of the pre-duplicated proto-chromosome and scenario leading to the medaka, *Tetraodon* and zebrafish genome

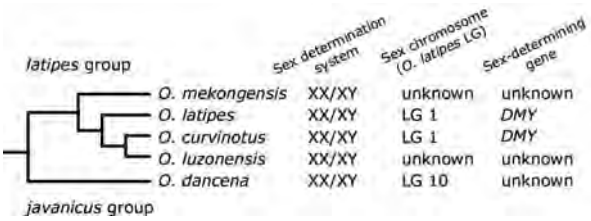

Figure 2. Phylogenetic relationships and sex determination mechanisms in *Oryzias* fishes

Ⅲ. National BioResource Project Medaka (NBRP Medaka)

In 2007, the NIBB was selected as the core facility of NBRP medaka. Our laboratory is taking an active part of this project. We provide, maintain and collect living resources such as standard strains, inbred strains, mutants, and frozen resources such as EST/cDNA and BAC/ Fosmid clones, as well as the integrated information on medaka with the goal of facilitating and enhancing the use of medaka as a model organism. NBRP Medaka is aiming to establish a first rate biological resource with the highest possible levels of accessibility and ease of use.

Figure 3. Medaka provided from NBRP Medaka

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DIVISION OF MOLECULAR ENVIRONMENTAL ENDOCRINOLOGY

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Synthetic chemicals found in the environment have the capacity to disrupt the development and function of the endocrine system in both wildlife and humans. This has drawn public concern since many of these chemicals may bind to estrogen receptors (ERs) 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.

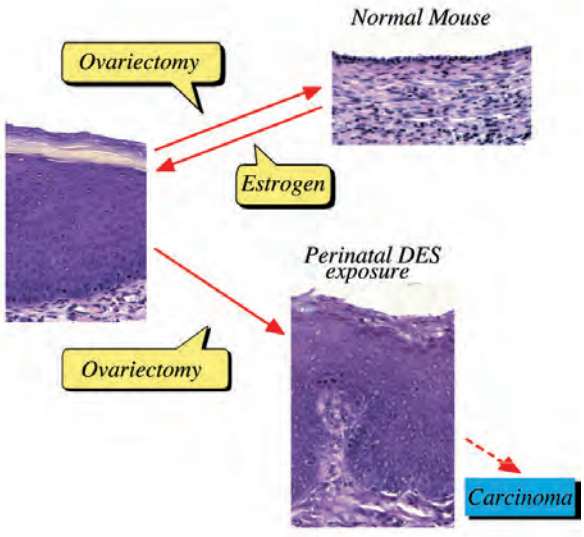

Figure 1. Scheme of estrogen-dependent and -independent vaginal epithelial cell proliferation in mice induced by perinatal estrogen exposure.

Ⅰ. Perinatal estrogen exposure induces persistent changes in reproductive tracts

The emerging paradigm of the "embryonic/fetal origins of adult disease" provides a powerful new framework for considering the effects of endocrine disrupters on human and animal health. In 1971, prenatal diethylstilbestrol (DES) exposure was found to result in various abnormalities of the reproductive tract in women and this syndrome was named the DES syndrome. Similar abnormalities have been demonstrated in experimental animals exposed perinatally to estrogens: for example, developmental estrogen exposure in mice induces persistent proliferation of vaginal epithelial cells. We found that the persistent changes in the vagina in mice exposed neonatally to estrogens result from the persistent phosphorylation of erbB2 and estrogen receptor α (ERα), sustained expression of EGF-like growth factors and phosphorylation of JNK1, IGF-I receptor and Akt. Recently, we found that Wnt 4, notch1, notch 3, and other genes also show persistent expression changes in neonatally DES-exposed mouse vaginae. Currently, we are analyzing the methylation status of genes showing altered expression in the mouse vagina. The number of DES-induced genes in the mouse vagina during the critical developmental exposure window was smaller than after the critical period.

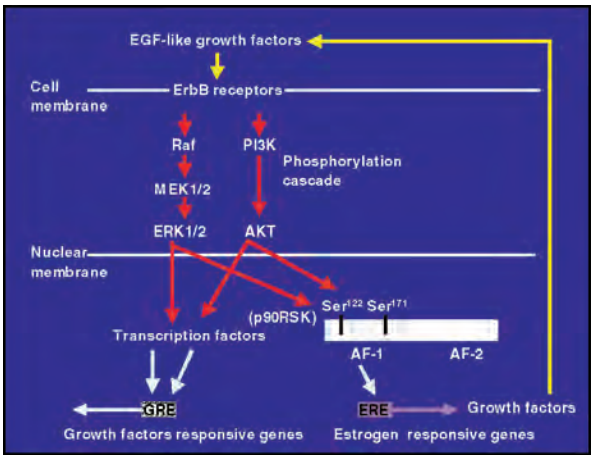

Figure 2. A hypothetical model for the estrogen-independent ER activation pathway in mouse vaginae.

Ⅱ. Microarray analysis of estrogen responsive genes

To understand the mechanisms through which estrogenic chemicals act on mouse reproductive organs, data documenting the temporal and spatial expression patterns of estrogen-responsive genes is essential. A large number of genes affected by estrogen treatment were identified in tissues of wild-type mice using a microarray approach. For most of the identified genes, expression was induced by 17 β-estradiol (E_2) in a dose-dependent manner. Subsequently, several environmental (xeno)estrogens were tested and

characteristic gene expression patterns were observed for each compound tested; these patterns were distinct from that obtained following E_2 exposure. We also found that xenoestrogenic chemicals and dioxin have distinct effects on the liver as well. Therefore, possible tissue-specific effects should be considered when elucidating the distinct effects of various EDCs.

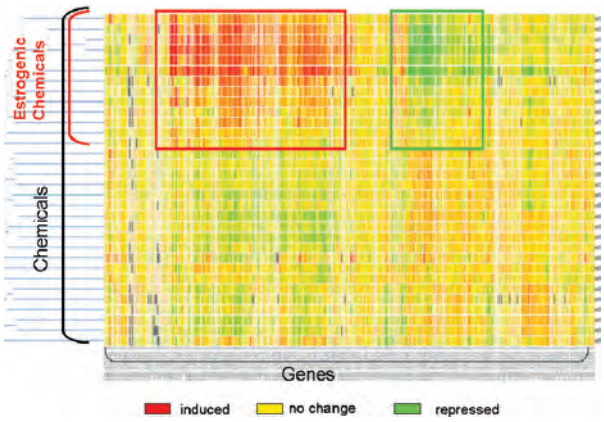

Figure 3. Scatter plot of average expression levels in control and chemical-treated uterus

Ⅲ. Steroid hormone receptors of reptiles, amphibians and fishes

Steroid and xenobiotic receptors (SXR) have been cloned from various animal species (fish, amphibian, reptiles, birds and mammals) by our group and we have demonstrated species-specific differences in their responses to various environmental and endogenous chemicals (receptor gene zoo). Thus, simple predictions of chemical effects based on data from a few established model species are not sufficient to develop real world risk assessments. ER and ER-like genes have been cloned from various animal species including rockshell, *Amphioxus*, lamprey, lungfish, sturgeon, roach, stickleback, mosquitofish, mangrove *Rivulus*, Japanese giant salamander, newt, *Silurana tropicalis*, American alligator, Nile crocodile, freshwater turtle, various snakes and vultures. Functional studies showed that the rockshell ER-like sequence does not bind estrogen but exhibits ligand-independent transactivation, whereas lamprey ER exhibited ligand-dependent transactivation, proving that primitive vertebrates, such as the Agnatha, have a functional ER.

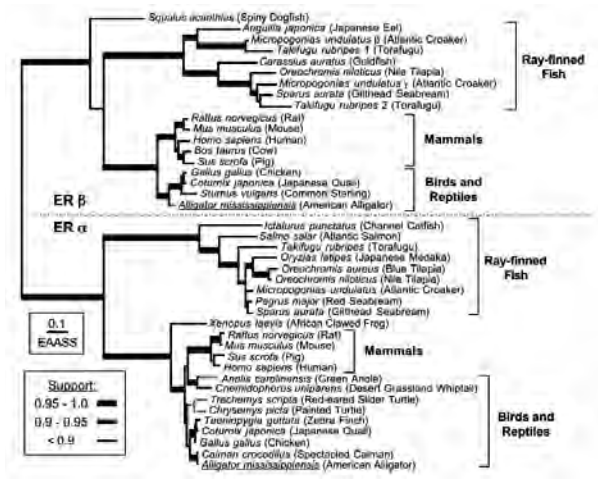

Figure. 4 Evolutionary relationships of estrogen receptor sequences

Ⅳ. Male production in Daphnids by juvenile hormones

Daphnia magna has been used extensively to evaluate the organism- and population-based responses of invertebrates to pollutants by their inclusion in acute toxicity or reproductive toxicity tests. These tests, however, provide no information about the mode of action of the tested compounds. Therefore, we applied an ecotoxicogenomic assessment of *D. magna*. We established a *Daphnia* EST database and developed an oligonucleotide-based DNA microarray with high reproducibility. Exposure of *D. magna* to several chemicals resulted in characteristic gene expression patterns that are chemical-specific, indicating that the established DNA microarray can be used for the classification of toxic chemicals as well as for the development of a mechanistic understanding of chemical toxicity in a common freshwater organism. *D. magna* reproduce asexually (parthenogenesis) when they are in an optimal environment for food, photoperiod and population density. Once environmental conditions become sub-optimal, they alter their reproductive strategy from asexual to sexual reproduction. Chemicals are able to affect sex determination of *D. magna* and we observed that juvenile hormone agonists (pesticides), for example, induce the production of male offspring. The molecular basis of environmental sex determination is largely unknown in these organisms. Therefore, we isolated sex determination-related genes to understand the molecular mechanisms of this phenomenon in *Daphnia*. DM-domain genes are well known as sex-related genes. We identified four DM-domain genes: DMRT11E, DMRT93B, DMRT99B and DSX. Quantitative gene expression analysis in daphnid gonads revealed that DMRT93B and DSX were expressed only in the testis. Recently, we have developed a method to inject silencing genes into *D. magna* embryos which will allow us to study gene function in more detail in this species.

Ⅴ. Gene zoo

We are establishing cDNA library banks of animal species in collaboration with the University of Pretoria, South Africa, the University of Florida, USA, San Diego Zoo, USA, 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 'X' 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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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 the 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 2006a).

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, which regulate the width of leaves, and the *ROTUNDIFOLIA3* (*ROT3*) and *ROT4* genes, which regulate the length of leaves. *AN* and *ROT3* genes control cell shape while *AN3* and *ROT4* genes regulate cell numbers in leaves (reviewed in Tsukaya 2006a). In addition to the polar-dependent leaf shape control, we have focused on the mechanisms of organ-wide control of leaf size, which are reflected in the 'compensation' phenomenon (Tsukaya 2006a). Furthermore, we have isolated and analyzed mutants with leaf polarity defects (*drl*: Cho et al. 2007) and leaf epidermis formation defects (*ale2*: Tanaka et al. 2007). Additionally, the accumulation of knowledge on the basic mechanisms of leaf shape control have enabled us to conduct Evo/Devo studies of the mechanisms behind leaf-shape diversity. Below is an overview of our research activities and achievements during 2007.

1-1 Polar growth of leaves in A. thaliana

AN is a member of *CtBP-BARS* gene family reported from animal genomes (reviewed in Tsukaya 2006b). Gene products of this gene family are very curious: they act as co-repressors in nuclei as a form of CtBP and also as Golgi regulators as BARS. Is AN a functional homolog of CtBP or of BARS? Our swapping experiments and two-hybrid analysis clearly showed that AN does not have any of the molecular functions of CtBP in *Drosophila melanogaster* (Stern et al. 2007). On the other hand, our detailed analysis of intracellular localization suggested that AN have a unique role (or roles) in Golgi-related function. Further analyses of AN functions are ongoing.

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

We have recently started to try to understand the genetic basis of the development of unifacial leaves that are known only 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 for the establishment of unifacial and normal bifacial leaves will provide good clues as to how leaf shape is diversified. For such purposes, comparative molecular-genetic and anatomical analyses between unifacial and bifacial leaf development have been recently undertaken using members of the genus *Juncus*.

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* show that a compensatory system (or systems) acts in leaf morphogenesis and an increase in cell volume might be triggered by a decrease in cell number (Tsukaya 2006a). Thus, leaf size is, at least to some extent, regulated at the organ level by the compensatory system or systems. 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.

As a result, we have succeeded in isolating specific mutants for number or size of leaf cells (Fujikura et al. 2007a, b). Moreover, we have isolated a number of new mutants that exhibit typical compensation syndrome, namely, decreased number of cells and increased cell volume: *fugu* (Ferjani et al. 2007). Combining these mutants as a resource, we have revealed that: (1) compensation syndrome results from the enhancement of a particular set of cell-expansion pathways which is required for normal leaf expansion; (2) compensation does not depend on the enhancement of endoreduplication; (3) compensation involves at least three different expansion pathways; and (4) compensation is not caused by the uncoupling of cell division from cell expansion at all. Further analyses on the mechanisms of compensation are in progress.

1-4 Size control of leaves and ploidy level

Why does a high-ploidy level cause increased cell/leaf size? The isolation of mutants with endoreduplication defects supplied us with a good clue for understanding the linkage mechanisms between the ploidy level and cell/organ size. *bin4* mutants cannot make their leaf cells higher than 8C and show severe dwarfism. If this dwarfism - and smaller leaves - are the direct result of lower ploidy level, tetraploidization of this mutant should suppress the dwarfism. We found that the tetraploid *bin4* showed partial recovery in cell/organ size, and this fact showed that ploidy level is a key factor for the determination of cell/organ size (Breuer et al. 2007). In addition, we found that the endoreduplication mutational defects were responsible for a curious enhancement of the effects of tetraploidization in terms of cell-size increase. Further studies of tetraploids of the other mutants are now ongoing to determine the precise relationship between the ploidy level and cell/organ size.

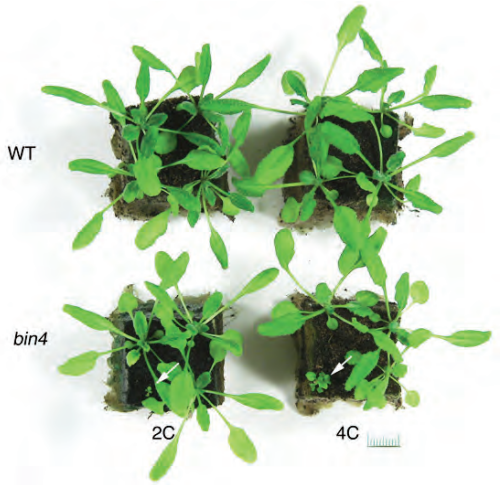

Figure 1. Severe dwarfism exhibited by the *bin4* mutant is partially recovered by tetraploidization. Arrows: homozygous individuals of the mutations. Bar, 1 cm.

Ⅱ. Biodiversity of leaf form

We are also interested in the biodiversity of wild plants. *Ainsliaea apiculata* is a perennial weed that is widely distributed in Japan. Some endemic varieties of this species, which are defined by characteristic leaf shapes, have been reported from Yakushima Island, Kyushu, Japan. We have re-examined the variations in the leaf shape of this species from Yakushima Island, in comparison with other localities in Japan, and found that the variations in the leaf shape were continuous with the other forms. Thus, these variants cannot be treated as distinct varieties. Yakushima Island was proved, however, to be a center locality of morphological variation in this species (Tsukaya et al. 2007a).

On the other hand, we have revealed that *Cayratia tenuifolia*, characterized by its leaf shape and flower/fruit characters, is a distinct species from the closely-related *C. japonica* (Okada et al. 2007). Cytological and molecular phylogenetic analyses of *Oxygyne shinzatoi* were also carried out (Tsukaya et al. 2007b; Yokoyama et al. 2008).

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- Stern, M.D., Aihara, H., Cho, K.-H., Kim, G.-T., Horiguchi, G., Roccaro, G.A., Guevara, E., Sun, J., Negeri, D., Tsukaya, H., and Nibu, Y. (2007). Structurally related *Arabidopsis* ANGUSTIFOLIA is functionally distinct from the transcriptional corepressor CtBP. Dev. Genes Evol. *217*, 759-769.
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- Tsukaya, H., Tsujino, R., Ikeuchi, M., Isshiki, Y., Kono, M., Takeuchi, T., and Araki, T. (2007a). Morphological variation in leaf shape in *Ainsliaea apiculata* with special reference to the endemic characters of populations on Yakushima Island, Japan. J. Plant Res. *120*, 351-358.
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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 mediated by phototropins and one of the simplest model systems to study photomorphogenesis. Because the phenomenon is cell autonomous, whole processes from photoperception 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 we are studying it because chloroplast movement and photomorphobgenesis share the same photoreceprors.

Ⅰ. Chloroplast relocation movement

We use the fern *Adiantum capillus-veneris* as a model plant 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 movement and for analyses of the genes' functions.

1-1 Velocity of signal transfer

Phototropins (phot1and phot2) and a 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 to chloroplasts remained to be clarified. We therefore studied the velocity of signal transfer using *Adiantum* gametophytes to find a clue to the identity of any possible candidates for the signal. The velocity in a long protonemal cell is confirmed to be different between the signal moving 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 both in the red and blue light-induced chloroplast movement. On the other hand, the velocity of signal transfer in two-dimensional gametophyte cells is the same (approximately 0.7 μm min^{-1}) irrespective of the direction of signal transfer or the wavelength (either red or blue light) that induces chloroplast movement. The velocities are slower than that of cytoplasmic streaming. The velocity of the signal transfer was very slow compared to our expectations, and we do not have any clear idea at the moment what is the signal and what is the mechanism of the transfer.

1-2 Chloroplast movement under cold condition

Chloroplasts movement was induced at about 4°C in *Adiantum* gametophytes. At low temperatures chloroplasts move to the anticlinal walls under either dark or light conditions. The position is similar to those of chloroplasts found in the dark or under strong light. Although the movement was even induced under darkness, it is not dark-induced movement, because the response could be induced in the mutant gametophytes defective of dark-induced chloroplast movement.

Ⅱ. Photoreceptor functions

2-1 Photoreceptors mediating nuclear movement in the fern *Adiantum*

In gametophyte cells of the fern *Adiantum capillus-veneris*, nuclei as well as chloroplasts change their position according to light conditions (Kagawa and Wada 1993, 1995). Nuclei reside on anticlinal walls in darkness and move to periclinal or anticlinal walls under weak or strong light conditions, respectively (Figure 1).

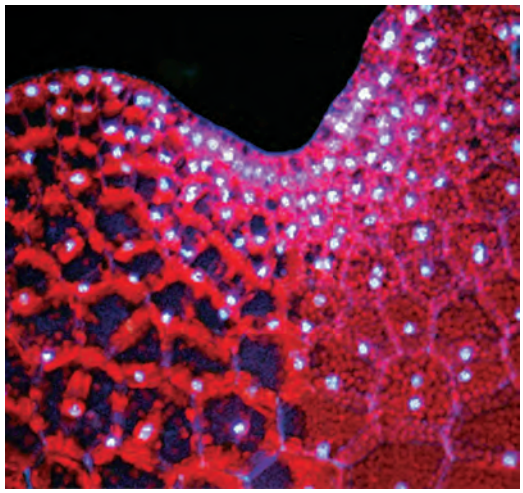

Figure 1. The left half of an *Adiantum* gametophyte cultured under weak white light was irradiated with strong white light to induce nuclear avoidance response and then fixed with glutaraldehyde and stained with DAPI. Nuclei irradiated with strong light moved to the anticlinal walls from the center of cells. The response was mediated by a blue light receptor, phototropin2 (Tsuboi *et al*. 2007).

This year we tried to identify the photoreceptor(s) that mediates nuclear movement in the gametophyte cells using photoreceptor mutants, *neo1* (neochrome1 defective), *phot2* (phototropin2 defective), and *neo1phot2* (Figure 2) and revealed that red and blue light-induced nuclear accumulation movement is mediated by neo1, and possibly phot1 and phot2, respectively, and that blue light-induced avoidance movement is mediated by phot2 (Tsuboi *et al*. 2007).

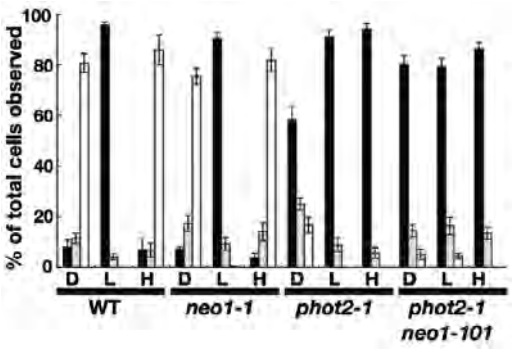

Figure 2. Positioning of nuclei of wild type, *neo1, phot2* and *neo1phot2* mutants in the dark and under weak or strong light conditions. Prothalli were dark-adapted for 1 day (D) or irradiated with weak white light (L: 20 - 30 mmol m-2 s-1) or strong white light (H: about 800 μmol m^{-2} s^{-1}) for 26 hrs. Percentages of nuclei at periclinal, anticlinal and intermediate positions are indicated with black bars, white bars, and gray bars, respectively.

It was also found that phot2 is necessary for dark positioning of nucleus (Tsuboi *et al*. 2007). It is curious that a photoreceptor is needed for a physiological response in the dark, but it is similar to the case of Arabidopsis chloroplast positioning wherein phot2 is indispensable in dark positioning. Thus, both the nuclear and chloroplast photorelocation movements may share common photoreceptor systems.

2-2 Aureochrome as a blue light receptor in *Vaucheria*

Vaucheria is a member of the group of stramenopile algae, which includes brown algae and diatoms and shows blue light responses, such as phototaxis, phototropism, photomorphogenesis and chloroplast relocation (Kataoka *et al*. 1975). In collaboration with Professor Kataoka at Tohoku University, we tried to find a blue light receptor(s) that might involve the blue light responses. We cloned two genes containing sequences of one basic-region/leucine-zipper (bZIP) domain and one light-oxygen-voltage (LOV) domain that binds a flavin mononucleotide (FMN). Its bZIP domain binds the target sequence TGACGT. We named them AUREOCHROME1 and 2 (aureo1 and aureo2). RNAi of AUREO2 induces sex organ primordial instead of branches, implicating AUREO2 as a sub-switch to initiate the development of a branch, but not a sex organ. These are the photoreceptors for the blue light-induced branching of *Vaucheria frigida*. AUREO sequences are also found in the genome of the marine diatom but are not in green plants (Takahashi *et al*. 2007).

2-3 Binding proteins to a putative blue light receptor in Arabidopsis

PAS/LOV protein (PLP) is a putative blue light receptor with a PAS domain at its N-terminal region and an LOV domain at its C-terminal region (Crosson *et al*. 2003). PLP interacting proteins were isolated by the yeast two-hybrid system and were studied in collaboration with Dr. Kiyosue at Kagawa University. Those were VITAMIN C DEFECTIVE 2 (VTC2, Jander *et al*. 2002), and VTC2 paralog (VTC2L) and BEL1-LIKE HOMEODOMAIN 10 (BLH10) (Hackbusch *et al*. 2004). The interaction of PLPA with VTC2L was weakened at the intensity of >100 mmole m^{-2} s^{-1} of blue light, while that of PLPB with VTC2L was undetectable at that intensity (Ogura *et al*. 2008).

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- Takahashi, F., Yamagata, D., Ishikawa, M., Fukamatsu, Y., Ogura, Y., Kasahara, K., Kiyosue, T., Kikuyama, M., Wada, M., and Kataoka, H. (2007). AUREOCHROME, a photoreceptor required for photomorphogenesis in stramenopiles. Proc. Natl. Acad. Sci. USA *104*, 19625 -19630.
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- Suetsugu, N., and Wada, M. (2007). Phytochrome-dependent photomovement responses mediated by phototropin family proteins in cryptogam plants. Photochem. Photobiol. *83*, 87-93.
- Suetsugu, N., and Wada, M. (2007). Chloroplast photorelocation movement mediated by phototropin family proteins in green plants. Biological Chemistry *388*, 927-935.
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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 appropriate photoenvironments. Our research is aimed at the elucidation of photoreceptive and signal transduction mechanisms of the light responses in microorganisms. This approach has led us to the discovery, characterization, and application of a remarkably unique light sensor molecule as described below.

I. Photoactivated Adenylyl Cyclase (PAC)

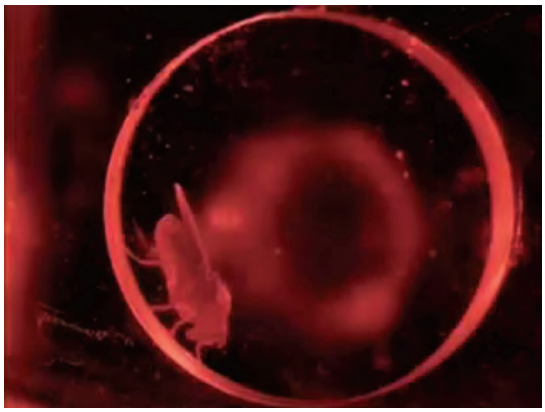
In 2002, we found a novel blue-light receptor with an effector role from *Euglena* (Iseki *et al*., Nature *415*, 1047-1051, 2002): *Euglena gracilis*, a unicellular flagellate (Figure 1), shows blue-light type photomovements. The action spectra indicate the involvement of flavoproteins as the photoreceptors 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. *Euglena gracilis*, a unicellular flagellate alga

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

In collaboration with Max-Planck-Institut für Biophysik, (Frankfurt) and other German groups, expression of PAC in cells was performed which allowed the manipulation of cAMP with exquisite spatiotemporal control. We functionally expressed PACs in two popular expression systems, *X. laevis* oocytes and HEK293 cells. Moreover, transgenic *D. melanogaster* flies demonstrated functional PAC expression by showing blue light–induced behavioral changes as described below.

To determine the reliance and kinetics of the light-induced change in behavior, the the grooming reflex15 was analysed: when covered with a fine powder, fruit flies instantaneously display vigorous and continuous grooming activity lasting up to 30 min. Monitoring this behavior for a total time of 5 min with irradiation alternating between dim white light and intense blue light for 1 min each, revealed high grooming activity in wild-type flies irrespective of stimulation by light (Figure 2). In contrast, neuronal expression of PAC (elav-Gal4/UAS-PAC) resulted in hyperactivity and a substantial decline in grooming activity under blue-light stimulation. When irradiation was switched back to dim white light, thus turning off blue light–induced PAC activity, flies returned to grooming behavior within several seconds These results demonstrate that transgenic expression of PAC in fruit flies results in a functional protein that is rapidly and reversibly activated by blue light. Moreover, the fast action observed at the on- and offset of irradiation demonstrates the feasibility of rapid control of cAMP levels in a freely moving animal.



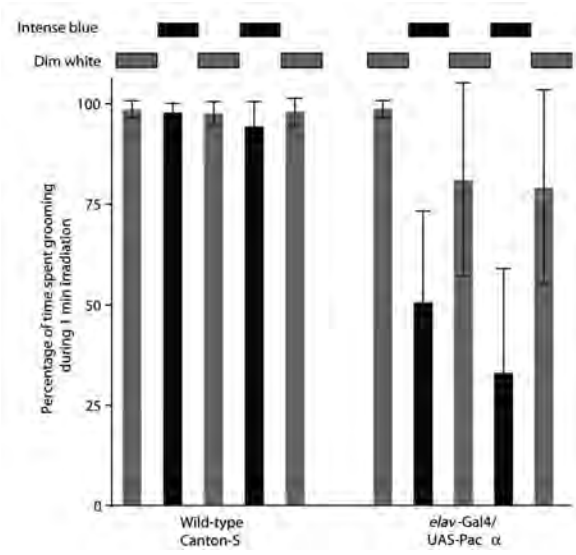


Figure 2. Photostimulation of PAC alters behavior in freely moving *D. melanogaster.*

1-2 Functional transplant of photoactivated adenylyl cyclase (PAC) into *Aplysia* sensory neurons.

In collaboration with Prof. T. Nagahama, (Faculty of Pharmaceutical Science, Toho University), functional transplant of photoactivated adenylyl cyclase (PAC) into *Aplysia* sensory neurons was performed to explore whether PAC can produce cAMP in the neurons by light stimulation. Serotonergic modulation of mechanoafferent sensory neurons in *Aplysia* pleural ganglia has been reported to increase intracellular cAMP level and promotes synaptic transmission to motor neurons by increasing spike width of sensory neurons. When cAMP was directly injected into the sensory neurons, spike amplitude temporarily decreased while spike width temporarily increased. We therefore explored these changes as indicators of appearance of the PAC function. PAC or the PAC expression vector (pNEX-PAC) was injected into cell bodies of sensory neurons. Spike amplitude decreased in both cases and spike width increased in the PAC injection when the neurons were stimulated with light (Figure 3), suggesting that the transplanted PAC works well in *Aplysia* neurons. These results indicate that we can control cAMP production in specific neurons with light by the functional transplant of PAC.

Publication List

〔Original papers〕

- Nagahama, T., Suzuki, T., Yoshikawa, S., and Iseki, M. (2007). Functional transplant of photoactivated adenylyl cyclase (PAC) into *Aplysia* sensory neurons. Neurosci. Res. *59*, 81-88.
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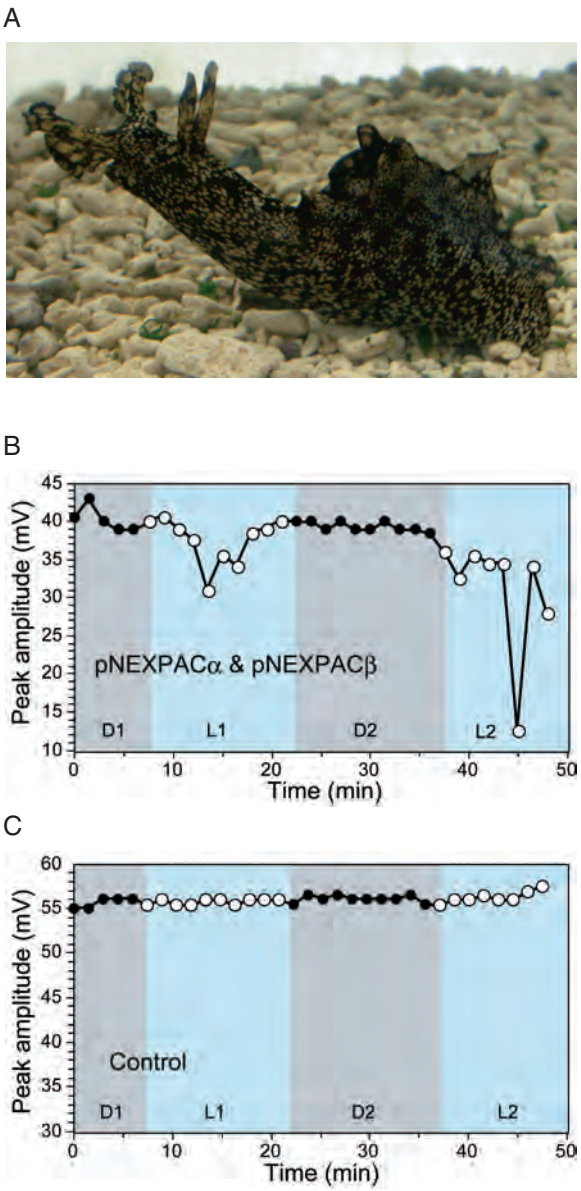

Figure 3. Effect of blue light on spike amplitude in the *Aplysia* sensory neurons into which plasmid vectors carrying PAC cDNAs were injected

DIVISION OF THEORETICAL BIOLOGY

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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 possible KaiC behavior 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*.

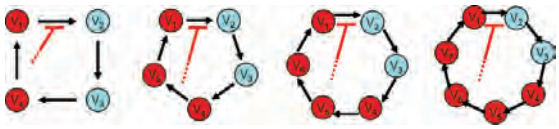


Figure 1. Schematic representation of closed circuit model and the condition for the possible oscillation by inhibition of the transition from state V_1 to V_2. Red-colored state (V_1, V_4-V_7) indicate that inhibition from the states can destabilize the system and possibly cause oscillation. Inhibition from the blue-colored state never induces oscillation.

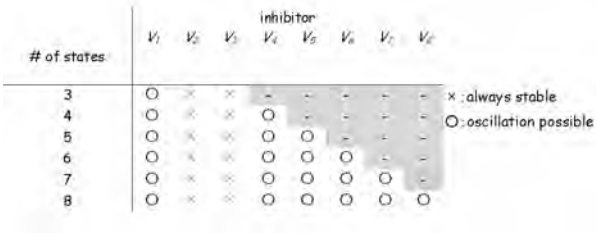


Table 1. Summary of the results of the general state transition model with conservation of molecules. The system could oscillate when the inhibiting state is more than two steps ahead of the inhibited reaction (from V_1 to V_2). If the inhibiting state is less than three steps ahead of the reaction,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

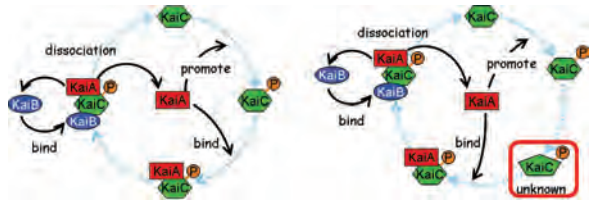


Figure 2. Schematic representations of "Basic model" (left) and "Multiple-phosphorelation-state model" (right). The basic model is determined from experimental results. It was proven that the model never shows oscillation. The multiple-phosphorelation-state model was developed based on the mathematical 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. This prediction has recently been confirmed by experimental methods.

Ⅱ. Mathematical models for pattern formation of dendrites of neurons

Dendrite is a part of a neuronal cell 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 the 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 the 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.

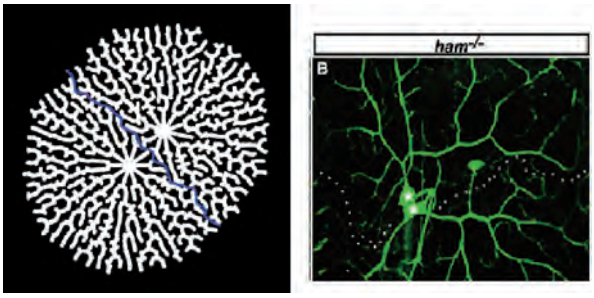


Figure 3. Examples of obtained dendrite patterns by computer simulation of the model (left) and the correspondence observed in an experiment (Grueber et al., 2003; Moore et al., 2002). Dendrites from the cells spread and cover the space. However, they never interfere with each other.

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 “cell compartment model”. 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. The model can represent characteristic features of the spatial regulation observed in actual dendrites: tiling and regeneration. We analyzed behavior of the model and determined the conditions for the dendrite formation. This work has been done in collaboration with Dr. T. Uemura and Dr. K. Sugimura of Kyoto University.

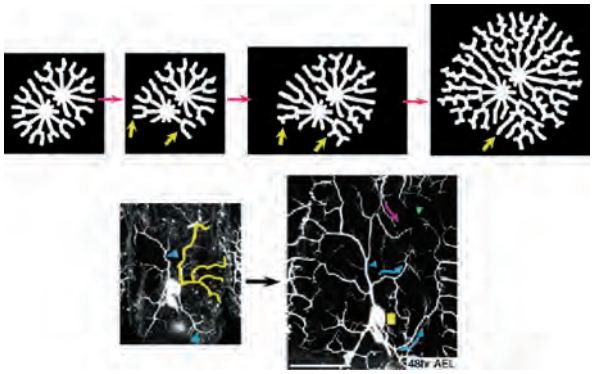


Figure 4. Regeneration after artificial severing. Patterns obtained by computer simulation of the model (up) and the corresponding pattern obtained in an experiment (down).

Publication List

〔Original papers〕

- Ishihara, S., Otsuji, M., and Mochizuki, A. (2007). Transient and steady state of mass-conserved reaction-diffusion systems. Phys. Rev. E *75*, 015203.
- Otsuji, M., Ishihara, S., Co, C., Kaibuchi, K., Mochizuki, A., and Kuroda, K. (2007). A Mass Conserved Reaction-Diffusion System Captures Properties of Cell Polarity. PLoS Comput. Biol. *3*, 1040-1054.
- Sugimura, K., Shimono, K., Uemura, T., and Mochizuki, A. (2007). Self-organizing mechanism for development of space-filling neuronal dendrites. PLoS Comput. Biol. *3*, 2143-2154.

LABORATORY OF GENOME INFORMATICS

Assistant Professor
UCHIYAMA, Ikuo

The accumulation of biological data has recently been accelerated by various high-throughput "omics" technologies such as genomics, transcriptomics, proteomics, and so on. The field of genome informatics is aimed at utilizing this data, or finding some principles behind the data, for understanding complex living systems by integrating the data with current biological knowledge using various computational techniques. In this laboratory we focus on developing computational methods and 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 user's 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. For researchers who are interested in ongoing genome projects, MBGD also provides a service called "My MBGD," 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.

The database now contains more than 500 published genomes and the number continues to grow. MBGD is available at http://mbgd.genome.ad.jp/.

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

As 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.

As the number of completed genomic sequences grows, comparison among closely related as well as distantly related genomes has become more important for understanding the function and evolution of various genomes. For evolutionary analysis of a target set of related organisms (ingroup), we often need another set of organisms outside of that group (outgroup) to correctly infer evolutionary processes. To incorporate this concept into the ortholog analysis, we have enhanced the DomClust algorithm to impose constraints on the resulting orthologous groups such that the outgroup species should come outside of the ingroup species (Figure 1). The resulting table has a nested structure when a duplication event occurs within the ingroup lineage.

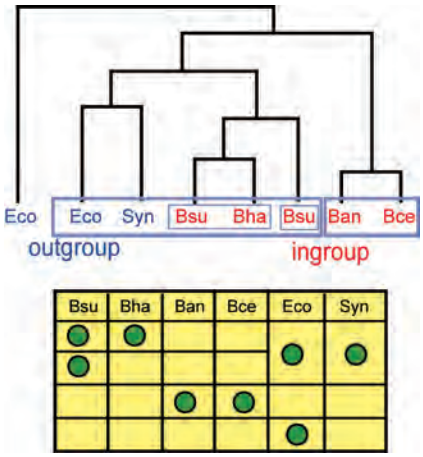


Figure 1. Converting from a gene tree (top) to a nested ortholog table (bottom)

Ⅲ. Identification of core structures conserved among moderately related microbial genomes

A growing body of evidence supports the theory that both horizontal transfer and vertical transfer have played significant roles in prokaryotic evolution. Despite the complexity of evolution suggested from these observations, it can be argued that prokaryotic phylogeny can still be

inferred using a certain subset of genes ("core genes") that have mainly transferred vertically throughout the evolutionary process. We are trying to identify a common "core structure" of 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 (Figure 2).

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 1400 genes for Bacillaceae and 1900 genes for Enterobacteriaceae. Despite the difference in the overall proportions of the core genes between these datasets, the proportion of the core genes in each functional category primarily exhibit a similar tendency: functional categories related to primary metabolism, genetic information processing, and cellular processes generally contain a higher proportion of core genes, while categories of membrane transport, signal transduction and secondary metabolism contain a lower proportion of core genes. In addition, it also turned out that these core structures contain most of the essential genes identified in *Bacillus subtilis* and *Escherichia coli*, respectively.

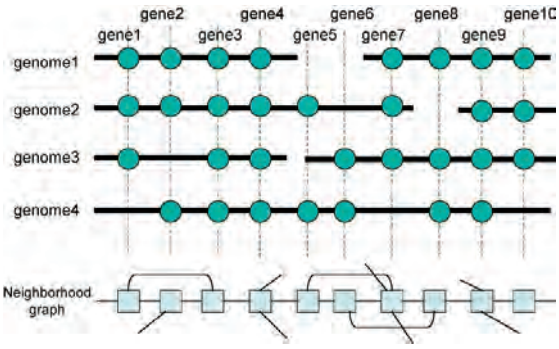


Figure 2. Construction of core genome alignment based on gene order conservation

Ⅳ. Research environment for comparative genomics

We have been developing a new system named RECOG (Research Environment for COmparative Genomics), which aims to extend the current MBGD system by incorporating the above-described approaches including the ingroup/outgroup distinction in ortholog grouping and the core structure extraction among related genomes. In addition, the RECOG system has several advanced features that allow users to perform more flexible phylogenetic pattern analyses.

The entire RECOG system employs client-server architecture. The RECOG server program has been developed based on the MBGD server and contains database construction protocol in MBGD including all-against-all similarity search calculation, as well as the extended version of the DomClust program. Unlike MBGD, however, users can install the RECOG server on their local machines to analyze their own genomic data. Alternatively, users can connect to the public RECOG server to analyze publicly available data.

The RECOG client program is a Java application that runs on a local machine by receiving data from any available RECOG server. The main window of the RECOG client consists of three parts: taxonomic tree viewer (left), ortholog table/phylogenetic pattern map viewer (center) and gene information viewer (right) (Figure 3). In the taxonomic tree viewer, users can specify either a set of organisms to be analyzed (as ingroup or outgroup), or conditions to filter phylogenetic patterns (i.e. presence or absence of each gene in each genome) to be displayed. The ortholog table viewer displays the entire ortholog table. By semantic zooming functionality, users can see from the entire picture of the phylogenetic pattern map to the full details of the ortholog table. In the gene information viewer, users can see detailed information about specified orthologous groups and genes belonging to each of the groups. Several sorting and filtering functions have been implemented for modifying the display of the ortholog table viewer, including sorting by functional category and gene name or by gene order of a specified genome, and filtering by keywords or by phylogenetic pattern conditions.

Figure 3. A snapshot of the main window of the RECOG system

Publication List

〔Original paper〕

● Uchiyama, I. (2007). MBGD: a platform for microbial comparative genomics based on the automated construction of orthologous groups. Nucleic Acids Res. *35*, D343-D346.

LABORATORY FOR SPATIOTEMPORAL REGULATIONS

Associate Professor
NONAKA, Shigenori

Technical Staff	KAJIURA-KOBAYASHI, Hiroko
NIBB Research Fellow	ICHIKAWA, Takehiko
Visiting Scientist	KANDA, Rieko
Secretary	KAMIYA, Akemi

In spite of superficially bilateral symmetry, our bodies are highly asymmetric along the left-right (L-R) axis, for example in 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 'the node', with hundreds of cilia moving in a clockwise rotational manner (Figure 1; Nonaka *et al.*, Cell *95*, 829-837,1998). The sum of the vortical motions of the cilia, however, generates a leftward flow of the surrounding fluid rather than a vortex. Because of the posteriorly tilted rotation axis, the node cilia can generate leftward force without pre-existing left-right asymmetry. (Figure 2; Nonaka *et al.*, PLoS Biology *3*, e268, 2005).

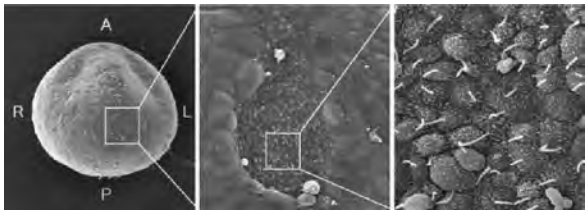


Figure 1. Left, ventral view of a 7.5-day mouse embryo. Middle, the node. Right, node cilia.

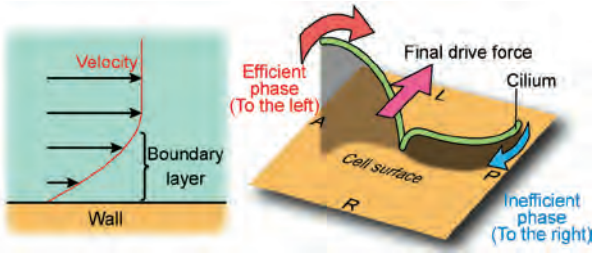


Figure 2. Mechanism to generate leftward flow by a rotating cilium. Left, fluid very close to the wall is generally reluctant to move (surface effect). Right, this constraint negatively affects water-dragging efficiency by the cilium. Given that the cilium rotates clockwise with a posteriorly tilted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

The leftward flow, known as nodal flow, determines subsequent L-R development. This idea has been confirmed by two experiments. Firstly, mutations without motile cilia in the node result in randomized L-R asymmetry. Secondly, and more importantly, embryos raised in a rightward artificial flow of culture medium develop reversed L-R asymmetry (Figure 3; Nonaka *et al.*, Nature *418*, 96-99, 2002).

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

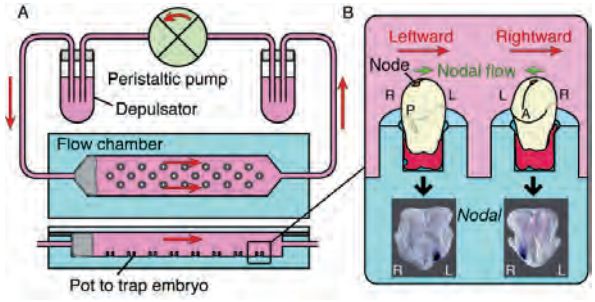


Figure 3. Flow culture experiment. A, A peristaltic pump and depulsators supply constant fluid flow in the chanber (red arrows). B, Embryos held in the pots receive the pump-driven flow on their surface. If the pump-driven flow reverse intrinsic nodal flow (green arrows), expression of nodal, a master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

Ⅱ. New microscopy

Long-term live imaging techniques of early mouse embryos promise to be extremely useful for our analyses of L-R development as well as for broader research interests. For this purpose, we are working to introduce a digital scanned light-sheet microscope (DSLM, Figure 4) that has been developed by Dr. Ernst Stelzer in European Molecular Biology Laboratory (EMBL).

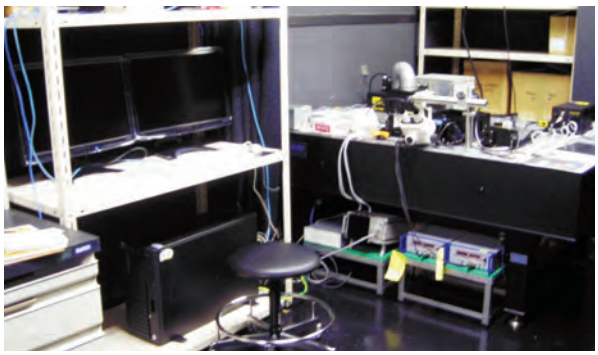


Figure 4. DSLM in construction

STRATEGIC PLANNING DEPARTMENT

Chair
NAGAHAMA, Yoshitaka

Vice-Chairs	UENO, Naoto
	NISHIMURA, Mikio
Professor	WADA, Masamitsu
Associate Professor	KODAMA, Ryuji
Postdoctoral Fellow	KURATA, Tomoko
Technical Assistants	MUKOHDA, Yasuyo
	OTA, Misaki
	TANAKA, Megumi
	EMDE, Jason R.

The Strategic Planning Department was founded in April 2005 as a central office for the efficient management of cooperational activities with other organizations, the distribution of scientific information to the public, planning and managing conferences, workshops and other extramural activities. The department also assists the director-general in preparing for NIBB's evaluation procedure and in planning a long-range strategy for the institute.

The main activities of the Department in 2007

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 at NIBB (Table 1)

2) Management of education-related programs
Internship Program 2007
The First International Practical Course (Jan. 2007)

3) Press Release
News on scientific achievements are sent to newspaper and magazine reporters via e-mail and [a? our?]password protected web page. For some of the releases, we arranged a press conference.

4) Editing of publications
Introductory Pamphlet of NIBB (in Japanese)
Introductory Pamphlet of NIBB (in English)
Educational Booklet "Organisms sustaining research" (in Japanese)
Bulletin of NIBB 2007/08 (in Japanese, in preparation)
Annual Report of NIBB 2007 (in English, with proof reading)

5) Production of posters and leaflets
Design and distribution of posters for international conferences and advertisements for the graduate school's entrance examination (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)

Name	*Date*	*Title*	*Organizer*
5th Okazaki Biology Conference	March 11-16	Speciation and Adaptation -Ecological Genomics of Model Organisms and Beyond-	K. Shimizu I. Dworkin
6th Okazaki Biology Conference	December 2-8	Marine Biology	N. Satoh B.J. Swalla D.R. Levitan
5th NIBB-EMBL Meeting	May 24-26	Cell and Developmental Biology	N. Ueno S. Cohen

Table 1. International conferences managed by Strategic Planning Department in 2007

5th Okazaki Biology Conference | 6th Okazaki Biology Conference | 54th NIBB Conference | Introductory pamphlet (Japanese) | Introductory pamphlet (English) | Educational booklet

Figure 1. Examples of posters, pamphlets and abstract books produced by the Strategic Planning Department in 2007

Research Support Facilities

Head
NISHIMURA, Mikio

● Large Spectrograph Laboratory

Professor (Adjunct)	WATANABE, Masakatsu
Technical Staff	HIGASHI, Sho-ichi
	NAKAMURA, Takanori
Technical Assistant	ICHIKAWA, Chiaki
Secretary	ISHIKAWA, Azusa

● Tissue and Cell Culture Laboratory

Assistant Professor	HAMADA, Yoshio
Technical Assistant	TAKESHITA, Miyako

● Computer Laboratory

Assistant Professor	UCHIYAMA, Ikuo
Technical Staff	MIWA, Tomoki
	NISHIDE, Hiroyo
	NAKAMURA, Takanori
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 researchers, 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 (cf. Watanabe, M. *In* "CRC Handbook of Organic Photochemistry and Photobiology, 2nd ed.". pp. 115-1~115-16, 2004).

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

Publication List on OLS Collaboration

〔Original papers〕

- Arimoto-Kobayashi, S., Sakata, H., Mitsu, K., and Tanoue, H. (2007). A possible photosensitizer: Tabacco-specific nitrosamine, 4-(N-methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), induced mutations, DNA strand breaks and oxidative and methylative damage with UVA. Mutation Reseach *632*, 111-120.
- Bolige, A., and Goto, K. (2007). Phytochrome-like responses in *Euglena*: A low fluence response that reorganizes the spectral dependence of the high irradiance response in long-day photoperiodic induction of cell division. J. Photochem. Photobiol. B. *86*, 97-108.
- Bolige, A., and Goto, K. (2007). High irradiance responses involving photoreversible multiple photoreceptors as related to photoperiodic induction of cell devision in Euglena. J. Photochem. Photobiol. B. *86*, 109-120.
- Negishi, T., Kawai, K., Arakawa, R., Higashi, S., Nakamura, T., Watanabe, M., Kasai, H., and Fujikawa, K. (2007). Increased levels of 8-Hydroxy-2'-Deoxyguanosine in drosophila larval DNA after irradiation with 364-nm laser light but not with X-rays. Photochem. Photobiol. *83*, 658-663.
- Shihira-Ishikawa, I., Nakamura, T., Higashi, S-i., and Watanabe, M. (2007). Distinct responses of chloroplasts to blue and green laser microbeam irradiations in the centric diatom *Pleurosira laevis*. Photochem. Photobiol. *83*, 1101-1109.
- Xie, X., Shinomura, T., Inagaki, N., Kiyota, S., and Takano, M. (2007). Phytochrome-mediated inhibition of coleoptile growth in rice: age-dependency and action spectra. Photochem. Photobiol. *83*, 131-138.

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; 1.6TBx10), a high-performance cluster system (DELL PowerEdge 1850; 2CPUx(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 the PCs in each laboratory, as well as all of the above-mentioned service machines, are connected to each other by a local area network (LAN), which is linked to the high performance multimedia backbone network of the 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 glasshouses 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 the 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 during 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 of 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 continue 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 for 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. Collaborative 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 encourage and 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 1. A laboratory room for computational studies

In 2007, we conducted the arrangements for the international meetings on systems biology, which will be held in April 2008 in Barcelona as a collaborative effort with EMBL. We have also held some small meetings 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
	NARUSE, Kiyoshi
Technical Staff	HAYASHI, Kohji
	ICHIKAWA, Yoko
	TAKAGI, Yukari
Supporting Staff	YASUDA, Mie
	NOGUCHI, Yuji
	KAWAMURA, Motofumi
	KOBAYASHI-NISHIMURA, Keiko
	OKUDA, Tadayoshi
	AJIOKA, Rie
	NAKASHIMA, Machiko

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 four associate professors.

Technical staff and supporting staff develop and promote research-supporting activities. A state-of-the-art facility 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 2,500 m^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.

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

Figure 2. Large scale autoclaves for sterilization

In 2007, 5,170 mice were brought into the CTAP in the Yamate area, and 30,870 mice (including pups bred in the facility) were taken out from the CTAP from November 1, 2006 to October 31, 2007.

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 2007, 85 mice were brought into the CTAP in the Myodaiji area, and 1,573 mice (including pups bred in the facility) were taken out from the CTAP from November 1, 2006 to October 31, 2007.

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.

Figure 3. Breeding equipment for zebrafish

In 2007 (from November 1, 2006 to October 31, 2007), 4,292 medaka and zebrafish (150 eggs, 1,310 embryos and 2,832 adults) were brought to the facility and 27,611 medaka and zebrafish (26,694 fertilized eggs and 917 adults, including animals bred in the facility) were taken out from the CTAP. In a laboratory for chick embryos 7,275 fertilized chicken eggs were brought in and 7,275 fertilized eggs and chicken embryos were taken out from the CTAP. These animals were used for research activities in neurobiology and developmental biology.

In 2007 NIBB was selected as a core facility of the National BioResource Project of Medaka. We have provided the standard strains, the inbred strains, natural and induced mutants and the transgenic lines as well as the genomic resources (such as 15,000 non-redundant cDNA/EST clones and BAC/Fosmid clones) covering 90% of medaka genome for the scientific community. We have provided the medaka bioresources to several countries, including Germany, United Kingdom, France, Norway, USA, Thailand, China, Taiwan, and Japan.

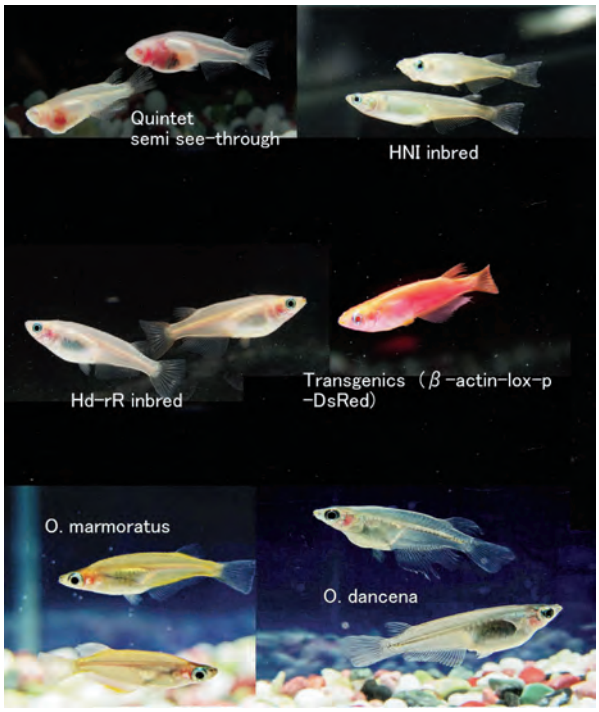


Figure 4. Medaka live resources provided from NBRP Medaka

Ⅲ. Academic activity

The associate professors of this center - E. Watanabe, T. Sasaoka, M. Tanaka and K. Naruse - are the principal investigators of the Laboratory of Neurophysiology, the Laboratory of Neurochemistry, the Laboratory of Molecular Genetics for Reproduction and the Laboratory of Bioresources, 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, the Laboratory of Molecular Genetics for Reproduction is studying the molecular mechanism of reproductive organ development and sex differentiation using mutagenized or transgenic medaka, while the Laboratory of Bioresources is studying the genome evolution, focusing mainly on the chromosome evolution among ray-finned fish and the evolution of sex-determination systems using medaka-related species. 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 Staff	MORI, Tomoko
	YAMAGUCHI, Katsushi
	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. A new Liquid Chromatograph/Q-TOF Mass Spectrometer (Waters Q-TOF Premier) was installed in 2007. The Center's instruments can be used by researchers from outside the institute upon proposal.

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 Analyzer (ABI PRISM 3130xl, 310)
Thermal Cyclers (Perkin Elmer PJ-9600, Takara TP-300, Biometra TGRADIENT)
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)
LC/Q-TOF MS (Waters Q-TOF Premier)
Inductively Coupled Plasma Atomic Emission Spectrometer (Seiko SPS1200A)
Spectrofluorometers (Hitachi 850, F-4500, Simadzu RF-5000)
Spectrophotometers (Hitachi 330, 557, U-2001, Varian Cary5G, 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 Analyzers (Fujifilm LAS 3000 mini)
Fluorescence Bio Imaging Analyzer (Takara FMBIO Ⅱ)
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)

Figure 1. Protein sequencers

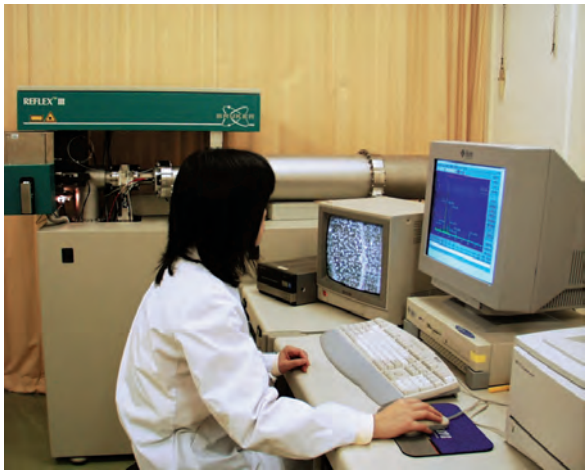


Figure 2. MALDI/TOF-MS

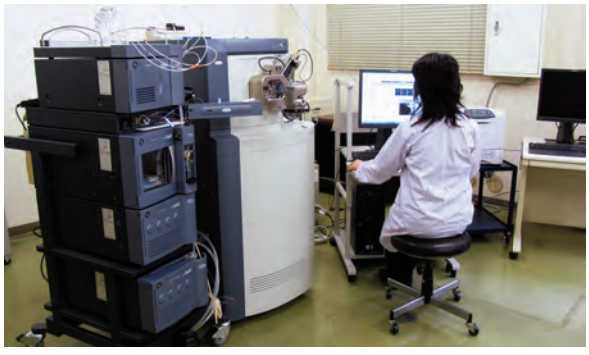


Figure 3. LC/Q-TOF-MS

TECHNICAL DIVISION

Head FURUKAWA, Kazuhiko

Common Facility Group

Chief MIWA, Tomoki

● Research Support Facilities

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

● Center for Analytical Instruments

Unit Chief MORI, Tomoko
Subunit Chief MAKINO, Yumiko
YAMAGUCHI, Katsushi
Technical Staff TAKAMI, Shigemi
Technical Assistants MORIBE, Hatsumi
KUROYANAGI, Asuka
IGHIKAWA, Mariko

● 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 Staff 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
Technical Staff MOROOKA, Naoki
SUMIKAWA, Naomi

● Environmental Biology

Subunit Chief MIZUTANI, Takeshi

Reception Office

Supporting Staff TSUZUKI, Shihoko
KATAOKA, Yukari
UNO, Satoko
KONDO, Yukie
MAEDA, Yuri

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 continually participate, through the Division, in self-improvement and educational activities to increase their capabilities and expertise in technical areas. Generally, technical staff members are attached to specific divisions so that they may contribute their special biological and biophysical knowledge and 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 maintained CFBI (Common Facilities Building I)-branch and LGER (Laboratory of Gene Expression and Regulation)-branch at the Myodaiji-Area. Ogawa, Sawada and Kamiya worked at the Yamate-Area. Kaneuji resigned from her job in March, while Kamiya moved to the Myodaiji-Area after March 2007.

The number of registrants and the number of users from January 2007 to December 2007 are presented in Table 1.

Users counted by the monitoring system going in and out of the controlled areas numbered 3,980 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.

Due to the recent decrease in the total number of users of the CRF and in order to prepare for a gene recombination experiment and an animal experiment using radioisotopes, we changed nuclides and the limit of radioactivity and prepared the rooms and applications for the genetic recombination and animal experiments using radioisotopes that began in August of 2007 (see Figure 3).

	Myodaiji-Area	Yamate-Area
registrants	142	111
users	66	42

Table 1. Numbers of registrants and users at Myodaiji-Area and Yamate-Area in 2007

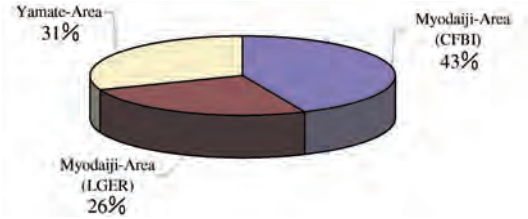


Figure 1. Percentage of users going in and out of each controlled area in 2007

	Myodaiji-Area		Yamate-Area	total
	CFBI-branch	LGER-branch		
users	1499	972	1092	3563
visitors	226	58	133	417
total	1725	1030	1225	3980

Table 2. Users and visitors who entered each controlled area in 2007

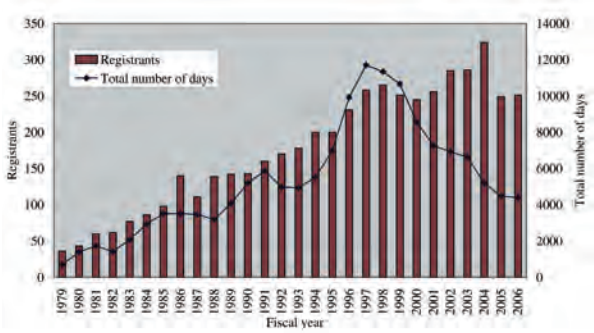


Figure 2. Annual changes of registrants and total number of days

	Myodaiji-Area		Yamate-Area	total
	CFBI-branch	LGER-branch		
^{125}I Receive	426	0	370	796
^{125}I Used	0	0	370	370
^{45}Ca Receive	0	0	-	0
^{45}Ca Used	0	0	-	0
^{35}S Receive	18500	0	166500	185000
^{35}S Used	0	0	159100	159100
^{32}P Receive	117750	1073000	1822250	3013000
^{32}P Used	449710	768690	1646135	2864535
^{14}C Receive	1850	0	0	1850
^{14}C Used	11360	0	0	11360
^{3}H Receive	223850	0	6475000	6698850
^{3}H Used	69828	0	6475000	6544828

Table 3. Balance of radioisotopes received and used (KBq) at each controlled area in 2007

Figure 3. These photographs show the gene recombination experiment and the animal experiment rooms at Myodaiji-Area.
Left: The gene recombination experiment room. This room has a safety cabinet and the biohazard protect type autoclave.
Right: Signs outside of the animal experiment and the gene recombination experiment room

The Fifth Okazaki Biology Conference
Speciation and Adaptation
-Ecological Genomics of Model Organisms and Beyond-

Organizing Chair : Kentaro Shimizu (University of Zurich, Switzerland)
Ian Dworkin (North Carolina State University, USA)

March 11 (Sun) – 16 (Fri), 2007

With the advent and widespread use of genomic tools, it is clear that the study of evolutionary genetics is undergoing a transformation. To promote the advancement and synthesis of the various fields of evolutionary and ecological genomics, the 5th Okazaki Biological Conference was held in March of 2007. The conference began with a social function to welcome 70 scientists from 10 countries and was followed by Plenary Lectures at the National Institute for Basic Biology (NIBB). Following the plenary lectures, the participants moved to Yamaha Resort Tsumagoi, where the scientists had opportunities to share their work during the oral platform sessions, in addition to poster sessions by many additional young scientists. The talks represented a broad overview of current areas of active research in ecological and evolutionary genomics, including studies of adaptation, speciation, domestication, genome duplication, co-evolution, canalization and theoretical population genetics.

Scientific sessions:

1. **Adaptive evolution**
2. **Genetics of speciation**
3. **Genome duplication and epigenetics**
4. **Artificial selection in domestication**
5. **Canalization, robustness and hidden genetic variation**
6. **Theory**
7. **Coevolution**

The real goal of such an intimate meeting was to encourage interaction between leading researchers and to facilitate interactions between scientists using different approaches but asking related questions and using similar model systems.. We believe that it is only when apparently disparate aspects of biology are integrated that we can hope to make real progress in our understanding of evolutionary mechanisms. During the conference, therefore, there was plenty of time for discussions on each of these subjects, both formally after each session with a panel discussion as well as more informally during social events and meals. The feedback from the participants on all aspects of the conference, including the scientific discussions, opportunities for developing new contacts, and the help of the supporting staff, was very positive.

Invited Speakers

AGUADE, Montserrat (Univ. Barcelona, Spain), ARAKI, Hitoshi (Oregon State Univ., USA), BARBASH, Daniel (Cornell Univ., USA), BRYSTING, Anne (Univ. Oslo, Norway), CAICEDO, Ana (Univ. Massachusetts, USA), COMAI, Luca (UC Davis, USA), COOP, Graham (Univ. Chicago, USA), DWORKIN, Ian (North Carolina State Univ., USA), FELIX, Marie-Anne (Institut Jacques Monod, France), GROSSNIKLAUS, Ueli (Univ. Zurich, Switzerland), HERMISSON, Joachim (Univ. Munich, Germany), HIRATE, Yoshikazu (Fred Hutchinson Cancer Research Center, USA), HOEKSTRA, Hopi (Harvard Univ., USA), IGIC, Boris (Univ. Illinois, Chicago, USA), KANAOKA, Masahiro (Univ. Washington, USA), KOBAYASHI, Yasushi (MPI for Developmental Biology, Germany), KUITTINEN, Helmi (Univ. Oulu, Finland), LAWTON-RAUH, Amy (Clemson Univ., USA), MABLE, Barbara (Univ. of Glasgow, UK), MACHADO, Carlos (Univ. Arizona, USA), OLSEN, Kenneth (Washington Univ., St. Louis, USA), PURUGGANAN, Michael (New York Univ., USA), RESCH, Alissa (NCBI, USA), RUTHERFORD, Suzannah (Fred Hutchinson Cancer Research Center, USA), SHIMIZU, Kentaro (Univ. Zurich, Switzerland), SHIMIZU-INATSUGI, Rie (Univ. Zurich, Switzerland), SHUSTER, Stephen (Northern Arizona Univ., USA), SIEGAL, Mark (New York Univ., USA), STEPHAN, Wolfgang (Univ. Munich, Germany), TANAKA, Kenta (Univ. Sheffield, UK), TIAN, Dacheng (Nanjing Univ., China), TSIANTIS, Miltos (Univ. Oxford, UK), VERGEER, Philippine (Univ. Leeds, UK), WIDMER, Alex (ETH Zurich, Switzerland), YANG, Hsiao-Pei (Cornell Univ., USA)

FUKATSU, Takema (AIST, Japan), HARUSHIMA, Yoshiaki (NIG, Japan), HASEBE, Mitsuyasu (NIBB, Japan), INNAN, Hideki (SOKENDAI, Japan), ISHIKAWA, Ryuji (Hirosaki Univ., Japan), IZAWA, Takeshi (NIAS, Japan), KUDOH, Hiroshi (Kobe Univ., Japan), MOCHIZUKI, Atsushi (NIBB, Japan), OKADA, Kiyotaka (Kyoto Univ., Japan), TAJIMA, Fumio (Univ. Tokyo, Japan), TAKAHASHI, Aya (NIG, Japan), TSUKAYA, Hirokazu (Univ. Tokyo, Japan), TSUMURA, Yoshihiko (FFRPI, Japan), YAHARA, Tetsukazu (Kyushu Univ., Japan)

The Sixth Okazaki Biology Conference
Marine Biology

Organizing Chair : Noriyuki Satoh (Kyoto University, Japan)
Billie J. Swalla (University of Washington, USA)
Don R. Levitan (Florida State University, USA)

December 2 (Sun) – 8 (Sat), 2007

We conceived this conference in order to bring together Marine Scientists from across the globe to discuss approaches to research in Marine Sciences. Most Marine Science is carried out at Marine Biological Laboratories, so we invited a number of Marine Laboratory Directors from Canada, France, Italy, Norway, Sweden and Japan to get an idea of what sort of studies are being conducted globally. Marine Biology covers an enormous number of research topics and we endeavored to bring together researchers in all of the main fields: reproductive biology, evolution and development, neurobiology, marine fungi/algae, marine genomics, behavior, ecology and conservation biology. This diverse group of speakers is united by their research on marine systems and the fact that they are internationally known for the quality of their research. We expected this group of brilliant leading Marine Scientists to find much in common to share about research techniques, approaches and future prospects and were delighted to see the many positive interactions that took place among the participants.

The diverse research presented at the OBC-6 Marine Biology Conference showed the vast potential of the marine environment in biological studies and highlighted current ecological problems that threaten the unprecedented biodiversity and untapped knowledge in the world's oceans. We heard many talks about the advances in genomics and marine exploration applied in novel and creative ways to understanding the marine environment and the biological organisms that live within it. The talks described research on a wide range of life forms from marine algae to invertebrates to vertebrates.

Towards the end of conference, it was suggested that an academic society for Marine Biologists in Japan be organized in the very near future. This may prove to be one of the most important achievements of this highly successful conference.

Scientific topics:

Reproduction
Evolution and Development
Neurobiology and Physiology
Marine Algae and Fungi
Marine Genomics
Behavior
Ecology

Speakers

BERNARDI, Giorgio (Stazione Zoologica Anton Dohrn, Italy), BOYEN, Catherine (CNRS & Univ. Paris 6, France), CHOURROUT, Daniel (Univ. Bergen, Norway), KLOAREG, Bernard (CNRS, Universit&eacute, France), KNOWLTON, Nancy (Smithsonian Inst., USA), LEVITAN, Don R. (Florida State Univ., USA), MATZ, Mikhail V. (Univ. Texas, Austin, USA), SARDET, Christian (CNRS UPMC, France), SPENCER, Andrew N. (Malaspina Univ.-College, Canada), SWALLA, Billie J. (Univ. Washington, USA), THORNDYKE, Michael C. (The Royal Swedish Academy of Sci., Sweden), VIZE, Peter D. (Univ. Calgary, Canada), WIDDER, Edith A. (Ocean Research & Conservation Assoc., USA)

AKASAKA, Koji (Univ. Tokyo, Japan), HARADA, Yoshito (Sugashima Marine Biological Laboratory, Japan), HIDAKA, Michio (Univ. Ryukyus, Japan), HOSHI, Motonori (Open Univ., Japan), INABA, Kazuo (Univ. Tsukuba, Japan), KIYOMOTO, Masato (Ochanomizu Univ., Japan), KUSAKABE, Takehiro G. (Univ. Hyogo, Japan), NAKAMURA, Masaru (Univ. Ryukyus, Japan), NARUSE, Kiyoshi (NIBB, Japan), NOZAKI, Masumi (Niigata Univ., Japan), OKAMURA, Yasushi (Okazaki Inst. Integrative Biosci., Japan), OTA, Kinya G. (RIKEN CDB, Japan), SAGA, Naotsune (Hokkaido Univ., Japan), SAKAMOTO, Tatsuya (Okayama Univ., Japan), SATO, Katsufumi (Univ. Tokyo, Japan), SATOH, Nori (Kyoto Univ., Japan), SAWADA, Hitoshi (Nagoya Univ., Japan), TAKEI, Yoshio (Univ. Tokyo, Japan), TSUKAMOTO, Katsumi (Univ. Tokyo, Japan), UEDA, Hiroshi (Hokkaido Univ., Japan), UENO, Naoto (NIBB, Japan), YASUI, Kinya (Hiroshima Univ., Japan)

The Fifth NIBB-EMBL Meeting
Cell and Developmental Biology

Organizing Chair : Naoto Ueno (NIBB)
Stephen Cohen (EMBL Heidelberg, Germany)

May 24 (Thu) – 26 (Sat), 2007

The 5th NIBB-EMBL joint meeting, entitled "Cell and Developmental Biology", was held in Okazaki on May 24-26, 2007. The scientific sessions were organized in consideration of the recent trend of understanding macro developmental phenomena at the micro/single cell level. During the two years since the 1st NIBB-EMBL joint meeting, "Developmental Biology", we have gradually realized the importance of understanding complex developmental processes as an integration of individual cell behaviors. During this most recent joint meeting, the cytoskeltal rearrangement underlying cellular morphogenesis and movement, the cellular mechanism of cell polarity, cell-to-cell interaction in organogenesis, and other related topics were discussed in a variety of developmental contexts. NIBB and EMBL share a common interest in these topics and it is hoped that further international collaboration between our institutions will result from 2007's fruitful and stimulating meeting.

Hoping to catch the big wave of genomics of many experimental organisms now sweeping the worldwide scientific community, we also aimed in this meeting to explore the possibilities of genome-wide biology unveiling a "Gene Regulatory Network" (GNR) of development. GRN is a key to understanding not only the structure of gene regulatory pathways but also the evolutional capacity that generates biodiversity. Several speakers covered this topic and presented insightful talks, generating a great deal of interest in the future of "Systems Biology" among those attending.

Speakers

BRUNNER, Damian (EMBL Heidelberg, GERMANY), EPHRUSSI, Anne (EMBL Heidelberg, GERMANY), FURLONG, Eileen (EMBL Heidelberg, GERMANY), KNOP, Michael (EMBL Heidelberg, GERMANY), SPITZ, Francois (EMBL Heidelberg, GERMANY), WITTBRODT, Jochen (EMBL Heidelberg, GERMANY), ROSENTHAL, Nadia (EMBL Monterotondo, ITALY)

AIZAWA, Shin (Riken CDB, JAPAN), HASEBE, Mitsuyasu (NIBB, JAPAN), HAYASHI, Shigeo (Riken CDB, JAPAN), KAGEYAMA, Yuji (NIBB, JAPAN), KAIBUCHI, Kozo (Nagoya Univ., JAPAN), KINOSHITA, Noriyuki (NIBB, JAPAN), KOBAYASHI, Satoru (NIBB, JAPAN), MURATA, Takashi (NIBB, JAPAN), NARUSE, Kiyoshi (NIBB, JAPAN), NOJI, Sumihare (Univ. Tokushima, JAPAN), OKADA, Kiyotaka (NIBB, JAPAN), SAGA, Yumiko (NIG, JAPAN), SATOH, Nori (Kyoto Univ., JAPAN), TABATA, Tetsuya (Univ. Tokyo, JAPAN), TAKADA, Shinji (NIBB, JAPAN), TAKAHASHI, Yoshiko (NAIST, JAPAN), TAKEDA, Hiroyuki (Univ. Tokyo, JAPAN), TAMURA, Koji (Tohoku Univ., JAPAN), TANAKA, Minoru (NIBB, JAPAN), UEMURA, Tadashi (Kyoto Univ., JAPAN), UENO, Naoto (NIBB, JAPAN), YAMAMORI, Tetsuo (NIBB, JAPAN)

Access

From Tokyo

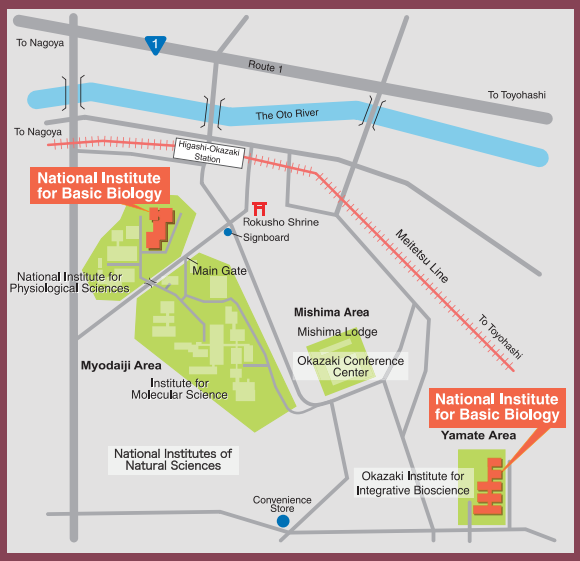
Take Tokaido Shinkansen and get off at Toyohashi, change to the Meitetsu Line and get off at Higashi Okazaki Station (approximately 20 minutes from Toyohashi).

From Osaka

Take Tokaido Shinkansen and get off at Nagoya, change to the Meitetsu Line and get off at Higashi Okazaki Station (approximately 30 minutes from Nagoya).

From Central Japan International Airport

Get on the Meitetsu bus bound for JR Okazaki Station and get off at Higashi Okazaki Station (approximately one hour from the airport). Alternatively, take the Meitetsu Line for Nagoya and change at Jingu-mae Station to a Toyohashi-bound train and get off at Higashi Okazaki Station (approximately 70 minutes from the airport).

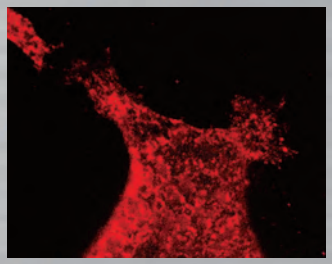


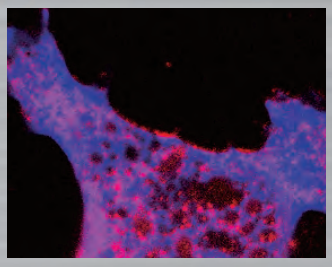
From Higashi Okazaki Station to Each Area

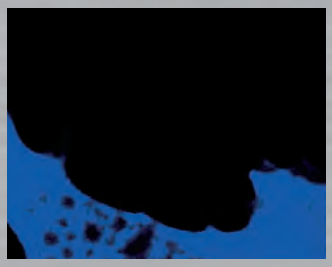
Turn left (south) at the ticket barrier and exit the station. The Institute is a 7-minute walk up the hill (Myodaiji-area) or 20-minute walk (Yamate-area).

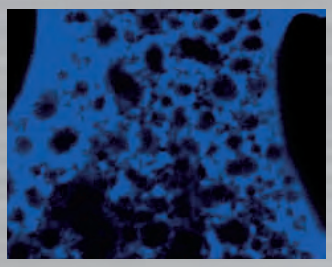
By car

Take the Tomei Expressway to the Okazaki Exit. Turn left at the City Office S.E. signal (approximately 10 minutes from the Exit).









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