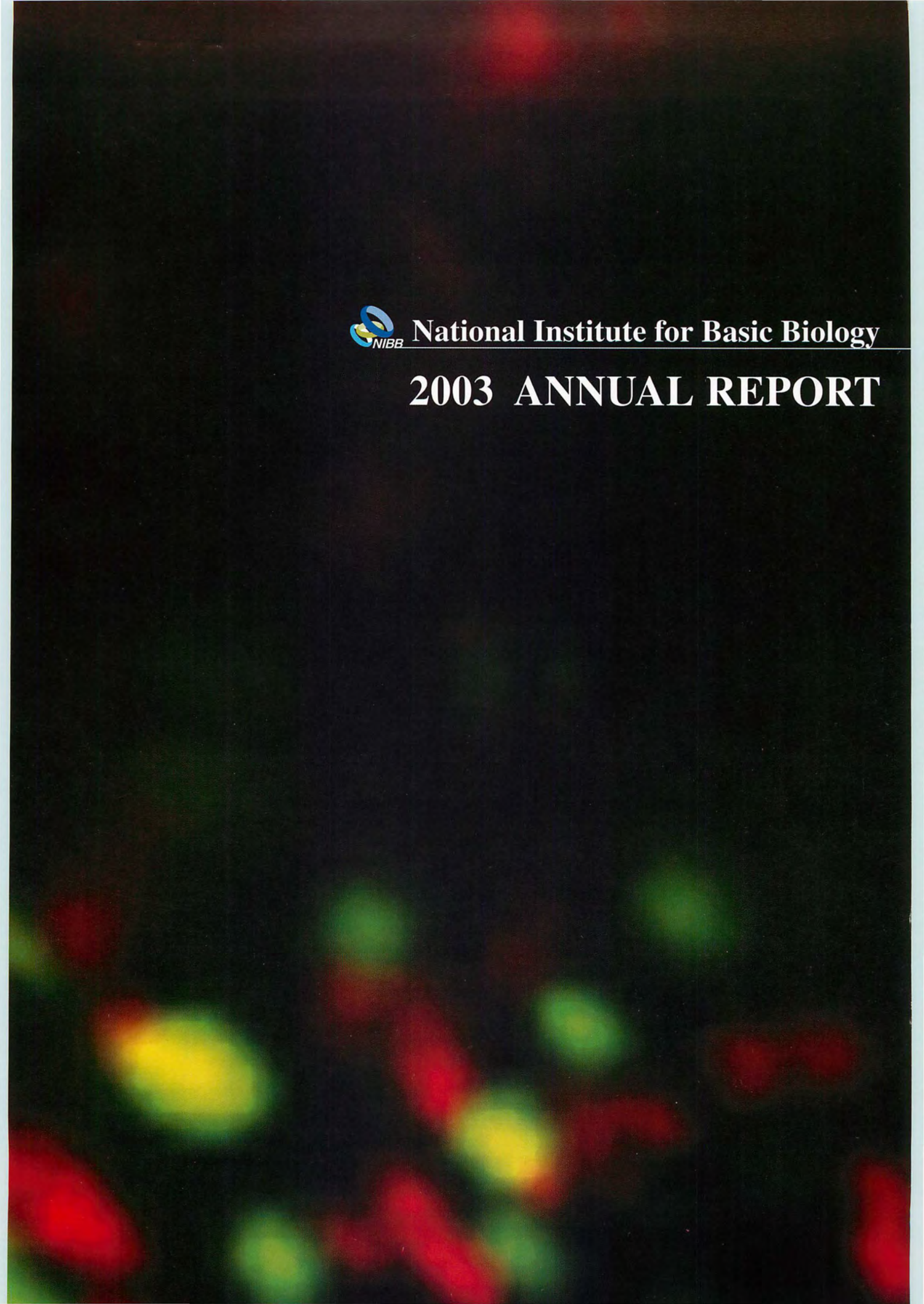

NIBB
National Institute for Basic Biology
2003 ANNUAL REPORT

CONTENTS

The cover photograph shows the interaction of Arabidopsis dynamin-related protein 3A (DRP3A), which is involved in both peroxisomal and mitochondrial division, with mitochondria. Green and red signals represent DRP3A and mitochondria, respectively. See also page 9.

INTRODUCTION

The National Institute for Basic Biology (NIBB) is a government-supported research institute established in 1977. In 1988, Department of Molecular Biomechanics in School of Life Science, the Graduate University for Advanced Studies, was established in the NIBB. The aim of the NIBB is the promotion and stimulation of studies in the field of Biology. As a Center of Excellence (COE), NIBB promotes not only the basic biology but also the modern biological sciences by conducting first-rate research on site as well as in cooperation with other universities and research organizations. Researchers at NIBB investigate cell structure and function, reproduction and development, neuronal and environmental biology, gene expression and regulation, molecular evolution of eukaryotic organisms and integrative and computational biology to elucidate the general and fundamental mechanisms underlying various biological phenomena.

In 2003, there were many doctors who were appointed as scientific staffs of NIBB. On April 1st, Profs. NAGATA, Shigekazu (Osaka University), OKADA, Norihiro (Tokyo Institute of Technology), and YANAGIDA, Mitsuhiro (Kyoto University) were appointed as adjunct Professors for Division of Cell Proliferation, Division of Cellular Communication, and Division of Cell Fusion, respectively, in the Department of Cell Biology. Prof. MORI, Yuji of The University of Tokyo was appointed as an adjunct Professor for Division of Behavior and Neurobiology in the Department of Regulation Biology on September 1st. Dr. SASAOKA, Toshikuni was appointed as an Associate Professor of Center for Transgenic Animals and Plants from National Center of Neurology and Psychiatry on August 1st. Drs. TAKAHASHI, Kazuhiko, OKUBO, Kataaki, HIWATASHI, Yuji, and SUZUKI, Kuninori were appointed as Research Associates of the NIBB in 2003.

In congratulation, Dr. HANAMURA, Kenji (NIBB Research Fellow) was promoted to an Assistant Professor of Gunma University School of Medicine. Drs. TAMADA, Atsushi and SHIMONO, Akihiko, who had been Research Associate in NIBB, were promoted as Research fellow of RIKEN Brain Science Institute and RIKEN Center for Developmental Biology, respectively.

An adjunct Professor, Dr. MURAKAMI, Fujio, and adjunct Associate Professors, ITO Kei, KIYOSUE, Tomohiro, and NAKAFUKU, Masato, left NIBB in March 31st, 2003 just after the completion of their 5-year appointments.

In addition, a number of postdoctoral fellows, technical assistants, secretaries, and graduate students after awarded PhD. degree left, and conversely people newly joined NIBB at the equivalent positions. In consequence, the total number of personnel working at NIBB has been kept at approximately 300 for several years.

As a COE of the biological research institute, NIBB is responsible for conducting research projects in cooperation with various research groups. As a part of such cooperative activities, NIBB has hosted International Conferences. The 49th NIBB International Conference entitled "Dynamic Vacuoles in Plants" was held in November 25-27 (Profs. Y. OHSUMI and M. NISHIMURA, organizers). This conference was sponsored by the Ministry of Education, Culture, Sports, Science and Technology. Moreover, NIBB continues to sponsor interdisciplinary symposia and study meetings on current topics by inviting leading scientists from around the world to the Institute. Based on this concept, NIBB will start from the upcoming 2004 to support a series of Okazaki Biology Conferences (OBC) through intimate cooperation with a variety of biological scientific societies. NIBB also provides a training course in biological sciences for young investigators.

NIBB together with the other members of Okazaki National Research Institutes (ONRI), the Institute for Molecular Science and the National Institute for Physiological Sciences, has been constructing a new campus to prepare the facility for Center for Integrative Bioscience (CIB). Also, NIBB built Center for Transgenic Animals and Plants as a research support facility in the new campus. Construction of the new campus still continues, and it is programmed that two divisions in NIBB and a laboratory in CIB will move there in 2004.

Finally, I would like to celebrate retirement of Dr. MOHRI, Hideo with appreciation of his deliberate and encouraging management as the President of ONRI. Together with his contribution as the previous Director-General of NIBB, the institute respectfully awarded a title for Professor Emeritus MOHRI on April 1st, 2003.

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

KATSUKI, Motoya, D.Sc.
Director-General

ORGANIZATION OF THE INSTITUTE

The National Institute for Basic Biology (NIBB) is an Institute in the Okazaki National Research Institutes (ONRI) that are composed of three independent organizations, NIBB, the Institute for Molecular Science (IMS) and the National Institute for Physiological Sciences (NIPS). They located on a hill overlooking the old town of Okazaki. NIBB was established in 1977 and its activities are supported by Monbukagaku-sho (the Ministry of Education, Culture, Sports, Science and Technology: Mext) of Japan. The Center for Integrative Bioscience that was established as a common facility of the ONRI in 2000 and began in 2001.

Policy and Decision Making

The Director-General oversees the operation of the Institute assisted by two advisory bodies, the Council and the Advisory Committee for Programming and Management. The Council, comprised of distinguished scholars representing various fields of science and culture, advises the Director-General on principles and policies governing the activities and operations of NIBB. The Advisory Committee, comprised of professors within the Institute and an equal number of leading biologists outside NIBB advises the Director-General, upon his request, on planning joint research programs and other important matters in NIBB, as well as on the scientific activities of the Institute. The Council makes a nomination of Director-General and Committee also makes recommendations on the Director-General and on faculty appointments, the Institute's annual budget and future prospects.

Administration

Administration of the Institute is undertaken by the Administration Bureau of the ONRI under the direct auspices of the Ministry of Education, Culture, Sports, Science and Technology.

Research

The Institute conducts its research programs through three departments and one laboratory subdivided into 17 divisions and the Center for Integrative and Computational Biology.

Each division has its own research project and is staffed by a professor, an associate professor and two research associates in principle. A division forms an independent project team. Six of the divisions are adjunct and headed by professors who hold joint appointments with other universities. Adjunct divisions have a resident research associate. This arrangement facilitates exchange in research activities in Japan. The Technical Department manages the activities of research technicians and helps to promote research activities of each division and to maintain the common research resources of the Institute. The Department also undertakes the technical education of staffs.

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

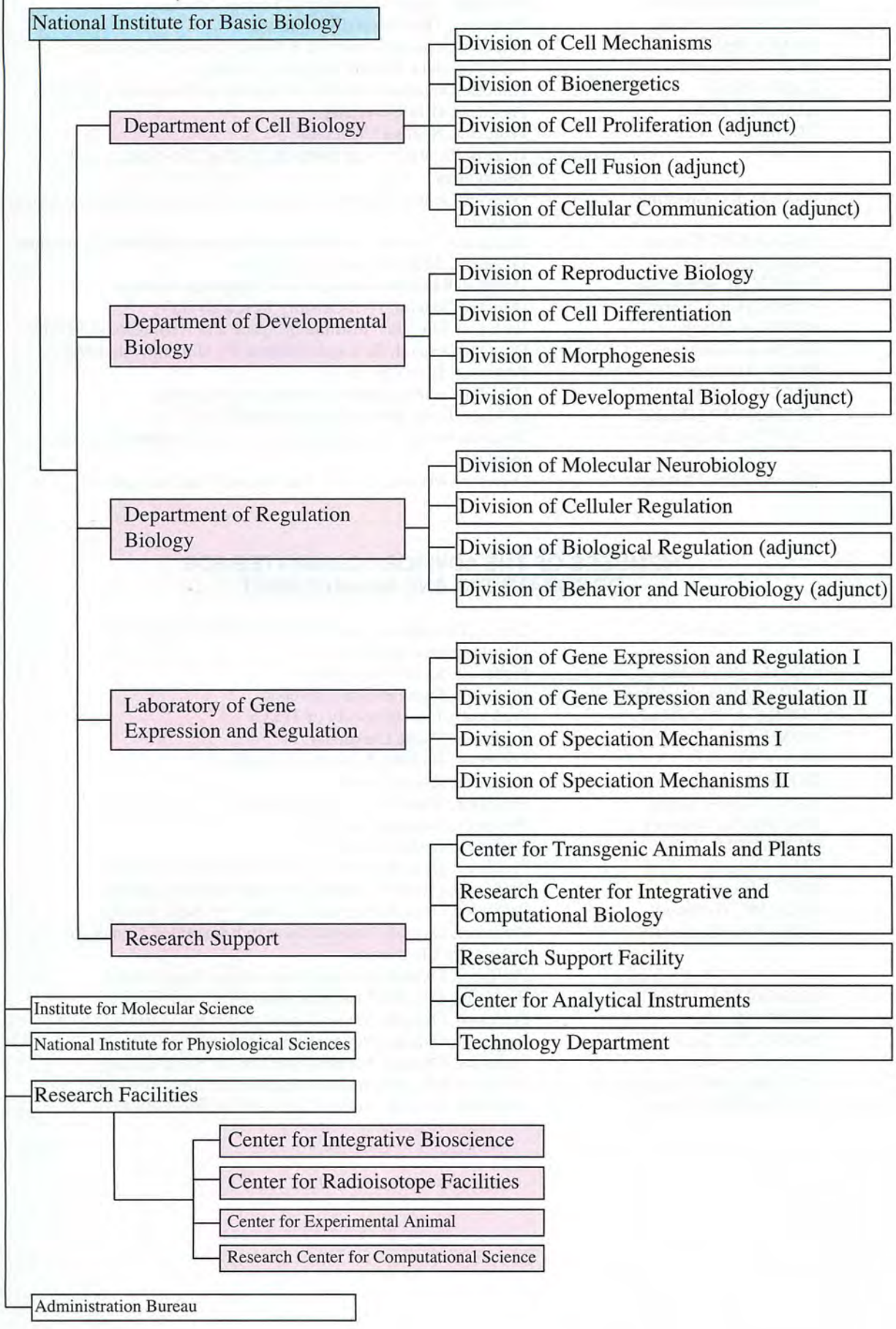
Research Support Facilities

The research support facilities of the NIBB consist of the Large Spectrograph Laboratory, the Tissue and Cell Culture Laboratory, the Computer Laboratory, the Plant Culture Laboratory, the Plant Cell Culture Laboratory, the Experimental Farm, the Laboratory of Stress-Resistant Plants and the Center for Transgenic Animals and Plants. In addition, five facilities are operated jointly with NIPS; they consist of the Electron Microscope Center, the Center for Analytical Instruments, the Machine Shop, the Laboratory Glassware Facilities and the Low-Temperature Facilities. The Radioisotope Facilities, the Computer Center and the Animal Care Facilities became common facilities of ONRI.

Campus

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

Okazaki National Research Institutes



MEMBERS OF THE COUNCIL

ANZAI, Yuichiro		President, Keio University
IKEHATA, Setsuho		President, Tokyo University of Foreign Studies
IWATSUKI, Kunio		Professor, The University of the Air
OSAKI, Hitoshi		Director-General, Center for National University Finance
OGAWA, Tomoko		Vice-President, Iwate College of Nursing
KAIFU, Norio		Director-General, National Astronomical Observatory of Japan
KUROKI, Toshio		President, Gifu University
GO, Mitiko		Professor, Nagoya University
	(present)	Dean & Professor, Nagahama Institute of Bio-Science and Technology
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HOSHI, Motonori		Professor, Keio University
HOTTA, Yoshiki		Director-General, National Institute of Genetics
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OHSUMI, Yoshinori	Professor, Okazaki National Institute for Basic Biology
KOBAYASHI, Satoru	Professor, Okazaki National Research Institutes, Center for Integrative Bioscience
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HORIUCHI, Takashi	Professor, Okazaki National Institute for Basic Biology
MURATA, Norio	Professor, Okazaki National Institute for Basic Biology
MOROHASHI, Ken-ichirou	Professor, Okazaki National Institute for Basic Biology
YAMAMORI, Tetsuo	Professor, Okazaki National Institute for Basic Biology

GRADUATE PROGRAMS

The NIBB sponsors two graduate programs.

1. Graduate University for Advanced Studies

NIBB constitutes the Department of Molecular Biomechanics in the School of Life Science of the Graduate University for Advanced Studies. The University provides a three year Ph.D. course. Those who have completed a master's course or equivalent at any university are eligible to apply.

The Department consists of the following Divisions and Fields:

DIVISIONS	FIELDS
Molecular Cell Biology	Biomolecular Systems Cell Dynamics
Developmental Gene Expression and Regulation	Gene Expression Morphogenesis Transgenic Biology
Regulation Biology	Biological Regulation Biological Information

2. Graduate Student Training Program

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

OFFICE OF DIRECTOR

Director-General: *KATSUKI, Motoya*
Associate Professor: *KODAMA, Ryuji*
UENO, Kohji
Research Associates: *OHNO, Kaoru*

Mechanisms working in the morphogenesis of the lepidopteran wings

KODAMA, Ryuji

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

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

The cell deaths in the degeneration region proceeds very rapidly and completes in a half to one day period in Pieris rapae or several other species examined. It was shown that the dying cells in the degeneration region have characteristics common with the apop-totic cell death in mammalian cells. 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 basal surfaces of the dorsal and ventral epithelium in the differentiation region.

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

In collaboration with the Division of Molecular Neurobiology, the localization of a sodium channel protein is being investigated using immunohistological staining. DAB stained Vibratome section was further fixed and embedded in epoxy resin and then sectioned with ultramicrotome and observed under a transmission electron microscopy. Electron microscopic observation was utilized in order to identify the stained cells and surrounding cells by their ultrastructural characteristics.

Scanning electron microscopic imaging of a mutant mouse phenotype is also being done according to a request of another laboratory.

Control of the distribution of palmitoylated proteins in neuronal growth cones

Kohji Ueno

Signalling proteins such as G proteins and G protein-coupled receptors are modified with palmitate via thioester linkages. Protein palmitoylation is thought to be important in the regulation of signal transduction. We have previously found that protein palmitoylase is expressed in neural cells during mouse embryogenesis. In developing neurons, growth associated protein (GAP)-43 and Go, which are palmitoylated proteins, are mainly concentrated in the growth cones. Addition of an inhibitor of protein palmitoylase to the medium of cultured primary neuronal cells reduces the axonal growth of neurons. From these findings, we speculated that the localization of the palmitoylated proteins in growth cones is critical for the development of axons.

In this study, we are attempting to elucidate the mechanism that determines the localization of the palmitoylated proteins in growth cones. For thisanalysis, we have established a method to chemically modify a biotinylated peptide composed of residues 1-15 of the GAP-43 N-terminal with fatty acids via thioester linkages. Cys 3 and Cys 4 of GAP-43 are modified with palmitate in developing neurons. By using the method, we have prepared the peptides which are modified with myristate (C14:0), palmitate (C16:0), palmitoleate (C16:1), stearate (C18:0) and arachidate (C20:0) via thioester linkages. The method could modify the peptide with not only saturated but also unsaturated fatty acids.

With the acylated peptides, we have assayed the binding activity of ERM (Ezrin/Radixin/Moesin) proteins, which are thought to be general cross-linkers between plasma membranes and actin filaments, because ERM proteins are reported to be concentrated in growth cones and ERM proteins contain a domain which has a similar structure to a fatty acid thioesters binding protein. Recombinant full-length ERM proteins, which were expressed in bacteria, had the binding activity with the palmitoylated peptides, whereas the binding activity were less with the synthetic peptides modified with myristate, palmitoleate, stearate and arachidate. These result suggested that ERM proteins have a potentiality to recognize the fatty acid residue of the acylated peptides. Recombinant erythrocyte membrane protein band 4.1, which contains a similar structure to ERM proteins, had low binding activity with the palmitoylated peptide. From these results we suggested that a domain of ERM proteins is responsible for the interaction with the palmitoylated peptide.

DEPARTMENT OF CELL BIOLOGY

Chairperson: UNO, Naoto

DIVISION OF CELL MECHANISMS
DIVISION OF BIOENERGETICS
DIVISION OF CELL PROLIFERATION (ADJUNCT)
DIVISION OF CELL FUSION (ADJUNCT)
DIVISION OF CELLULAR COMMUNICATION (ADJUNCT)

The department consists of two regular divisions and three adjunct divisions. The department conducts studies on molecular dynamics of the cell in eukaryotes such as organelle differentiation, autophagy, cell motility, cytokinesis and neural development.

DIVISION OF CELL MECHANISMS

Professor:	*NISHIMURA, Mikio*
Associate Professor:	*HAYASHI, Makoto*
Research Associates:	*MANO, Shoji*
	YAMADA, Kenji
Technical Staff:	*KONDO, Maki*
NIBB Research Fellow:	*SHIRAHAMA, Kanae (~April 30)*
JSPS Postdoctoral Fellows:	*NITO, Kazumasa*
	KUROYANAGI, Miwa (April 1~)
	ARAI, Yuko (April 1~)
Graduate Students:	*FUKAO, Youichiro (~March 31)*
	KAMADA, Tomoe
	HATSUGAI, Noriyuki
Technical Assistants:	*NAKAMORI, Chihiro*
	YAGI, Mina
	YOSHINORI, Yumi
	SUZUKI, Iku (April 1~)
Secretaries:	*UEDA, Chizuru*
	IYODA, Yuri

Higher plant cells contain several distinct organelles that play vital roles in cellular physiology. During proliferation and differentiation of the cells, the organelles often undergo dynamic changes. The biogenesis of new organelles may occur, existing organelles may undergo a transformation of function, while other organelles may degenerate. Because the dynamic transformation of organellar function (differentiation of organelles) is responsible for flexibility of differentiation events in higher plant cells, the elucidation of regulatory mechanisms underlying organelle transformation are currently studied in this division.

I. Reversible transformation of plant peroxisomes

Dramatic metabolic changes which underlie the shift from heterotrophic to autotrophic growth occur in greening of seed germination. Accompanying these metabolic changes, many constitutive organelles are functionally transformed. Etioplasts differentiate into chloroplasts and mitochondria acquire the ability to oxidize glycine. Glyoxysomes which are microbodies engaged in the degradation of reserve oil via β-oxidation and the glyoxylate cycle, are transformed into leaf peroxisomes that function in several crucial steps of photorespiration. After the functional transition of glyoxysomes to leaf peroxisomes during the greening of pumpkin cotyledons, the reverse transition of leaf peroxisomes to glyoxysomes occurs during senescence. The functional transformation between glyoxysomes and leaf peroxisomes is controlled by gene expression, alternative splicing, protein translocation and protein deg-radation. We now engage in proteomic and transcriptomic analyses of the reversible peroxisomal transition in *Arabidopsis* cotyledons.

II. Transcriptomics and proteomics 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 carboxyl terminus of the mature proteins. The permissible combinations of amino acids for PTS1 in plant cells are [C/A/S/P]-[K/R]-[I/L/M]. In contrast, PTS2 is involved in a cleavable amino terminal presequence of peroxisomal proteins that are synthesized as precursor protein with larger molecular mass. PTS2 consists of a consensus sequence [R]-[L/Q/I]-X5-[H]-[L].

We identified 256 gene candidates of PTS1- and PTS2-containing proteins and other 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. Statistical analyses revealed that the peroxisomal genes could be divided into five groups in terms of their transcription. One group showed ubiquitous expression in all organs examined, while the other four were classified as showing organ-specific expression in seedlings, cotyledons, roots and in both cotyledons and leaves. These data proposed more detailed description of differentiation of plant peroxisomes (Fig. 1).

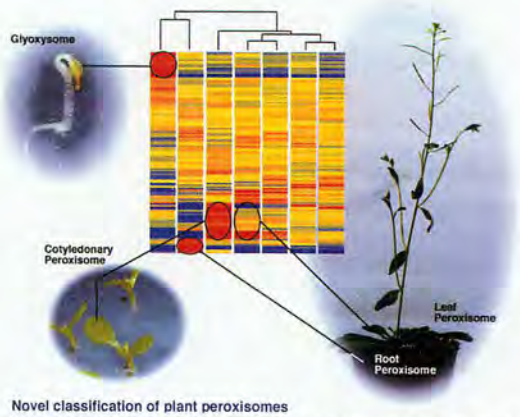

Fig. 1 Novel nomenclature of plant peroxisomes identified by peroxisomal gene-specific transcriptomics.

In parallel, we made two-dimensional protein map of glyoxysomes and leaf peroxisomes isolated from *Arabidopsis*. Peptide MS fingerprinting analyses allowed us to identify novel proteins exists in either glyoxysomes or leaf peroxisomes. Some of these proteins contain no obvious PTS1 and PTS2. Of these, we characterized GPK1 as a novel protein kinase in glyoxysomes.

III. Involvement of the same dynamin molecule in peroxisomal and mitochondrial division in higher plants

To better understand peroxisome biogenesis, we mutagenized seeds of transgenic *Arabidopsis*, GFP-PTS1, in which peroxisomes with normal size and number can be visualized with GFP, and screened a

number of *Arabidopsis* mutants with aberrant peroxisome morphology (*apm* mutants) based on the different pattern of GFP. The *apm* mutants were classified into four classes. These were mutants with (1) long peroxisomes, (2) giant peroxisomes, (3) GFP fluorescence in the cytosol as well as in peroxisomes, and (4) other distributions of GFP.

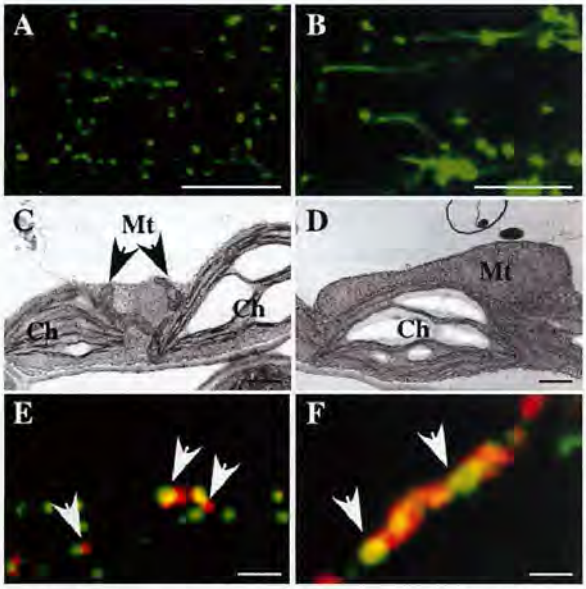

Fig. 2 Phenotype of *apm1* mutant and subcellular localization of APM1/DRP3A protein.
(A) and (B) show GFP-labelled peroxisomes in GFP-PTS1 as a parent plant (A) and *apm1* mutant (B). (C) and (D) represent electron microscopic observation of leaf cells in GFP-PTS1 (C) and *apm1* mutants (D). Mt: mitochondrion, Ch: chloroplast. (E) and (F) show the co-localization of DRP3A protein with peroxisomes (E) and mitochondria (F). Spherical spots in green indicate DRP3A proteins. Red signals show peroxisomes (E) and mitochondria (F), respectively. Arrow heads represent the sites of interaction with DRP3A proteins. Each bar indicates 50 μm for (A) and (B), 1 μm for (C) and (D), and 10 μm for (E) and (F).

In one of these mutants, *apm1*, the peroxisomes are long and reduced in number, apparently as a result of inhibition of division (Fig. 2). *APM1* gene encodes DRP3A (Dynamin related protein 3A). Interestingly, mutations in APM1/DRP3A also caused aberrant morphology of mitochondria. The growth of *Arabidopsis*, which requires the cooperation of various organelles including peroxisomes and mitochondria, is repressed in *apm1*, indicating that the changes of morphology of peroxisomes and mitochondria reduce the efficiency of metabolism in these organelles. These findings indicate that the same dynamin molecule is involved in peroxisomal and mitochondrial division in higher plants.

IV. ER derived organelles for transport of proteins to vacuoles.

Precursor-accumulating (PAC) vesicle, mediates the transport of storage protein precursors from endoplasmic reticulum (ER) to protein storage vacuoles in maturing pumpkin seeds. PAC vesicles had diameters of 300 to 400 nm, are derived from ER and contained an electron-dense core of storage proteins. PV72, a type I integral membrane protein was found on the membrane of the PAC vesicles. PV72 has been shown to bind to the precursor of pumpkin 2S albumin, implying that PV72 functions as a vacuolar sorting receptor (VSR) for storage proteins in pumpkin seeds. *Arabidopsis* has seven homologue of PV72. The homologue closely related to PV72 was designated AtVSR1. We used a reverse-genetic approach to explore the function of AtVSR1. Two T-DNA insertion mutants (*atvsr1-1* and *atvsr1-2*) missort storage proteins by secreting them from cells, and abnormally accumulate the precursors of storage proteins, together with the mature forms of these proteins in the seeds. These finding demonstrate a receptor-mediated transport of seed storage proteins to protein storage vacuoles in higher plants.

ER bodies are another ER-derived compartment specific to the *Brassicaceae*, including *Arabidopsis*. ER bodies are rod-shaped structures (5 μm long and 0.5 μm wide) that is surrounded by ribosomes. ER bodies can be visualized in transgenic plants of *Arabidopsis* (*GFP-h*) expressing green fluorescent protein fused with an ER retention signal (GFP-HDEL). ER bodies were widely distributed in the epidermal cells of whole seedlings. In contrast, rosette leaves had no ER bodies. *nai1* is an *Arabidopsis* mutant in which ER bodies were hardly detected in whole plants. Analysis of the *nai1* mutant reveales that a β-glucosidase with an ER-retention signal (KDEL), called PYK10, is the main component of ER bodies. The putative biological function of PYK10 and the inducibility of ER bodies in rosette leaves by wound stress suggest that the ER body functions in the defense against herbivores.

V. Maturation of vacuolar/lysosomal proteins by vacuolar processing enzyme in animal and plant cells.

Vacuolar processing enzyme (VPE) belongs to the cysteine protease family C13. This family is found in various eukaryote organisms including higher plants and animals. VPE was originally identified as an enzyme responsible for the processing and maturation of seed storage proteins in plants. VPE exhibit substrate specificity toward an asparagine residue, the amino acid well conserved at the P1 position in the processing sites of various vacuolar/lysosomal proteins. Plant VPE homologues were separated to two subfamilies: one seed type and the other vegetative type. We have identified one seed type (*βVPE*) and two vegetative type (*αVPE* and *γVPE*) VPE genes from *Arabidopsis*. We isolated six *Arabidopsis* mutants that accumulate detectable amounts of the precursors of the storage proteins. All mutants had a defect in the *βVPE* gene, indicating that βVPE is involved in the processing of storage proteins *in vivo*. We further generated various mutants lacking different VPE isoforms: αVPE, βVPE and/or γVPE. *αvpe-1/βvpe-3/γvpe-1* triple mutant seeds

accumulate no properly processed mature storage proteins. Instead, large amounts of storage protein precursors are found in the seeds of this mutant. In contrast to *βvpe-3* seeds, which accumulate both precursors and mature storage proteins, the other single (*αvpe-1* and *γvpe-1*) and double (*αvpe-1/γvpe-1*) mutants accumulate no precursors in their seeds at all. Therefore, the vegetative type VPEs, αVPE and γVPE, compensates for the deficiency in βVPE in *βvpe* mutant seeds.

To explore the physiological function of VPE in mammals, we generated and characterized VPE-deficient mice. VPE was abundantly expressed in kidney and localized in the late endosomes of the proximal tuble cells. Disruption of the *VPE* gene led to the enlargement of lysosomes in these cells in an age-dependent manner, which suggests that the materials to be degraded are being accumulated within the lysosomal compartments. The processing of the lysosomal proteases, cathepsins B, H, and L, from the single-chain forms into the two-chain forms was completely defected in the deficient mice. Thus, the VPE deficiency caused the accumulation of macromolecules in the lysosomes, highlighting a pivotal role of VPE in the endosomeal/lysosomal degradation 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 also characterized a mitochondrial co-chaperonin (Cpn10), chloroplast co-chaperonins (Cpn20 and Cpn10) and a small heat shock protein from *Arabidopsis*. In 2003, we started to characterize HSP90s. Their evolutional and functional characterization is now under experiments.

Publication List:

Fukao, Y., Hayashi, M., Hara-Nishimura, I. and Nishimura, M. (2003) Novel glyoxysomal protein kinase, GPK1, identified by proteomic analysis of glyoxysomes in etiolated cotyledons of *Arabidopsis thaliana*. *Plant Cell Physiol.* **44**, 1002-1012.

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DIVISION OF BIOENERGETICS

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	KUMA, Akiko (~May 2003)
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	KAWAMATA, Tomoko [3]
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	IMAMURA Yuko
	TAKEUCHI, Yuka
	TSUKESHIBA Kumi
Secretary:	*HARA, Yoko*

[1] *Study abroad from Mar. 2003*
[2] *Graduate University for Advanced Studies*
[3] *KOBE Univ.*

This division aims to understand the autophagy in respects to its molecular mechanism and its physiological role in yeast and higher eukaryotes. Cells execute degradation processes of their constituents together with biosynthetic processes. These two processes are well coordinated to regulate the biological activities. In other word, we must shed light on degradation process to fully understand the cell, because the study on the degradation has been retarded compared to the biosynthetic process. Autophagy is a major route for bulk degradation of cytoplasmic constituents and organelles in lysosome/vacuole, and is well conserved in eukaryotes.

I. Background

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

II. In vitro reconstitution of Atg8-PE conjugation system

In yeast, *S. cerevisiae*, Atg8 plays an important role during autophagosome formation. We have previously reported that the Atg8 is covalently attached to phosphatidylethanolamine (PE) via a ubiquitin-like conjugation system. The C-terminal Arg of newly synthesized Atg8 ($Atg8^{R117}$) is removed by Atg4 protease to expose a Gly residue at the C-terminus ($Atg8^{G116}$). The $Apg8^{G116}$ is then activated by Atg7 (E1 enzyme) and transferred to Atg3 (E2 enzyme). Following these reactions, the $Apg8^{G116}$ conjugates to PE through an amide bond between its C-terminal Gly and the amino group of PE. The subsequent deconjugation reaction by Atg4 is necessary for the normal progression of autophagy. We developed *in vitro* Atg8-PE reconstitution system. The Atg8-PE was successfully reconstituted simply with $Atg8^{G116}$, Atg7 and Atg3 by using *in vivo* in *E. coli* and *in vitro* system. These results suggested that Atg7 and Atg3 are necessary and sufficient for the Atg8-PE conjugation reaction. The *in vitro* Atg8-PE reconstitution system using recombinants and liposomes demonstrated that the efficiency of Atg8-PE conjugation was strongly affected by lipid composition. Further, the Atg8 was linked to the PE in liposomes, but not to the PE in the presence of detergent, suggesting that the lipid bilayer of membrane is essential for the Atg8-PE conjugation.

III. Molecular dissection of Atg12

Atg12 is activated by Atg7, transferred to Atg10 and attached to Atg5 in a manner similar to the ubiquitination. Although Atg12 has scarcely sequence similarity with ubiquitin, its secondary structure was predicted to have ubiquitin-like domain in the C-terminus region. We prepared N-terminally truncated Atg12 mutant of the yeast *S. cerevisiae* according to the predicted secondary structure. Truncated form of Atg12 having only a predicted ubiquitin-like domain conjugated with Atg5 was still active in autophagy. While a truncated Atg12 mutant lacking the first beta strand in the ubiquitin-like domain didn't conjugate with Atg5. These results showed that the ubiquitin-like domain of Atg12 is necessary and sufficient for conjugation and autophagy. Furthermore, we altered several hydrophobic amino acid residues in the ubiqutin-like domain of Atg8 and found a certain amino acid residue is critical for autophagy, even though still active for conjugation with Atg5.

IV. Preferential degradation by autophagy

In contrast to the ubiqutin/proteasome pathway, autophagy is thought to be non-selective protein degradation process. We surveyed the changes of protein profiles after nitrogen starvation of wild-type and Δ*atg7* cells using 2D-PAGE, and we found that cytosolic acetaldehyde dehydrogenase (Ald6p; Figure 1) was degraded more rapidly than other cytosolic proteins in an autophagy dependent manner. We observed that Ald6p was enclosed in the autophagosome and delivered to the vacuole preferentially. The Ald6p may be harmful to cells during nitrogen starvation, since disruption of *ALD6* improved the loss of viability of the Δ*atg7* mutant. This is the first report of the preferential degradation of a detrimental protein via autophagy.

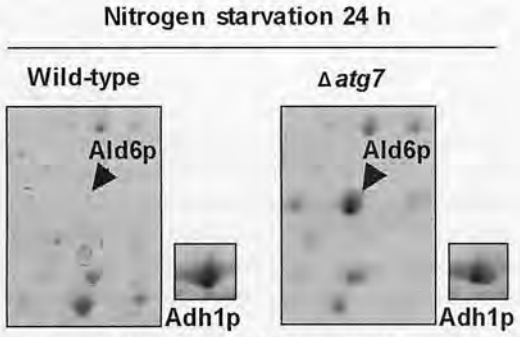


Figure 1. Ald6p was degraded by autophagy. Protein spots from 2D-PAGE using soluble lysates of nitrogen-starved cells were shown. Ald6p, cytosolic acetaldehyde dehydrogenase; Adh1p, alcohol dehydrogenase (control).

V. Involvement of early secretory pathway

In addition to Atg proteins, we have previously identified the involvement of secretory proteins in autophagy. Autophagosome formation is completely blocked when some early Sec proteins such as Sec24p, are defective, which are involved in the formation of the COPII coated vesicles from ER to Golgi. The autophagic defect in *sec24* deleted mutant cells was, however, suppressed upon the recovery of ER-Golgi secretory flow by the overexpression of its homologue, Sfb2p. We found that the autophagic defect is not observed in *sec13* and *sec31* mutants, a phenomenon that can be explained by the fact that starvation stress suppresses the secretory defect of these mutants. These observations indicate that the active flow in the early secretory pathway plays an important role in autophagy. Both autophagy and its closely related cytoplasm to vacuole-targeting (Cvt) pathway occur through a pre-autophagosomal structure (PAS), and since the PAS and the functional Cvt pathway exist in all *sec* mutants, the early secretory pathway must be involved specifically in autophagy, subsequent to PAS formation.

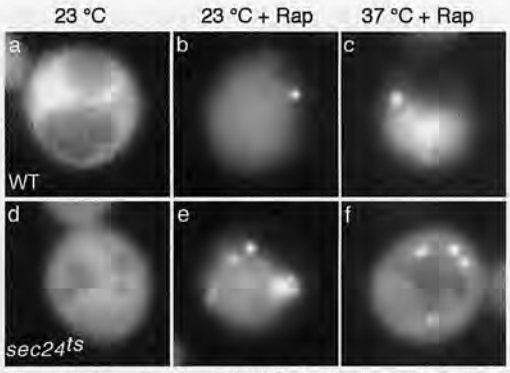
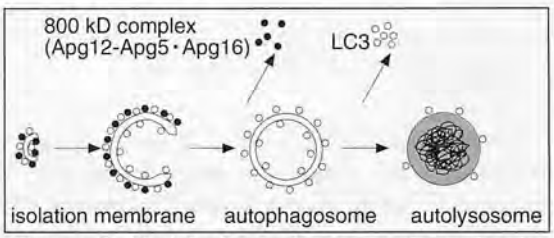


Figure 2. Localization of GFP-Atg8p in *sec24* mutant cells. Wild-type (KVY55) and *sec24* (MHY24) cells haboring pRS316 GFP-Atg8p were used. GFP-Atg8p was visualized under growing condition (a, d), starvation condition at permissive temperature (b, e) and starvation condition at non-permissive temperature (c, f).

VI. Mammalian Atg16-like protein: a novel WD-repeat protein

We have shown that, in yeast and mammalian cells, the Atg12-Atg5 protein conjugate, which is formed by a ubiquitin-like system, is essential for autophagosome formation. In yeast, the Atg12-Atg5 conjugate interacts with a small coiled-coil protein, Atg16, to form a ~350-kDa multimeric complex. We demonstrate that the mouse Atg12-Atg5 conjugate forms a ~800-kDa protein complex containing a novel WD repeat protein (Fig. 3). As the N-terminal region of this novel protein shows homology with yeast Atg16, we have designated it mouse Atg16-like protein (Atg16L). Atg16L, however, has a large C-terminal domain containing seven WD repeats, absent from yeast Atg16. Atg16L interacts with both Atg5 and additional Atg16L monomers; neither interaction, however, depends on the WD-repeat domain. In conjunction with Atg12-Atg5, Atg16L associates with the autophagic isolation membrane for the duration of autophagosome formation. As these features are similar to yeast Atg16, we concluded Atg16L is the functional counterpart of the yeast Atg16. We also found that membrane targeting of Atg16L requires Atg5, but not Atg12. As WD repeat proteins provide a platform for protein-protein interactions, the ~800-kDa complex is expected to function in autophagosome formation, further interacting with other proteins in mammalian cells.



Figgure 3. Model of autophagosome formation in mammalian cells.
The Atg12-Atg5 conjugate and Atg16L localize to the isolation membrane throughout its elongation process. LC3 is recruited to the membrane in the Atg5-dependent manner. Atg12-Atg5 and Atg16L dissociate from the membrane upon completion of autophagosome formation, while LC3 remains on the autophagosome membrane. Atg5 and its modification by Atg12 are required for elongation of the isolation membrane.

VII. Mice with a fluorescent marker for autophagy

In yeast, autophagy is required for cell survival during starvation and is necessary for spore formation. In contrast, the role of autophagy in mammals is still poorly understood. Although the possible involvement of autophagy in development, cell death and pathogenesis has been repeatedly pointed out, systematic analysis has not been performed, mainly due to a limitation of monitoring methods. Our recent studies have made available several marker proteins for autophagosomes. To understand where and when autophagy occurs in vivo, we have generated transgenic mice systemically expressing GFP fused to LC3, which is a mammalian homologue of yeast Aut7/Atg8. Cryosections of various organs were prepared and the occurrence of autophagy was examined by fluorescence microscopy (Fig. 4). Active autophagy was observed in various tissues, such as the skeletal muscle, liver, heart, exocrine glands, thymic epithelial cells, lens

epithelial cells and podocytes. Autophagy is differently induced by nutrient starvation in most tissues. In some tissues, autophagy even occurs spontaneously. Our results suggest that the regulation of autophagy is organ-dependent and the role of autophagy is not restricted to the starvation response. This transgenic mouse is a useful tool to study mammalian autophagy.

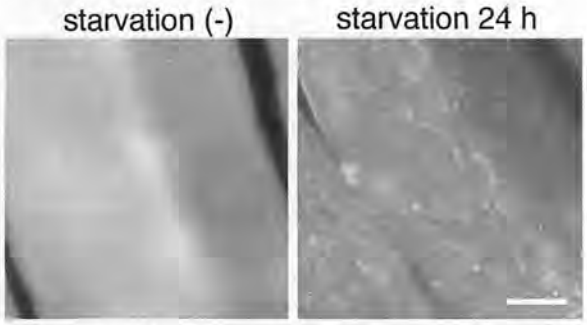


Figure 4. Muscle autophagy in response to food withdrawal. GFP images of gastrocnemius muscles at 0 h and 24 h starvation. Small dots indicate autophagosomes. Bar, 10 μm.

VIII. Monitoring of autophagic process in a whole plant

So far, autophagy in plant has been described by morphological studies. Recent genome-wide search revealed significant conservation in autophagy genes between yeast and plant. But little is known about the physiological roles and molecular mechanisms underlying autophagy in higher plants. To elucidate the plant autophagy, we focused ubiquitination-like Atg8 lipidation system. In yeast, Atg8 binds to autophagosomes and is delivered to the vacuole through autophagic process.

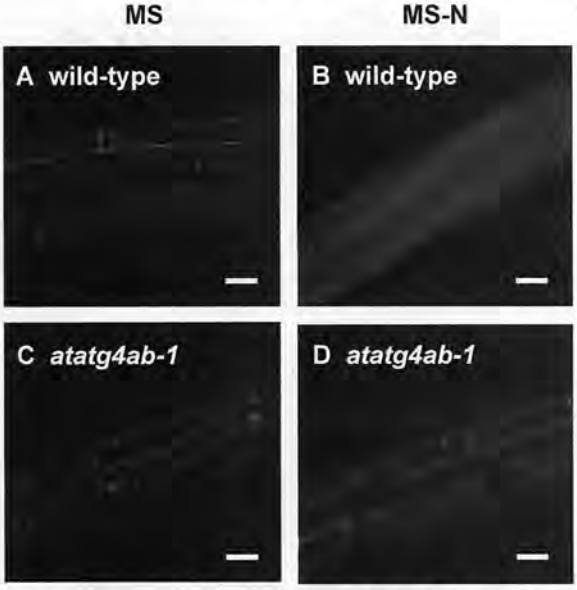


Figure 5. Localization of GFP-AtATG8a fusion protein in Arabidopsis roots. The roots of 1-week-old transgenic seedlings were grown on MS and MS-N medium. For starvation, seedlings grown on MS medium were transferred to MS-N medium for additional 7 days. (A, B) Wild-type Arabidopsis roots stably expressing GFP-AtATG8a. (C, D) *atatg4ab-1* disruption mutant roots stably expressing GFP-AtATG8a. Bar: 20 μm.

Therefore, Atg8 is a useful molecular marker for monitoring autophagic process. To establish a system monitoring autophagy in a whole plant, we generated transgenic Arabidopsis expressing GFP-AtATG8 fusion protein. In wild-type plants, GFP-AtATG8s were observed as ring structures in the cytoplasm (Figure 5 panel A), and delivered to the lumens of vacuole under nitrogen-starvation condition (Figure 5 panel B). On the basis of analogy of yeast, we regarded these ring structures as autophagosomes. In contrast, in double disruptant of *AtATG4*s which are required for C-terminal cleavage of Atg8, GFP-AtATG8s were not localized on autophagosomes (Figure 5 panel C) and were not delivered to the vacuole under nitrogen-starvation condition (Figure 5 panel D). These results indicate that AtATG8 is a suitable marker for monitoring autophagy in plant.

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DIVISION OF CELL FUSION (ADJUNCT)

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Research Associate: *TAKAHASHI, Kazuhiko*
Graduate Student: *SASAKI, Takeshi*
Technical Assistant: *MIURA, Seiko*

The above members of this adjunct division have started a new project in this institute in April 2003.

I. Introduction

During the long history of evolution of vertebrates, they acquired extensive diversity in characters such as morphology, ecology and behavior. Some examples, such as conserved morphological traits of coelacanths, show that phenotypic change during the course of evolution can be sometimes very slow, whereas other examples suggest, by contrast, that their speciation and diversification can be often accomplished very rapidly by the process called "adaptive radiation". What factors affect rates of phenotypic change during the evolution of vertebrates? Although many examples of adaptive radiation (and slow evolution) have been reported so far for various groups of vertebrate, its mechanism has been hardly clarified, especially from the molecular perspective.

Morphological diversification of organisms is accomplished by differentiation of developmental systems, which are controlled by regulation of numerous morphogenetic genes. This implies that, in order to obtain insights into the mechanisms of adaptive radiation of vertebrates, we absolutely need the viewpoint of developmental biology. However, the fields of evolutionary biology and developmental biology have not been necessarily interactive enough so far in spite of that such an idea has been becoming popular in recent years. Our division aims to understand the processes and mechanisms of diversification of vertebrates during their evolution based on molecular approaches of evolutionary developmental biology (evo-devo).

II. Evolutionary history of cichlids in African Great Lakes

Cichlid flocks of the East African Great Lakes, which consist of Lakes Victoria, Malawi, and Tanganyika, have attracted the interest of evolutionary biologists for more than a century (Figure 1). These species exhibit extraordinary levels of diversity and high species endemicity to each lake as the result of independent explosive adaptive radiation (Figure 2).

Our group conducted phylogenetic analyses of these fish using insertions of retroposons as markers for elucidation of their evolutionary history. These studies confirmed that cichlids in Lakes Victoria and Malawi are closely related, and that species in both lakes are related to only a portion of the lineages found in Lake Tanganyika, the oldest Great Lake estimated at 9-12MY (Figure 3). Evidence from the above analyses suggested that the fauna in Lake Tanganyika as well as its ancestor and other riverine lineages have experienced at least two waves of a rapid radiation event more than several million years ago. Interestingly, such radiations seem now ongoing in the Victorian fauna (see the next section for the detail) and the major groups of cichlids in Lake Malawi, too. These results suggest that explosion of speciation (adaptive radiation) has been a common phenomenon during the course of evolution of cichlids is East Africa.

Given the above results, the next primary problem to be solved in order to clarify the background of their evolution is how their genetic and developmental systems have changed during the processes of adaptive radiation.

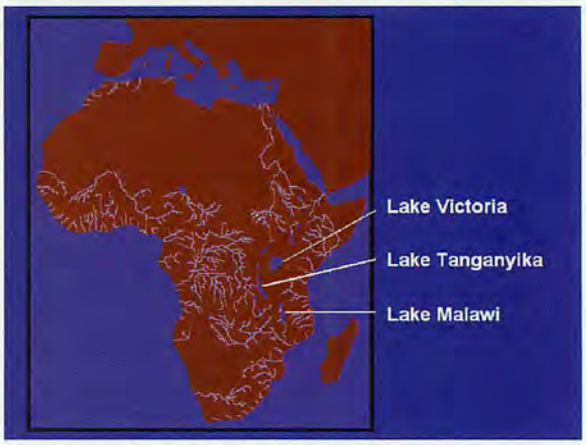

Figure 1. African Great Lakes

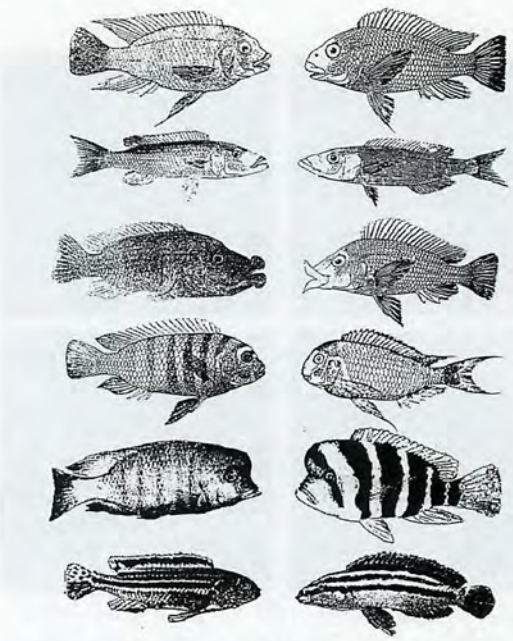

Figure 2. Morphological diversity and convergent evolution of cichlids in African Great Lakes. Left column, species in Lake Malawi; right column, species in Lake Tanganyika. [Figure 1 in Kocher, T. D., J. A. Conroy, K. R. McKaye and J. R. Stauffer (1993) Mol. Biol. Evol. 2: 158-165]

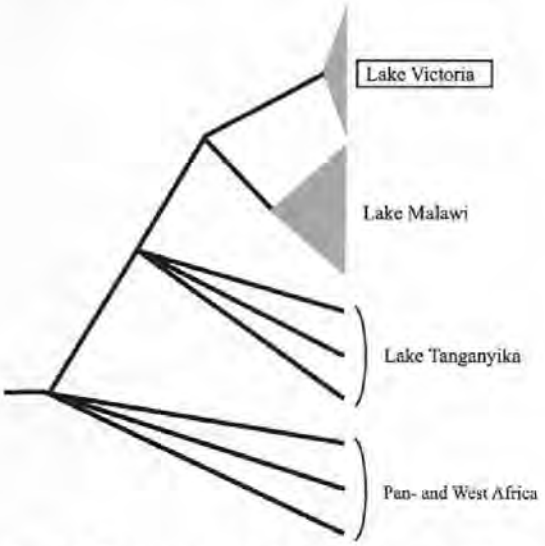

Figure 3. Phylogenetic relationships among cichlids in Africa as revealed by molecular markers.

III. Analysis of Cichlids in Lake Victoria for elucidation of molecular mechanisms of speciation and diversification

Among the cichlid faunas in African Great Lakes, the one in Lake Victoria is considered to be the youngest since this lake is suggested to have once been dried up about 12,000 years ago. Such a recent origin of this fauna is consistent with the observation by our group that most of selectively-neutral polymorphic alleles are retained both within- and among species within this lake (Figure 4). This means that, if a certain allele is uniquely fixed at a certain locus in natural populations of a certain species, we can assume the past and/or present existence of a selective pressure on this gene and hence can regard it as a candidate gene that was responsible for the phenotypic differentiation of this species from other related species. Therefore, investigation of cichlids in Lake Victoria is advantageous for the purpose of elucidation of molecular mechanisms of speciation and their following diversification during the adaptive radiation.

One of the most attractive diversification of the above cichlids is observed in morphology of jaws. Since they exhibit remarkable specialization to various food resources such as algae, shrimps, fry, planktons and fish, morphology of their jaws shows extensive diversity among species. Another interesting point is that all the cichlids in Lake Victoria, as well as those in Lake Malawi, show mouth brooding of their eggs and fry. Thus, their jaw shape might be also related to their breeding habit. Therefore, the mechanism of diversification of jaw shapes among species may be one of the most important factors to be considered for the present purpose.

One of our strategies to identify genes that were responsible for diversification of jaw shapes among Victorian cichlids is to use transgenic techniques. For a detailed observation of morphogenesis of jaws, injection of the GFP (Green Fluorescent Protein) gene with a promoter known to be specific to this organ may be useful. Another advantage of establishing a transgenic system is that we can newly identify genes that are specifically expressed in jaws by using gene-trap and enhancer-trap systems. However, a problem with application of this methodology to cichlids is that transgenic techniques have been hardly established for these fish so far. One solution to this problem is to take advantage of a transposon-mediated transgenic system, which was recently established for zebrafish and shown to be highly efficiently integrated into the genome compared to other conventional systems. In order to apply this transgenic system to the above cichlid fish, we have started control experiments using zebrafish.

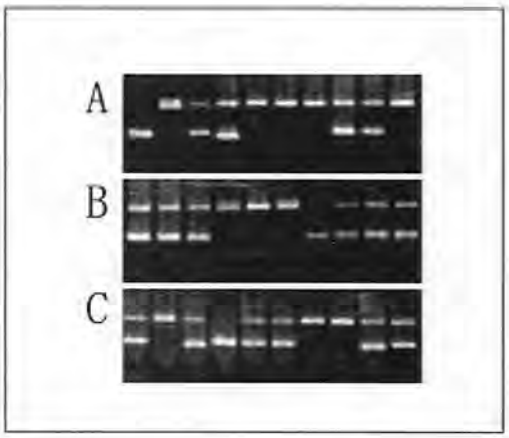

Figure 4. Polymorphism of insertion of a retroposon sequence at a single locus is shared among cichlids species in Lake Victoria. Panels A-C correspond to different species, and each panel includes results of PCRs from ten different individuals. In each panel, an upper band is observed only if the sequence of the retroposon exists at one or both of the alleles at the investigated locus whereas a lower band is observed if one or both of the alleles did not contain a sequence of the retroposon. Individuals showing both bands indicate heterozygote.

Original papers:

Terai Y, Takahashi K, Nishida M, Sato T, Okada N (2003) Using SINEs to probe ancient explosive speciation: "Hidden" radiation of African cichlids? *Mol. Bio. Evol.* **20**: 924-930.

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DEPARTMENT OF DEVELOPMENTAL BIOLOGY

Chairperson: MOROHASHI, Ken-ichirou

DIVISION OF REPRODUCTIVE BIOLOGY
DIVISION OF CELL DIFFERENTIATION
DIVISION OF MORPHOGENESIS
DIVISION OF DEVELOPMENTAL BIOLOGY (ADJUNCT)

The department is composed of three divisions and one adjunct division. Department members conduct molecular analysis on various aspects of developmental phenomena including (1) gonadal sex differentiation and gametogenesis, (2) gene regulation in cell differentiation and growth, and (3) molecular basis of body plans.

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	OKUBO Kataaki
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	YI, Mei-Sheng
	BHANDARI, Ramji
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	UNO, Eri
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The pituitary-gonadal axis plays an important role in regulating gametogenesis in vertebrates. Gonadotropins typically act through the biosynthesis of gonadal steroid hormones which in turn mediate various stages of gametogenesis. Their effects are particularly profound in teleost fishes which provide excellent models for investigating the basic hormonal mechanisms regulating gonadal sex differentiation and gametogenesis Our research focuses on (1) the identification of regulators and steroidal mediators involved in sex determination, gonadal sex differentiation and gametogenesis, and (2) the mechanisms of synthesis and action of these mediators.

I. Sex-determining gene of medaka

Although the sex-determining gene *SRY/Sry* has been identified in mammals, no comparable genes have been found in non-mammalian vertebrates. To identify such a sex-determining gene, a positional cloning approach is suitable. The medaka, *Oryzias latipes*, has two major advantages for genetic research: a large genetic diversity within the species and the existence of several inbred strains. As in mammals, sex determination in medaka is male heterogametic, although the Y chromosome is not cytogenetically distinct. Alteration of phenotypic sex with no reproductive consequences, and recombination over the entire sex chromosome pair, suggest that there are no major differences, other than a sex-determining gene, between the X and Y chromosomes.

Medaka possesses a stable genetic XX/XY sex determining system. Using positional cloning and detailed sequence analysis of BAC clones by shotgun sequencing, we identified *DMY* (DM domain gene on the Y chromosome) as a strong candidate for the sex-determining gene of medaka. *DMY* encodes a protein of 267 amino acids including the highly conserved DM domain. The DM domain was named after a related DNA binding motif found in two proteins, *dsx* and *mab-3*, involved in sexual development in *Drosophila* and *C. elegans*, respectively. Two naturally occurring *XY* female medaka showed different mutations in the *DMY* gene. One of these mutants was found to carry a mutation causing a frameshift and premature termination of the DMY protein. When mated, all XY offspring with the mutant Y were female. The other mutant had a severe depression in *DMY* expression in the embryo and 60% of its XY offspring with the mutant Y developed as females. More recently, we showed that a 117-kilobase genomic DNA fragment carrying *DMY* was sufficient to induce testis differentiation and subsequent male development. These loss- and gain-of-function studies indicate that *DMY* is the sex-determining gene of medaka. *DMY* provides the first example of a sex-determining gene in non-mammalian vertebrates. *O. curvinotus* also has the *DMY* gene on the Y chromosome, which is homologous to the Y chromosome of medaka, and that *DMY* is expressed in XY embryos. A phylogenetic tree based on the amino acid sequence including the DM-domain shows that *DMY* was derived from *DMRT1* immediately before speciation of *O. latipes* and *O. curvinotus* (Fig. 1). The branch length of *DMY* is longer than that of *DMRT1* in the phylogenetic tree. This means that *DMY* has more mutations than *DMRT1* and suggests that *DMY* accumulated these mutations after it acquired its sex-determining function. These results suggest that a new sex-determining gene generated by a gene duplication event in the sex chromosome tends to evolve fast.

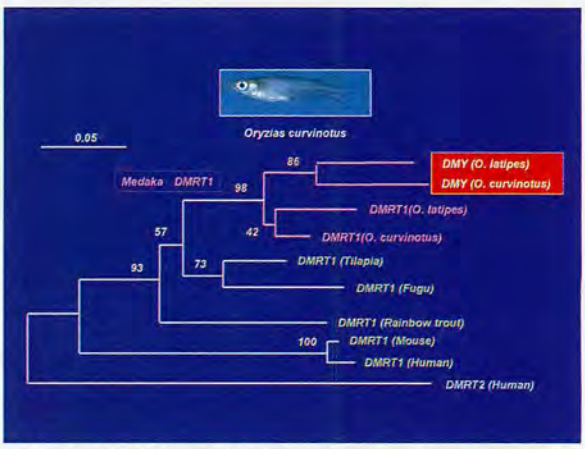

Fig. 1 A neighbor-joining tree based on the amino acid sequences from positions 21 to 109 of DMY proteins in O. *latipes* and *O. curvinotus*. The scores above each branch represent bootstrap values of 1000 times.

II. Endocrine regulation of gonadal sex differentiation

Nile tilapia, *Oreochromis niloticus*, is an excellent example of the precise nature of steroidogenic actions during gonadal sex differentiation. In this fish, all genetic female (XX) or male (XY) broods can be obtained by artificial fertilization of normal eggs (XX) and sex-reversed, pseudo male sperm (XX), or normal eggs (XX) and super male sperm (YY), respectively. Fertilized eggs hatch after 4 days at 26°C. On the day of hatching, primordial germ cells (PGCs), are located in the outer layer of the lateral plate mesoderm around the hind gut. At 3 days post-hatching, PGCs are located in the gonadal anlagen after the formation of the coelomic cavity in the lateral plate mesoderm rather than through active mi-gration.

In tilapia, ovarian differentiation is initially marked by the appearance of an narrow space in the stromal tissue representing the formation of the ovarian cavity. Using all-female tilapia fries, we showed that steroid-producing cells in XX gonads prior to and during sex differentiation express all of the steroidogenic enzymes required for estradiol-17β biosynthesis from cholesterol. Transcripts of estrogen receptors (ER) α and β first appear in both female and male gonads of fries at 5-10 days after hatching. These results, together with evidence of masculinization of genetic females by fadrozole (an aromatase inhibitor) or tamoxifen (an estrogen receptor antagonist), strongly suggest that endogenous estrogens act as the natural inducers of ovarian differentiation in tilapia. In contrast, the ability of steroid-producing cells to synthesize steroid hormones in the testes only appears at the time of testicular differentiation, suggesting that unlike in females, in males sex steroids do not play any major roles in testicular differentiation in tilapia. This was further confirmed by the absence of transcripts of androgen receptors in gonads of genetic males during sex differentiation. We have cloned several genes which were reported to be involved in gonadal sex differentiation of other vertebrates including *Sox9*, *Dax1*, *Ad4BP/SF-1*, etc. Among these genes, *DMRT1* was the only gene that was shown to be expressed male-specifically in the gonads (Sertoli cells) during sex differentiation, suggesting an important role for *DMRT1* in testicular differentiation.

III. Endocrine regulation of spermatogenesis

Using an organ culture system for eel testes consisting of spermatogonia and inactive somatic cells, we have shown that the hormonal regulation of spermatogenesis in eel testes involves the gonadotropin stimulation of Leydig cells to produce 11-ketotestosterone (11-KT), a potent androgen in fish (Fig. 2). In turn, 11-KT activates Sertoli cells to stimulate the production of activin B. Addition of recombinant eel activin B to the culture medium induced proliferation of spermatogonia, producing late type B spermatogonia, within 15 days in the same manner as did 11-KT. cDNAs encoding two androgen receptors (ARα and ARβ) have been cloned, for the first time in any vertebrates, from eel and tilapia testes. *In situ* hybridization reveals that although both AR mRNAs are present in eel testes prior to HCG injection, only ARα transcripts increase during HCG-induced spermatogenesis suggesting that ARα and ARβ play different roles in spermatogenesis. Activin B binds to activin type I and II receptors on spermatogonia to stimulate *de novo* synthesis of G1/S cyclins and CDKs leading to the initiation of mitosis. Interestingly, cyclin A1 transcripts are first detected in primary spermatocytes during HCG-induced spermatogenesis.

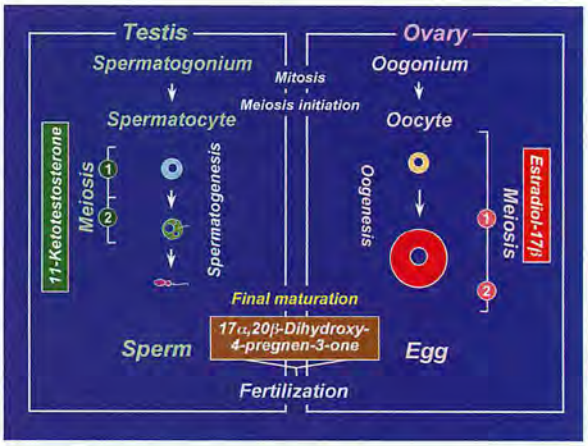

Fig. 2 Hormonal regulation of gametogenesis in fish. Sex steroid hormones produced by gonadal somatic cells under the influence of pituitary gonadotropins stimulate various stages of gametogenesis including spermatogenesis, oogenesis, and final gamete maturation.

IV. Endocrine regulation of oocyte growth and maturation

Gonadotropins (FSH and LH) require two major steroidal mediators, estradiol-17β and 17α, 20β-dihydroxy-4-pregnen-3-one (17α, 20β-DP) to act as critical hormones to execute oocyte growth and maturation, respectively (Fig. 2). Two cell-type models in which the thecal layer provides precursor steroids to the granulosa layer, have been demonstrated for estradiol-17β and 17α, 20β-DP production. Ad4BP/SF-1 serves as a transcriptional regulator for ovarian cytochrome P450 aromatase (P450arom) which converts testosterone to estradiol-17β in granulosa cells during vitellogenesis. LH induces a distinct shift in steroidogenesis, i.e. from estradiol-17β to 17α, 20β-DP as well as the steroidogenic enzyme genes from P450arom to 20β-hydroxysteroid dehydrogenase (20β-HSD), in the granulosa layers of ovarian follicles prior to oocyte maturation. The triggering of the steroidogenic shift by LH is manifested through two molecular mechanisms, the first being the subjugation of the expression of Ad4BP/SF-1 vis a vis P450arom, and the second, the induction of over-expression of 20β-HSD probably via CREB.

Unlike estradiol-17β (genomic action), 17α, 20β-DP binds to a novel, G-protein-coupled membrane receptor (non-genomic action), leading to the *de novo* synthesis of cyclin B, the regulatory component of maturation-promoting factor (MPF), which activates a preexisting 35-kDa cdc2 kinase via phosphorylation of its threonine 161 by a threonine kinase (MO15), thus producing the 34 kDa active cdc2. Upon egg activation, MPF is inactivated by degradation of cyclin B. We showed that the 26S proteasome initiates cyclin B degradation through the first cut of its NH_2 terminus at lysine 57.

An endocrine-disrupting chemical, diethylstilbestrol (DES), a nonsteroidal estrogen, triggers oocyte maturation in fish. The morphology (the time course of the change in germinal vesicle breakdown) and an intracellular molecular event (the *de novo* synthesis of cyclin B) induced by DES were indistinguishable from those induced by 17α, 20β-DP. A synergistic action of DES on 17α, 20β-DP-induced oocyte maturation was observed. Both 17α, 20β-DP- and DES-induced oocyte maturation was inhibited by an antibody against the 17α, 20β-DP receptor. These results suggest that DES may act on the 17α, 20β-DP receptor as an agonist of 17α, 20β-DP.

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Although sexual dimorphism manifests most obviously in the gonads (testis and ovary), it can be observed in the other parts of the entire animal body. For instance, it is well known that several tissues such as the external genitalia, muscle, and brain exhibit sexual dimorphisms in terms of their structures and functions. This process of sex differentiation can be divided into three steps. The first occurs at fertilization, during which the sexes of fertilized eggs are determined genetically by combination of sex chromosomes. As the second step, the individuals carrying XY and XX sex chromosomes develop the testis and ovary, respectively. Sex differentiation of the gonads usually proceeds during fetal stages. Thereafter, the gonads control the sexes of the whole body through the function of sex steroids synthesized in the sexually differentiated gonads. Therefore, the gonadal sexes are quite important for sex differentiation of the animals.

It has been established that a number of transcription factors play crucial roles in the process of gonadal differentiation. Some of these factors, such as SRY, WT1, DAX-1, SOX9 and ARX were identified as the products of genes responsible for human diseases that display structural and functional defects in tissues including the gonads. The functions of the other transcription factors such as Ad4BP/SF-1, Emx2, M33, and Lhx9 were defined by the phenotypes of the gene disrupted mice. In addition, the expression profiles with respect to their distribution and sexual dimorphism strongly suggested the functional significance at the early stage of gonadal differentiation. However, it remains to be clarified how the transcription factors above regulate their target gene transcription and how the genes encoding the transcription factors are regulated by upstream regulators. Studies from above two directions are quite important to define the gene regulatory cascade and the molecular mechanisms that supports sex differentiation of the gonad.

I. Function of AhR (dioxin receptor) in the ovary.

It has been well documented that dioxins acts as an endocrine disruptor through estrogenic action. However, the molecular mechanisms underlying this action have been largely unknown. We have clarified it by focusing on the reproductive defects displayed in the AhR gene disrupted mouse. The gene disrupted female mice showed significantly reduced fertility probably due to disordered estrus cycle, low concentration of ovarian estradiol, and reduced numbers of ovulated eggs. Since estradiol is a representative sex steroid of female mainly produced in the ovary by successive reactions of steroidogenic enzymes, the expression of these steroidogenic genes were investigated, and found that the expression of cytochrome P450 aromatase catalyzing the final step of estrogen synthesis is affected significantly in the gene disrupted mouse. Showing a good correlation, estrogen treatment rescued the reduced ovulation capacity. This remarkable phenotype suggested that AhR regulates the *P450 aromatase* gene transcription. Indeed, reporter gene analysis indicated that the mouse and human *P450 aromatase* genes were activated by AhR.

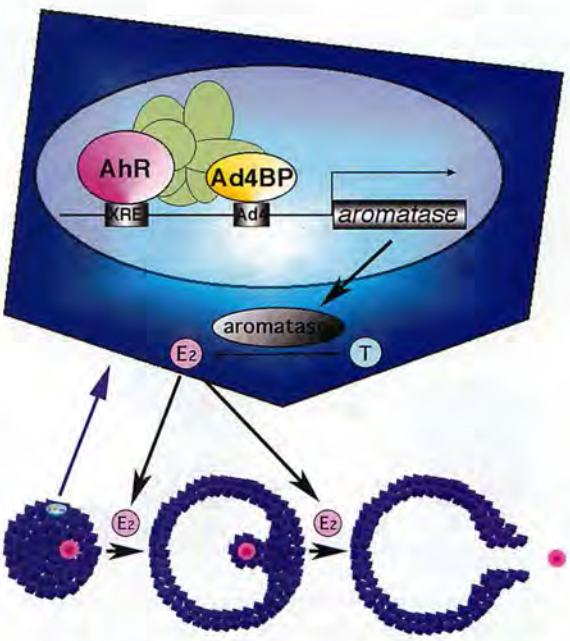

Fig. 1, During maturation process of eggs, estrogen (E2) stimulates follicular growth and rapture. AhR together with Ad4BP/SF-1 were elucidated to regulate *P450 aromatase* gene expression.

Our previous study revealed that Ad4BP/SF-1, a member of nuclear receptor, is expressed specifically in the steroidogenic tissues including the ovary, and implicated in regulation of the steroidogenic genes. Therefore, it was investigated whether AhR activates the *P450 aromatase* gene transcription synergistically with Ad4BP/SF-1. Interestingly, reporter gene assays showed synergy between the two factors. Showing a good correlation, these two factors were indicated to interact mutually, and moreover chromatin immunoprecipitation assays with anti-AhR and anti-Ad4BP/SF-1 antibodies revealed that both factors bind their recognition sequences on the *aromatase* promoter and formed a protein complex in the ovarian cells. Thus, together with the *AhR* KO phenotype, it was concluded that AhR regulates the *P450 aromatase* gene transcription with Ad4BP/SF-1, and thereby regulates estrogen synthesis in the ovary (Fig. 1). This finding clearly elucidated that dioxins through binding to AhR exert estrogenic action by upregulating *P450 aromatase* gene expression in the ovary. (This study was performed as collaboration with Prof. Fujii-Kuriyama (Tsukuba University).)

II. Characterization of factors interacting with Ad4BP/SF-1

In order to define the function of Ad4BP/SF-1, we have screened a two-hybrid library prepared form mouse embryonic gonads. UBC9, PIAS1 and PIAS3, all of which have been indicated as components for sumoylation reaction (conjugation of SUMO (small ubiquitin-like modifier)), were isolated as the interacting proteins. Thus, we investigated whether Ad4BP/SF-1 is sumoylated *in vivo* and *in vitro*. Immunoprecipitation study from cultured cells showed that at least a part of Ad4BP/SF-1 is sumoylated in cultured cells. *In vitro* reconstitution study using purified components revealed clearly that sumoylation occurs at two lysine residues, K119 and K194, of Ad4BP/SF-1. Moreover, interestingly, sumoylation at K194 is essential for the second sumoylation at K119, suggesting that conjugation of SUMO induces conformational alteration of Ad4BP/SF-1.

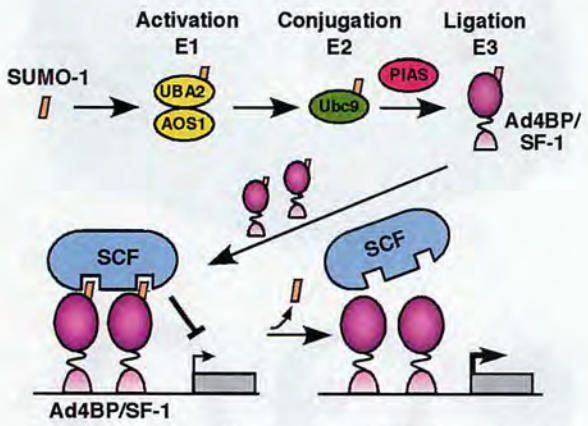

Fig. 2, Sumoylation (conjugation of SUMO) consists of three step reactions successively catalyzed by UBA2-AOS1 (E1), UBC9 (E2), and PIAS (E3). Reporter gene assays indicated that unsumoylated form of Ad4BP/SF-1 enhances synergistic transcription. This observation suggested the presence of a putative synergy control factor (SCF).

With respect to the physiological function of SUMO, we have investigated whether sumoylated Ad4BP/SF-1 differentially activates transcription, since sumoylation consensus sequence is overlapped with that of synergy control region originally defined as a suppression domain for synergistic transcriptional activation. Ad4BP/SF-1 carrying mutation at the sumoylation site enhanced synergistic transcription with reporter gene driven by multiple Ad4 binding sites. It was established that *Müllerian inhibiting substance* (*MIS*) gene is regulated by SOX9 synergistcally with Ad4BP/SF-1. Interestingly, SOX9 has a sumoylation consensus sequence and in fact sumoylated as well. Therefore, we asked whether sumoylation has effects on the synergy between the two transcription factors. Expectedly, unsumoylated forms activated the synergistic transcription of the MIS gene more than wild type. To define the molecular mechanisms underlying the synergy enhancement, we asked with *in vitro* sumoylated Ad4BP/SF-1 whether sumoylation affects DNA binding activity and interaction with SOX9. However, sumoylation was found to have no effect on both aspects, strongly suggesting the presence of a putative synergy control factor (Fig. 2). Although the physiological significance of sumoylation has been controversial, identification of factors interacting preferentially with the sumoylated Ad4BP/SF-1 such as the synergy control factor will probably give us quite valuable information. (This study has been performed as collaboration with Profs. Kikuchi (Hiroshima Univ.), Shirakawa (Yokohama City Univ.), and Yamanaka (Kyushu Univ.).)

III. Function of growth factors during early stages of gonad differentiation.

The gonad as well as the reproductive tracts, kidney, and adrenal cortex are derived from a part of the intermediate and lateral plate mesoderms. In addition, the mesonephros essential for the early gonadal development also originates from the intermediate mesoderm. Although the mesonephros was elucidated as the source of certain cell types constituting the testis, its contribution to gonad formation has been largely unknown. We examined in detail the expression profiles of *Wnt4*, *Fgf8*, and *Fgf9* in the developing mesonephros of chick embryos, and found that the expressions of these factors are spatially and temporally correlated with those of the marker genes for the gonad and/or adrenal cortex such as *Ad4BP/SF-1* and *Dmrt-1* (*doublesex- and mab-3-related transcription factor*). As was shown previously with the rat fetuses, it was confirmed with the chick embryos that these two tissues are derived from a single cell population, the adreno-gonadal primordium. Thereafter, the primordium divided into two cell populations, one of which is the gonadal primordium and the other of which is the

adrenal primordium. All of these cells are identified as immunoreactive cells for Ad4BP/SF-1. The expression of the growth factors, Wnt4, Fgf8, and Fgf9, are detectable in the nephroginous mesenchyme (feature mesonephros) and developing nephric tubules at the stage of the early gonad and adrenocortical development. Interestingly, the expressions of Wnt4 and Fgf9 are correlate with those of Ad4BP/SF-1 and Dmrt1. Therefore, it was assumed that these growth factors regulate the expression of the gonadal and adrenocortical marker gene expressions.

In order to define it, *Wnt4* and *Fgf9* were misexpressed with replication competent virus system in the gonad and adrenal cortex of the early developmental stages. Misexpression of *Wnt4* expanded the expression of *Ad4BP/SF-1* as the marker for the adrenogonadal primordium while failed to expand the expression of Dmrt-1. Interestingly, misexpression of *Fgf9* expanded the expression of *Dmrt-1* and probably thereby resulted in additional gonad formation. In fact, the expressions of marker genes for the gonad were observed even in the additional gonad (Fig. 3). Moreover, chemical inhibitor for Fgf receptor downregulated the *Dmrt-1* expression. Both gain-of-function and loss-of-function treatments above induced no obvious structural alteration of the adrenal cortex, indicating that Fgf9 induced gonad development probably through stimulating cell proliferation or alternatively through trans-determining cell fate into the gonad. This study demonstrated that *Wnt4* and *Fgf9* expressed in the developing mesonephros are involved in the early stages of the gonad and adrenocortical development through distinct functions.

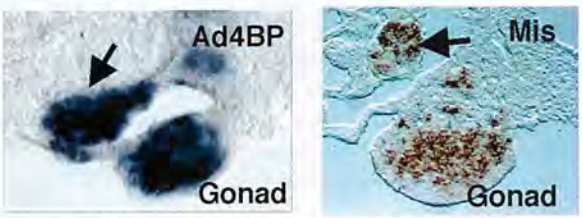

Fig. 3, Misexpression of Fgf9 induced additional gonad formation as indicated by arrows. Ad4BP/SF-1 and Mis are both expressed in the additional gonads.

In the case of mammals, a gene disruption study indicated that *Fgf9* is essential for testicular differentiation. However, in the chick the misexpression of *Fgf9* was unable to induce the expression of the testicular marker gene in the female gonad. This clear discrepancy might be due to the difference of experimental conditions, one of which is gene disruption (loss-of-function) study and the other is misexpression (gain-of-function) study, or due to difference of animal species. (This study has been performed as collaboration with Dr. Yoshioka in Hyogo Univ. of Teacher Education.)

In addition to the studies described above, we have investigated tissue-specific enhancers localized in the *Ad4BP/SF-1* gene. Recently, we identified a fetal adrenocortical-specific, pituitary-specific, and ventromedial hypothalamic nucleus-specific enhancers, and their fine structures have been clarified. Investigation of the factors to bind the enhancers will be essential to clarify the mechanism underlying the tissue differentiation.

It has been known that several gene disruption studies revealed their implication into gonad differentiation and gonad sex differentiation. However, functional and genetic correlation among the genes have remained to be investigated. As the investigation of the genetic correlation, analyses for genetic interaction have been performed with a certain lines of gene disrupted mice, of which genetic backgrounds have been fixed into C57B/6 and FVB in addition to the original 129J. Some of the analyses already indicated the presence of the genetic interaction, and the molecular basis for the interaction is under investigation.

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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, transcription factors which act cell-autonomously are thought to be essential for the determination of cell fates. Our main interest is to know how pattern formation and morphogenesis during development is regulated by these growth factors and transcription factors. We address this problem using several model animals, including frog, fly and ascidian, employing embryology, genetics, molecular and cellular biology, and biochemistry. In addition, we have recently introduced genome-wide approaches to elucidate precise genetic program controlling early development.

I. Gastrulation movement regulated by Wnt signaling

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

We have previously demonstrated that *Xenopus prickle* (*Xpk*), *a Xenopus* homologue of a *Drosophila* PCP gene, is an essential component for gastrulation cell movement. Both gain-of-function (GOF) and loss-of-function (LOF) of *Xpk* severely perturbed gastrulation and caused *spina bifida* embryos without affecting mesodermal differentiation. We also demonstrated that XPK binds to *Xenopus* Dsh as well as to JNK. This suggests that XPK plays a pivotal role in connecting Dsh function to JNK activation. To understand the molecular mechanism, we identified proteins which binds to XPK by yeast two-hybrid screening. One of XPK binding protein was found to be a member of the Ste20 kinase family and named as *Xenopus* prickle-interacting kinase, XPIK. Developmental expression pattern of XPIK is reminiscent of that of XPIK, suggesting functional interaction between XPK and XPIK. GOF and LOF of XPIK resulted in perturbation of gastrulation, leading to shortened or *spina bifida* embryo. Furthermore, we have found that XPIK is not only sufficient to activate JNK in embryo, but also required for full activation of JNK by Dishevelled. These suggest that XPIK also plays an essential role in connecting extracellular Wnt signal to JNK activation through Dishevelled and XPIK.

In addition, we have been attempting to identify novel regulatory components controlling gastrulation cell movements by an expression cloning method based on morphology of dorsal marginal zone explant (Keller's explant) and of embryo. After 1,500 clones were examined by overexpression in the dorsal region of embryos, approximately 5% of clones were found to perturb normal gastrulation cell movements. Functional relevance of identified genes to cell movements is currently under investigation.

II. Regulation of actin cytoskeletal dynamics during *Xenopus* gastrulation

Because gastrulation movements are accompanied by dynamic changes in cell polarity, morphology, and motility, it is very likely that actin cytoskeleton is carefully regulated. Thus, we analyzed the regulatory mechanism of actin cytoskeletal dynamics during this process. Among several factors implicated in the regulation of the actin cytoskeleton, we decided to focus on myristoylated alanine-rich C kinase substrate (MARCKS). MARCKS is an actin-binding, membrane-associated protein previously implicated in the regulation of F-actin dynamics. It has been investigated mainly in tissue culture cells and biochemical analyses. *MARCKS* knock-out mice has been reported to show neural tube defects. Yet, its molecular mechanism has not been elucidated. We demonstrate that MARCKS is essential for gastrulation movements and neural tube closure in *Xenopus* embryos. Cell biological analyses

revealed that MARCKS knock-down with Morpholino oligo (MO) significantly reduced cortical actin formation and caused defects in cell polarity, adhesion, motility and protrusive activity, leading to these developmental defects. We also showed that the Wnt pathway dramatically promoted the formation of lamelipodia- and filopodia-like protrusions and MARCKS is required for this activity. These findings show that the Wnt signaling pathway regulates cortical actin dynamics and MARCKS is requisite for the Wnt function. We conclude that MARCKS is essential for dynamic morphogenetic movements during embryogenesis.

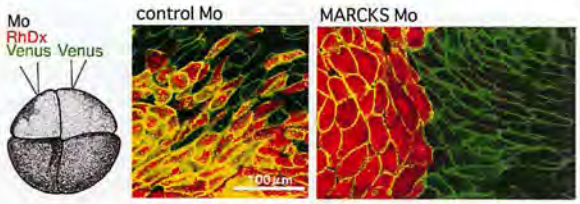

Figure 1 MARCKS is required for convergent extension movements. *MARCKS* Mo or control Mo, Rhodamine dextran (RhDx), and Venus mRNA were co-injected into one of the two dorsal blastomeres. Venus mRNA alone was injected into the other dorsal blastomere. In the absence of *MARCKS* Mo, red cells and non-red cells were polarized and intercalated. In contrast, *MARCKS* Mo-injected cells were not polarized and did not participate in the intercalation.

III. Genetic screening for novel DPP/BMP signaling components utilizing *Drosophila* model system

Drosophila is one of the ideal model organisms to dissect signal transduction pathway by genetic methods. We have carried out dominant suppressor screening for a transgenic mutant fly that expresses activated DPP/BMP type-I receptors in wing imaginal discs. We isolated 19 suppressor mutants, *Suppressor of constitutively activated Dpp singnaling* (*Scad*). Alleles of *punt*, *Mad*, *shn* and *dCrebA* were found in isolated Scad mutants. Most of the Scad mutants encode a nuclear protein suggesting these molecules regulates DPP signaling at nuclear level.

We now focus to study one of the mutants *Scad67*. *Scad67* was also isolated by a Mexican group and named as *tonnalli* (*tna*). We also isolated vertebrate homologs of *Scad67/tna*, TONAS-1 and TONAS-2. The most characteristic feature of these proteins is the existence of a single SP-RING finger motif in the middle. The SP-RING motif was originally found in the PIAS family SUMO-E3 ligase proteins. We found that TONAS has SUMO-E3 ligase activity and TONAS facilitates specific SUMO-2/3 conjugation to TONAS itself. TONAS also shows strong activity of nuclear body formation in cultured cells. TONAS effectively recruits transcriptional regulators including PML, CBP and P300 to the nuclear body.

It has been shown that the Trithorax group componets are essential factors in ATP-dependent chromatin remodeling complex. Our results suggest a role for Scad67/TNA and TONAS in the connection of the Trithorax/SWI/SNF chromatin remodeling complex to CBP/P300, the relocation of these protein complexes into the nuclear substructure, and the regulation of gene expression.

IV. Brachyury downstream notochord differentiation in the ascidian embryo

Ascidians, urochordates, are one of the three chordate groups, and the ascidian tadpole is thought to represent the most simplified and primitive chordate body plan. It contains a notochord, which is a defining characteristic of chordate embryo composed of only 40 cells. To understand the morphogenesis in this simple system, we have focused on a gene, *Brachyury*, which is known to play an important role in the notochord development. In ascidian, *Brachyury* is expressed exclusively in the notochord and the misexpression of the *Brachyury* gene (*Ci-Bra*) of *Ciona intestinalis* is sufficient to transform endoderm into notochord. This gene encodes a sequence-specific activator that contain a T-box DNA-binding domain, and in vertebrates, it is initially expressed throughout the presumptive mesoderm and gradually restricted to the developing notochord and tailbud. The phenotype of the *Brachyury* mutants in mice and zebrafish revealed that this gene is essential for notochord differentiation. Our goal is to elucidate the down stream pathway of this important gene in ascidian in order to set the stage for understanding not only the formation and function of the notochord but how this important structure has evolved. We conducted the subtractive hybridization screens to identify potential *Brachyury* target genes that are induced upon *Ci-Bra* overexpression. Out of 501 independent cDNA clones that were induced, 38 were specifically expressed in notochord cells. We characterized of subcellular-localizations of the 20 GFP fusion gene products in the notochord cell. These products observed after electroporating the embryos at one-cell stage with GFP fusion gene containing notochord specific promoter. They showed various subcellular localizations in the notochord cells of the tadpole tails. In addition to investigate the actual functions of the genes during the notochord formation, functional analyses were performed by injecting of antisence morphollino oligos.

V. Comprehensive analysis of developmentally regulated genes using cDNA microarray

In order to examine the global expression profile during early development of *Xenopus laevis*, we have collected massive EST sequences from three normalized cDNA libraries of early gastrula, neurula and tailbud stage. To date, more than 100,000 ESTs (~30% of *Xenopus laevis* ESTs registered in the public database) were produced. Using these cDNA clones, we generated the NIBB 40k cDNA macroarray and 4.6k non-redundant cDNA microarray. With the DNA

arrays, we conducted a series of large-scale screening of the genes which expressions are regulated by some transcription factors and signaling factors (Xnr-1, FAST1, FGF etc.). Consequently, we have been able to isolate lots of the candidate genes that encode a variety of signal transduction and transcription regulatory components, and also cytoskeletal components, suggesting dynamic cellular changes in the early development of *Xenopus laevis*. This also proves DNA array to be an effective screening assay for novel genes which function during early development process of *Xenopus laevis*.

Adding the EST sequences, the assembled sequences and their annotation information are available through the web at NIBB *Xenopus laevis* EST database XBD2 (Fig.2). XDB2 also provides the whole mount *in situ* hybridization images as the spatial expression pattern (Fig.3).

Figure 2 XDB2 (http://xenopus.nibb.ac.jp).

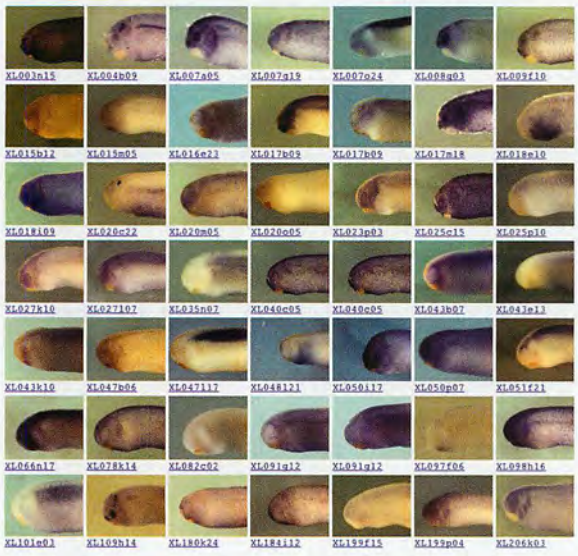

Figure 3 Whole mount *in situ* hybridization images.

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DEPARTMENT OF REGULATION BIOLOGY

Chairperson: NODA, Masaharu

DIVISION OF MOLECULAR NEUROBIOLOGY
DIVISION OF CELLULAR REGULATION
DIVISION OF BIOLOGICAL REGULATION
AND PHOTOBIOLOGY (ADJUNCT)
DIVISION OF BEHAVIOR AND NEUROBIOLOGY (ADJUNCT)

The department is composed of two regular divisions and two adjunct divisions. The study of this department is directed towards molecular mechanisms for the development and functioning of the central nervous systems in vertebrates, and also those for the signal transduction of plants and microorganisms with respect to the environmental factors, such as light, temperature and salinity.

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	TAKAO, Motoharu (Apr. '03~)
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	ETANI, Kazuma (Apr. '03~)
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We have been studying the molecular and cellular mechanisms underlying the development of the vertebrate central nervous system. It covers all the major events including the patterning of the nervous system, neuronal differentiation, axonal navigation and targeting, synapse formation and plasticity, and neuronal regeneration, mainly in the visual system. The scope of our interests also encompasses various functions of the matured brain, including sensation, behavior, learning and memory.

I. Regional specification in the retina and topographic retinotectal projection

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

Since 1992, we have been devoting our efforts to searching for molecules with asymmetrical distribution in the embryonic chick retina, and to characterization of their roles in the topographic retinotectal projection. In 1996, we first identified two winged-helix transcriptional regulators, *CBF-1* and *CBF-2*, expressed in the nasal and temporal retina, respectively. Misexpression experiments in the retina using a retroviral vector showed that these two transcription factors determine the regional specificity of the retinal ganglion cells, namely, the directed axonal projections to the appropriate tectal targets along the anteroposterior axis. Secondly, we identified a novel retinoic acid-generating enzyme, RALDH-3, which is specifically expressed in the ventral region of the retina, together with a dorsal-specific enzyme RALDH-1.

Furthermore, we recently identified a novel secretory protein, Ventroptin, which has BMP-4 neutralizing activity. *Ventroptin* is expressed in the retina with a ventral high-dorsal low gradient at early stages. This expression pattern is complementary to that of *BMP-4*. At later stage (E6~), a nasal high-temporal low gradient expression pattern of it is also detected. *Ventroptin* thus shows a double-gradient expression profile along the dorsoventral and nasotemporal axes. Misexpression of *Ventroptin* altered expression patterns of several topographic genes and projection of the retinal ganglion-cell axons to the tectum along the both axes.

In this year of 2003, we revealed that misexpression of *CBF-1* represses the expression of *EphA3* and *CBF-2*, and induces that of *SOHo-1*, *GH6*, *ephrin-A2* and *ephrin-A5*. *CBF-1* controls *ephrin-A5* by a DNA binding-dependent mechanism, *ephrin-A2* by a DNA binding-independent mechanism, and *CBF-2*, *SOHo-1*, *GH6* and *EphA3* by dual mechanisms (Fig. 1A). *BMP-2* expression begins double-gradiently in the retina from E5 instead of *BMP-4* in a complementary pattern to the *Ventroptin* expression. Ventroptin antagonizes BMP-2 as well as BMP-4. CBF-1 interferes in BMP-2 signaling and thereby induces expression of *ephrin-A2*. Our data suggest that *CBF-1* is located at the top of the gene cascade for the regional specification along the nasotemporal (NT) axis in the retina (Fig. 1B) and distinct BMP signals play pivotal roles in the topographic projection along both axes.

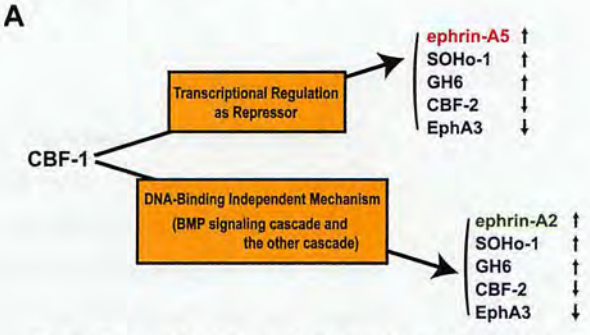
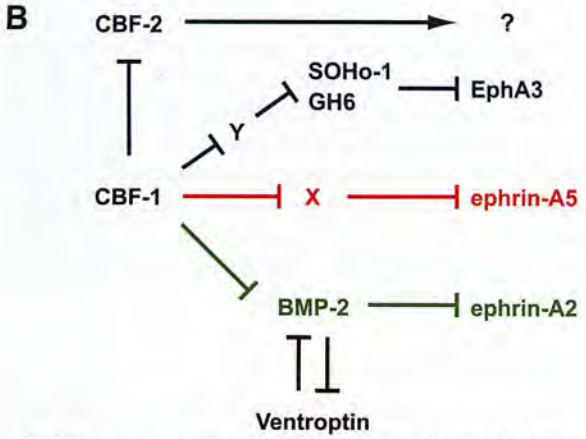


Fig.1. The molecular mechanisms by which CBF-1 controls the expression of topographic molecules. (A) Schematic representation of modes of actions of CBF-1. You can see that one Eph-ephrin system is controlled as a set by each mode of CBF-1 action.



(B) Expressional regulation of the topographic molecules along the NT axis by CBF-1. *EphA3* and ephrins are directly implicated in the control of axon guidance. *CBF-1* and *Ventroptin* repress expression of *BMP-2* by inhibiting BMP signaling as an interrupter and antagonist, respectively, and induce *ephrin-A2* expression. *CBF-1* represses the transcription of negative regulators, *X* and *Y*. *SOHo-1* and *GH6* inhibit the expression of *EphA3*. *CBF-1* also represses *CBF-2* expression, however, its downstream target genes have not been identified yet.

Currently, with respect to the other identified molecules, we are conducting misexpression experiments using chick embryos and generating knockout and transgenic mice to elucidate the molecular functions. We expect that our studies will lead to dissection of the molecular mechanism underlying the retinal patterning and topographic retinotectal projection, and ultimately to uncovering the basic principles for establishing complicated but extremely precise neural networks.

II. Axonal morphogenesis and behavior

During development, cells undergo dynamic morphological changes by rearrangements of the cytoskeleton including microtubules. However, molecular mechanisms underlying the microtubule remodeling between orientated and disorientated formations are almost unknown. We found that novel subtypes of collapsin response mediator proteins (CRMP-As) in addition to the originals (CRMP-Bs), which occur from the alternative usage of different first coding exons, are involved in this conversion of microtubule patterns.

Overexpression of *CRMP2A* and *CRMP2B* in chick embryonic fibroblasts induced orientated and disorientated patterns of microtubules, respectively. Moreover, sequential overexpression of another subtype overcame the effect of the former expression of the countersubtype. Overexpression experiments in cultured chick retinae showed that CRMP2B promoted axon branching and suppressed axon elongation of ganglion cells, while CRMP2A blocked these effects when co-overexpressed (Fig. 2). Our findings suggest that the opposing activities of CRMP2A and CRMP2B contribute to the cellular morphogenesis including neuronal axonogenesis through remodeling of microtubule organization.

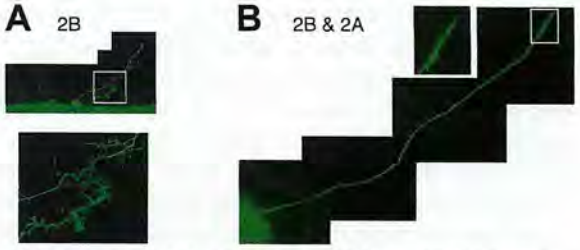


Fig.2. Antagonistic effects of CRMP2A and –2B on axon morphology. Retinae from chick E6 embryos were electroporated with IGG vector to express CRMP2B (A) and CRMP2B + CRMP2A (B), and cultured for 2 days.

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

Protein tyrosine phosphorylation plays crucial roles in various biological aspects including all stages of brain development. In 1994, we found that PTPζ/RPTPβ/Ptprz, a nervous system-rich receptor-type PTP, is expressed as a chondroitin sulfate proteoglycan in the brain. *Ptprz* is expressed from the early developmental stage to the adulthood in neurons as well as astrocytes. This suggests that this molecule plays variegated roles in the brain development and brain function.

We found in 1996 that Ptprz binds pleiotrophin (PTN)/HB-GAM and midkine (MK), closely related heparin-binding growth factors which share many biological activities. The chondroitin sulfate portion of Ptprz is essential for the high affinity binding (Kd = ~0.25 nM) to these growth factors, and removal of chondroitin sulfate chains results in a marked decrease of binding affinity (Kd = ~13 nM).

To identify the substrate molecules of Ptprz, we recently developed the yeast substrate-trapping system. This system is based on the yeast two-hybrid system with two essential modifications: Conditional expression of v-src to tyrosine-phosphorylate the prey proteins and screening using a substrate-trap mutant of PTP as bait. Using this system, we successfully isolated a number of candidate clones for substrate molecules (ex. GIT1/Cat-1) and continuously-interacting molecules (ex. PSD-95/SAP90) for Ptprz. We are now continuing efforts to characterize these candidate clones.

In addition, to know the physiological roles of Ptprz *in vivo*, we generated *Ptprz*-deficient mice in which the Ptprz gene was replaced with the *LacZ* gene in 1997. We are currently studying the phenotype of *Ptprz*-deficient mice biochemically, anatomically, physiologically and ethologically.

We reported this year that mice deficient in *Ptprz* do not show mucosal damage by VacA, although VacA is incorporated into the gastric epithelial cells to the same extent as in wild-type mice (Fig. 3). The vacuolating cytotoxin VacA produced by *Helicobacter pylori* causes massive cellular vacuolation *in vitro* and gastric tissue damage *in vivo*, leading to gastric ulcers, when administered intragastrically. Primary cultures of gastric epithelial cells from *Ptprz*$^{+/+}$ and *Ptprz*$^{-/-}$ mice also showed similar incorporation of VacA, cellular

vacuolation and reduction in cellular proliferation, but only *Ptprz*$^{+/+}$ cells showed marked detachment from a reconstituted basement membrane 24 h after treatment with VacA. VacA bound to Ptprz, and the levels of tyrosine phosphorylation of Git1, a Ptprz substrate, were higher after treatment with VacA, indicating that VacA behaves as a ligand for Ptprz. Furthermore, PTN, an endogenous ligand of Ptprz, also induced gastritis specifically in *Ptprz*$^{+/+}$ mice when administered orally. Taken together, these data indicate that erroneous Ptprz signaling induces gastric ulcers.

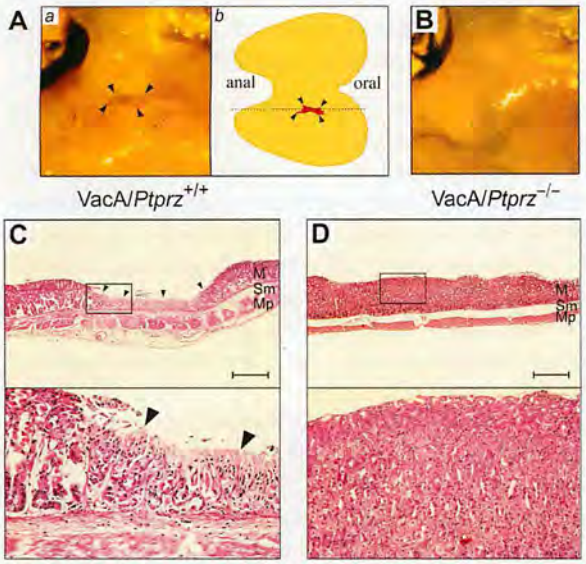


Fig.3. Pathological analyses of gastric tissues treated with VacA. (**A**,**a**) Stereomicroscopic appearance of the inside of stomachs from *Ptprz*$^{+/+}$ mice 48 h after administration of VacA (500 μg per kg body weight). (**A**,**b**) Diagrammatic representation of a showing the gastric ulcer (red area with arrowheads). (**B**) Appearance of the inside of stomachs from *Ptprz*$^{-/-}$ mice treated equally. (**C**,**D**) Gastric sections stained with hematoxylin and eosin. Loss of epithelial cells and gastric gland structure (arrowheads) was observed in the mucosal layer in *Ptprz*$^{+/+}$ mice (**C**) but not *Ptprz*$^{-/-}$ mice (**D**). The lower panels are enlargements. Scale bars: 500 μm. M, Mucosa; Sm, Submucosa; Mp, Muscularis propia.

IV. Na^+-level sensing in the brain

Sodium-level sensing system in the brain is essential for the regulation of the sodium and water balance in body fluids. Previously, we demonstrated that Na_x (also called *Na$_x$2/NaG*) gene is expressed in the circumventricular organs and Na_x-deficient mice ingest salt in excess. In Na_x-deficient mice, *c-fos* expression in the subfornical organ (SFO) and organum vasculosum laminae terminalis (OVLT) was markedly elevated as compared with wild-type mice. Stimulation of the SFO/OVLT of wild-type animals by infusion of a hypertonic sodium solution into intracerebroventricule (ICV) leads to avoidance of salt intake. In contrast, Na_x-deficient mice did not show such aversion.

We showed that Na_x is a sodium channel which is sensitive to the increase of extracellular sodium level. Entry of sodium ions occurred in response to a rise of the extracellular sodium concentration ($C_{1/2}$= 159 mM). In contrast, these responses were not observed in Na_x-immunonegative cells or neurons of Na_x-deficient mutant origin. Transfection of Na_x cDNA conferred the sodium sensitivity on Na_x-deficient cells. All of the GABA-immunopositive neurons isolated from the SFO responded to the extracellular sodium increase. Based on these findings, we proposed that GABAergic inhibitory neurons expressing Na_x control the activity of the SFO and suppress the salt-intake behavior of animals under thirst conditions.

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DIVISION OF CELLULAR REGULATION

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The major thrust of our research efforts is directed towards the comprehensive understanding of the molecular mechanisms that governs the responses of plants and microorganisms to new environmental. In particular, our attention is focused on the perception and transduction of various stress signals, such as extreme temperatures, osmosis and salinity. Another line of our research is focused on the repair mechanisms from the damage to the photosynthetic machinery under severe stress conditions. In 2003, significant progress was made in the following areas.

I. Discovery of four kinds of the two-component system responsible for perception and transduction of hyperosmotic and salt signals in *Synechocystis*.

Living organisms respond and acclimate to hyperosmotic and salt stress by changing the gene expression. We previously discovered that the cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*) responds differently to hyperosmotic stress and salt stress with respect to regulation of gene expression and changes in the cytoplasmic volume. These results suggested that this organism might recognize the two kinds of stress as different signals and perceived them by different sensors.

To investigate the components involved in the perception and transduction of hyperosmotic and salt signals in this organism, we examined all knockout mutants for 43 histidine kinases (Hik) and 42 response regulators (Rre) by a DNA microarray technique and found, in contrast to the prediction, that the four kinds of two-component signalling pathways of Hik33/Rre31, Hik34/Rre1, Hik16/Hik41/Rre17 and Hik10/Rre3 are involved in perception and transduction of both hyperosmotic and salt signals. However, each of the two-component signaling pathways except Hik16/Hik41/Rre17 was found to regulate the expression of genes that were induced specifically by either hyperosmotic stress or salt stress, although they also regulated the expression of genes which were commonly induced by these two stress signals (Fig. 1). For example, Hik34 perceives and transduces both hyperosmotic and salt signals to the response regulator Rre1 to induce the expression of different sets of genes. Although the number of genes which are regulated by the Hik10/Rre3 pathway is small, this two-component system induces different genes in response to different stimuli. These findings clearly demonstrate that the two-component systems constitute complex signal-integration pathways rather than a simple "one stimulus-one output" scheme.

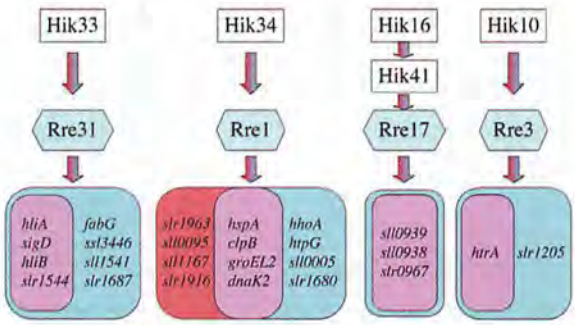

Fig.1. Schematic presentation of four kinds of two-component system involved in the perception and transduction of hyperosmotic and salt signals. Genes whose expression is induced by hyperosmotic stress, by salt stress, and commonly by the two kinds of stress via each two-component system are included in blue, red and purple rectangles, respectively.

II. Discovery of four histidine kinases as sensors of H_2O_2 signals in *Synechocystis*.

Hydrogen peroxide (H_2O_2) is produced as an inevitable consequence of aerobic life. Microorganisms detect increases in the concentration of H_2O_2 and regulate the expression of certain genes to enhance tolerance against this oxidative stress. DNA microarray analysis of genome-wide expression of genes revealed

that incubation of *Synechocystis* cells with 0.25 mM H_2O_2 dramatically changed the pattern of gene expression. This treatment induced the expression of 77 genes and repressed the expression of 55 genes with induction factors > 4.0. A half of the H_2O_2-inducible genes were specifically induced by H_2O_2 but by no other stress, such as heat, cold, light, salt and hyperosmotic stress. These findings suggest that the oxidative stress may be distinct from the general stress. Screening for mutants in the disruptant library of histidine kinases that exhibit null response to the H_2O_2-inducible expression of genes identified four histidine kinases, Hik33, Hik34, Hik2 and Hik41, as sensors of H_2O_2 signals. They regulate the expression of 25, 1, 6, and 2 H_2O_2-inducible genes, respectively (Fig. 2).

In *Bacillus subtilis*, the transcription factor PerR perceives H_2O_2 signals and regulates the expression of a group of H_2O_2-responsible genes. We also examined the genome-wide patterns of gene expression in the mutant of PerR homolog in *Synechocystis* by DNA microarray analysis. We found that PerR regulates the expression of five H_2O_2-inducible genes. These findings indicate that histidine kinases act as major sensors of H_2O_2 signals in *Synechocystis*.

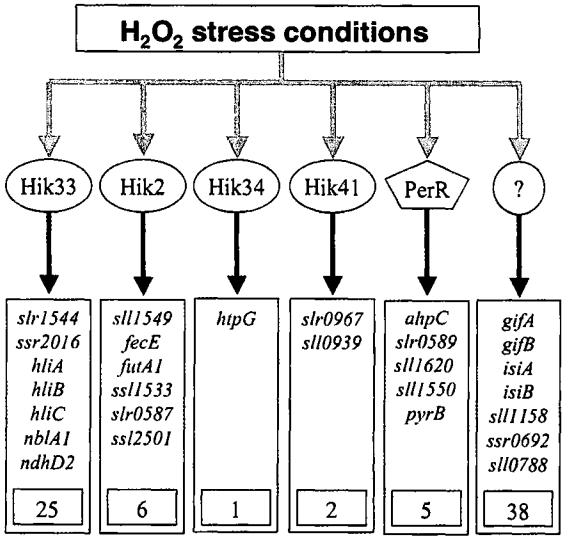

Fig.2. Schematic presentation of four histidine kinases and a transcription factor PerR involved in the perception of H_2O_2 stress and regulation of gene expression. Numbers in each box correspond to the number of H_2O_2-inducible genes with induction factors > 4.0 whose expression was regulated by each histidine kinase or the transcription factor PerR.

III. Multi-stress sensing by Hik33 in *Synechocystis*.

Our previous study indicated that Hik33 is involved in the regulation of gene expression by cold and hyperosmotic stresses. We applied the DNA microarray technique to examine whether Hik33 also perceives stress signals other than cold and hyperosmotic stresses. The results clearly showed that Hik33 is involved also in the perception of salt, H_2O_2, and strong-light stresses. However, it does not regulate the gene expression caused by heat and nutritional stresses. Nevertheless, Hik33 regulates distinct sets of genes under different stress conditions. Therefore, it is possible that Hik33 senses the various kinds of stress to differentially regulate the gene expression in a stress-specific manner. However, it should be noted that Hik33 regulates the expression of genes which are induced commonly by various kinds of stress. Interestingly, Rre31 which is a cognate response regulator of Hik33 in the gene expression due to hyperosmotic and salt stress is not involved in the signal transduction pathway of cold and H_2O_2 stresses. These results may suggest that Hik33 perceives various kinds of stimulus and sorts their signals to the stimulus-specific downstream pathways. These findings suggest that the two-component signal-transduction system is more complex than a currently accepted scheme where a single sensory histidine kinase is tightly coupled with a cognate response regulator.

IV. Environmental stress inhibits the repair of photosystem II by suppressing the transcriptional and translational activities.

Strong light impairs the photosynthetic machinery, in particular, photosystem II (PSII), *via* a process known as photodamage or photoinhibition and the PSII reaction center protein D1 is most sensitive target of photodamage. However, photodamaged PSII can be repaired by replacement of the photo damaged D1 with light-dependently synthesized D1 *de novo*.

Under natural conditions organisms are exposed to combinations of various kinds of environmental stress, such as light, salt, oxidative, heat, and cold stress, that may synergistically act on the damage to PSII. We have found that the effects *in vivo* of light on PSII are completely different from the effects of the other kinds of stress. Strong light induces photodamage to PSII, whereas the other kinds of stress inhibit the repair of the photodamaged PSII and do not accelerate damage to PSII directly. Results of molecular-biological analysis reveals that these kinds of stress inhibit either or both of the transcription and translation of several genes, in particular, *psbA* genes for D1, whose turnover is essential for the repair of PSII. These results suggest that stress inhibits the repair of PSII via suppression of the activities of both transcriptional and translational machineries. We also elucidated that the requirement of light for the repair can be explained by sustenance of the intracellular concentration of ATP via the photosynthetic transport of electrons.

V. Transformation of plants to enhance the stress tolerance of reproductive organs to salt and cold stress.

We showed previously that transformation with the *codA* gene for choline oxidase allows plants to synthesize glycine betaine (GB) and enhances their ability to tolerate various kinds of stress during germination and vegetative growth. In these years, we

examined tolerance of transformed plants to salt stress at the reproductive stages, i.e., the stages at which plants are most sensitive to environmental stress. Salt-shock treatment of wild-type plants for three days resulted in the abortion of flower buds and decreased the number of seeds per silique. Microscopic examination of floral structures revealed that salt stress inhibited the development of anthers, pistils and petals. In particular, the production of pollen grains and ovules was dramatically inhibited. These effects of salt stress were significantly reduced by transformation with the *codA* gene, and our observations suggest that the enhanced tolerance of the transgenic plants was a result of the accumulation of GB in the reproductive organs.

The *cis*-unsaturated molecular species of phosphatidylglycerol (PG) in chloroplasts have been implicated in the chilling tolerance of plants. We established homozygous lines of transgenic tobacco (*Nicotiana tabacum*) that overexpressed a cDNA for glycerol-3-phosphate acyltransferase, a key enzyme in the determination of the extent of *cis*-unsaturation of the PG, from a chilling-sensitive squash (*Cucurbita moschata*). In transgenic plants, the proportion of saturated plus *trans*-monounsaturated molecular species of PG increased from 24% to 65%. However, this change did not affect the architecture of the chloroplasts. Chilling stress also damaged inflorescences much more severely in transgenic plants than wild-type plants (Fig. 3). These observations allowed us to conclude that decreases in the proportion of *cis*-unsaturated PG enhanced the sensitivity to chilling of reproductive organs.

Fig.3. The sensitivity of inflorescence of tobacco plants to low temperature 5°C. Left: Wild-type plants which contain a high level of the *cis*-unsaturated molecular species of phosphatidylglycerol (PG). Right: Transgenic plants which contain a low level of the *cis*-unsaturated molecular species of PG. The results of this study indicate that changes in the level of unsaturated PG molecular species have a great impact on the chilling sensitivity of plants in a broad range of the life cycle.

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Szalontai, B., Kota, Z., Nonaka, H., Murata, N. (2003) Structural consequences of genetically engineered saturation of the fatty acids of phosphatidylglycerol in tobacco thylakoid membranes. An FTIR Study. *Biochemistry*, **42**, 4292-4299.

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(2) Review articles

Mikami, K., Murata, N. (2003) Membrane fluidity and the perception of environmental signals in cyanobacteria and plants. *Prog. Lipid Res.*, **42**, 527-543.

Mikami, K., Suzuki, I., Murata, N. (2003) Sensors of abiotic stress in *Synechocystis*. Topics in Current Genetics: Plant Response to Abiotic Stress, vol. 4 (eds., Hirt, H., Shinozaki, K.), Springer, Berlin, p.103-119.

DIVISION OF BIOLOGICAL REGULATION AND PHOTOBIOLOGY (ADJUNCT)

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

I. Chloroplast relocation movement

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

1- 1 *Arabidopsis*

Chloroplasts accumulate at the cell surface under weak light and escape from the cell surface to the anticlinal wall under strong light to optimize photosynthesis. We identified the photoreceptors in Arabidopsis. Phototropin2 (phot2) mediates the avoidance response under strong light and phot1 and phot2 mediate, redundantly, the accumulation response under weak light. However, components of signal transduction pathways still remain to be identified. A mutant called *chup1* is deficient in chloroplast movement and consequently chloroplasts gather at the bottom of cells. *CHUP1* is a novel gene that has a hydrophobic region in the N-terminus and an actin binding domain, a prolin-rich region, and two leucin zippers. It was confirmed that the actin-binding domain binds F-actin. When fusion proteins of the N-terminal, hydrophobic region of *CHUP1* with GFP were expressed transiently in *chup1* mutant cells, fluorescence of the fusion proteins were found in the outer part of chloroplasts, likely on the outer membrane of chloroplasts. CHUP1 is proposed to play an important role on chloroplasts movement of both accumulation and avoidance responses.

1- 2 *Adiantum phy3*

Adiantum phytochrome3 (PHY3) is a unique chimeric protein with phytochrome structure in the N-terminal half and phototropin structure in the C-terminal half. Transient expression of *PHY3* or modified *PHY3* genes in *rap* (red light-induced aphototropic) mutants of *Adiantum* reveal that phy3 is the photoreceptor of red light-induced chloroplast photorelocation movement, and that the phy3 signal might be transferred through the C-terminal, phototropin region.

The phot region of phy3 has not yet been shown to function as a blue light photoreceptor, but the above method of transient expression of modified *PHY3* in *rap* and *phot* mutants may provide experimental evidence of this possibility soon.

II. Gene targeting and gene silencing

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

2- 1 *Miniature transposable element*

Transposable elements constitute a large portion of eukaryotic genomes and contribute to their evolution and diversification. Miniature inverted-repeat transposable elements (MITE) constitute one of the main groups of transposable elements. MITEs have been found in wide range of organisms but active MITEs have not been identified. We found a new class of MITEs in rice and named them *miniature Ping* (*mPing*). *mPing* was identified as the first active MITE from any organism and the first active DNA transposon from rice. *mPing* is a short 430 base pairs element with 15 base pair terminal inverted repeats that lacks a transposase. *mPing* elements are activated in calli derived from anther culture and excise efficiently from original sites to reinsert into new loci. The *mPing*-associated *Ping* element which has a putative transposase sequence was also found and shown to transpose within the rice genome.

Rice is the most agriculturally important crop in the world. m*Ping*/*Ping* transposon system is a useful molecular tool for gene isolation and gene knockout in rice.

2- 2 *Expression sequence tag in* Adiantum

To understand the genetic information of a fern, *Adiantum capillus-veneris*, a normalized cDNA library was constructed from prothallia grown under white light and analysis of expressed sequence tags (EST) were carried out. Approximately 10,000 clones were sequenced and clustering of these obtained sequences was performed. As a result, 7,132 non-redundant groups were generated. These groups were subjected to similarity searches to identify putative function. Approximately 1,600 EST groups were found to be

similar to sequences of genes registered in the public database. About 1,100 EST groups showed similarity to sequences of unknown function. The remaining EST groups showed no significant similarity and were classified as novel sequences.

List of publication:

Original articles

Stoelzle, S., Kagawa, T., Wada, M., Hedrich, R., Dietrich, P. (2003) Blue light activates calcium-permeable channels in Arabidopsis mesophyll cells via the phototropin signaling pathway. *Proc. Natl. Acad. Sci. USA* **100**, 1456-1461.

Sato, Y., Wada, M., Kadota, A. (2003) Accumulation response of chloroplasts induced by mechanical stimulation in bryophyte cells. *Planta* **216**, 772-777.

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Oikawa, K., Kasahara, M., Kiyosue, T., Kagawa, T., Suetsugu, N., Takahashi, F., Kanegae, T., Niwa, Y., Kadota, A., Wada, M. (2003) CHUP1 is essential for proper chloroplast positioning. *Plant Cell* **15**, 2805-2815.

Srinivas, A., Behera, R. K., Kagawa, T., Wada, M., Sharma, R. (2003) High pigment1 mutation negatively regulates phototropic signal transduction in tomato seedlings. *Plant Physiol.* in press

Lamparter, T., Kagawa, T., Brücker, G., Wada, M. (2003) Positive and negative tropic curvature induced by microbeam irradiation of protonemal tip cells of the moss *Ceratodon purpureus*. *Plant Biology* in press.

Review articles

Sato, Y., Kadota, A., Wada, M. (2003) Chloroplast movement: dissection of events downstream of photo- and mechano-perception. *J. Plant Res.* **116**, 1-5.

Wada, M., Kagawa, T., Sato, Y. (2003) Chloroplast movement. *Annu. Rev. Plant Biol.* **54**, 455-468.

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Wada, M., Kanegae, T. (2004) Photomorphogenesis of Ferns. In: Photomorphogenesis in Plants 3ed Edition. Ed. by Schäfer and Nagy, Kruwer Academic Publishers, in press.

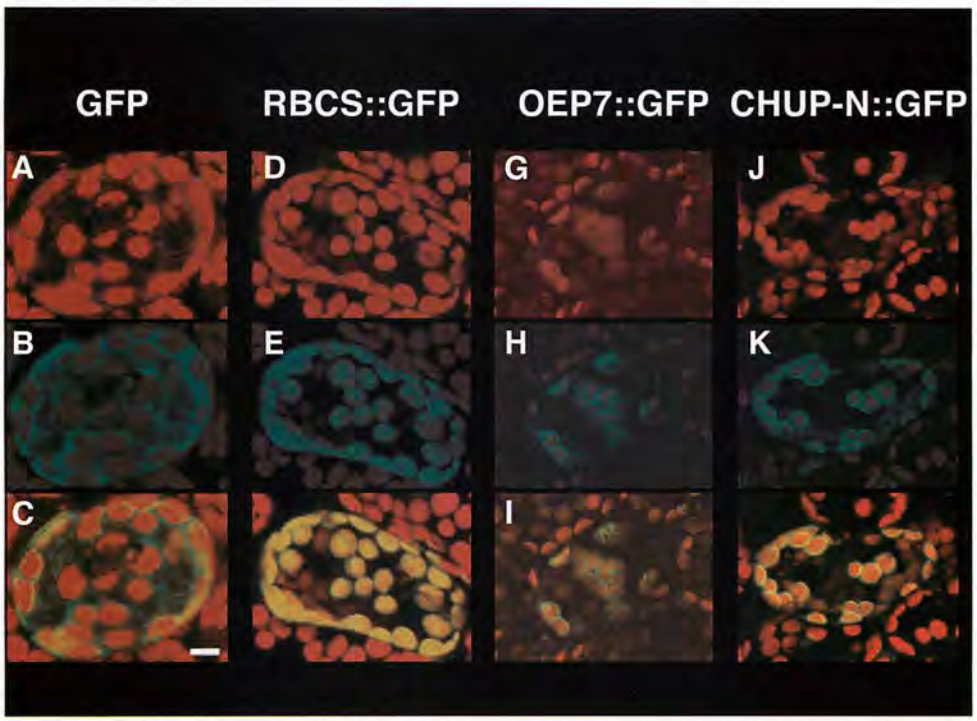

Figure Localization of N-CHUP-GFP in Arabidopsis palisade mesophyll cells as confocal microscopic images of the optical sections transiently expressing GFP (A-C), pt-sGFP (D-F), AtOEP7-GFP (E-G) and N-CHUP-GFP (H-J). The green and red colors show fluorescence from GFP and autofluorescence from chloroplasts, respectively. Images of green and red channel were merged (C, F, I and L). Scale bar: 10 μm. OEP is a membrane protein known to distribute at the chloroplast outer membranes (Kind gift of Dr. Yasuo Niwa).

DIVISION OF BEHAVIOR AND NEUROBIOLOGY (ADJUNCT)

Professor (Adjunct):	*MORI, Yuji*
Associate Professor (Adjunct):	*TSUKAMURA, Hiroko*
Research Associate:	*IMAMURA, Takuya*

This adjunct division will start at April, 2004.

LABORATORY OF GENE EXPRESSION AND REGULATION

Chairperson: YAMAMORI, Tetsuo

DIVISION OF GENE EXPRESSION AND REGULATION I
DIVISION OF GENE EXPRESSION AND REGULATION II
DIVISION OF SPECIATION MECHANISMS I
DIVISION OF SPECIATION MECHANISMS II

The laboratory consists of four regular divisions and conducts researches into regulatory mechanisms of gene expression in microorganisms, plants and animals.

DIVISION OF GENE EXPRESSION AND REGULATION I

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	HOSHINO, Atsushi
	TSUGANE, Kazuo
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	TANAKA-FUKADA, Sachiko
NIBB Research Fellow:	*CHOI, Jeong-Doo*
JSPS Postdoctoral Fellows:	*PARK, Kyeung-Il*
	*EUN, Chan-Ho **
Postdoctoral Fellows:	*JOHZUKA-HISATOMI, Yasuyo*
	*ISHIKAWA, Naoko ***
Visiting Scientist:	*KOUMURA, Toshiro ****
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	*IKEUE, Natsuko [2)] *****
	*TAKAGI, Kyouko [3)] ******
Technical Assistants:	*MORITA, Yasumasa*
	NAGAHARA, Miki
	SAITOH, Miho
Secretary:	*SANJO, Kazuko*

** from October, 2003*
*** until March, 2003*
**** from June, 2003*
***** from April until November, 2003*
****** from November, 2002*

[1)] Graduate University for Advanced Studies
[2)] from Graduate School of Tokyo University
[3)] from Graduate School of Hokkaido University

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

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. A large number of *Ipomoea* species can be found in the Americas, particularly in Mexico. Among the genus *Ipomoea*, three morning glories, *Ipomoea nil* (the Japanese morning glory), *Ipomoea purpurea* (the common morning glory), and *Ipomoea tricolor*, were domesticated well as floricultural plants, and many mutants displaying various flower pigmentation patterns were isolated. Among these morning glories, *I. nil* and *I. purpurea* belong to the same subgenera *Ipomoea*, whereas *I. tricolor* was classified into another subgenera, *Quamoclit*. Both *I. nil* and *I. tricolor* display blue flowers (see Figure 2A) that contain the peonidin (3' methoxyl cyanidin) derivative named Heavenly Blue Anthocyanin (HBA), and *I. purpurea* produces dark purple flowers containing a cyanidin derivative that lacks one glucose molecule and a methyl residue from HBA. All of them produce dark-brown seeds. As floricultural plants, spontaneous mutants of *I. purpurea* and *I. tricolor* displaying either white or reddish flowers were obtained while various spontaneous mutants of *I. nil* exhibiting many different flower colorations were generated.

Although *I. nil* has long been believed to have originated from Southeast Asia, a hypothesis that *I. nil* may have arrived in Asia from tropical America was also recently proposed. In either case, the plant had been introduced into Japan from China approximately in the 8th Century as a medicinal herb. The seeds were used as a laxative, and the plant became a traditional floricultural plant in Japan around the 17th Century. *I. nil* has an extensive history of genetic studies, and a number of its spontaneous mutants related to the color and shape of the flowers have been isolated. It was also used extensively for physiological studies of the photoperiodic induction of flowering.

The wild-type *I. purpurea* plant, which originated from Central America, was probably introduced to Europe in the 17th Century, and cultivars with white and red flowers were already recorded in the late 18th Century. Like *I. purpurea*, *I. tricolor* originated from Central America, and several spontaneous mutants exhibiting various flower pigmentations were obtained in the mid-20th Century.

As Figure 1 shows, the spontaneous mutants of *I. nil*, *I. purpurea*, and *I. tricolor* carrying the *magenta*, *pink*, and *fuchsia* alleles, respectively, produce reddish flowers containing pelargonidin derivatives, and all of them are deficient in the gene for flavonoid 3'-hydroxylase (F3'H). The magenta allele in *I. nil* is a nonsense mutation caused by a single C to T base transition generating the stop codon TGA, and the cultivar Violet carries the same mutation. Several tested *pink* mutants in *I. purpurea* carry inserts of the 0.55-kb DNA transposable element *Tip201* belonging to the *Ac/Ds* superfamily at the identical site. No excision of *Tip201* from the *F3'H* gene could be detected, and both splicing and polyadenylation patterns of the *F3'H* transcripts were affected by the *Tip201* integration. The *fuchsia* allele in *I. tricolor* is a single T insertion generating the stop codon TAG, and the accumulation of the *F3'H* transcripts was drastically reduced by the nonsense-mediated RNA decay.

The *I. tricolor* spontaneous mutant Blue Star carrying the mutable *ivory seed-variegated* allele exhibits pale-blue flowers with a few fine blue spots and ivory seeds with tiny dark-brown spots (Figure 2). The mutable allele is caused by an intragenic tandem duplication of 3.3 kb within a gene for transcriptional activator

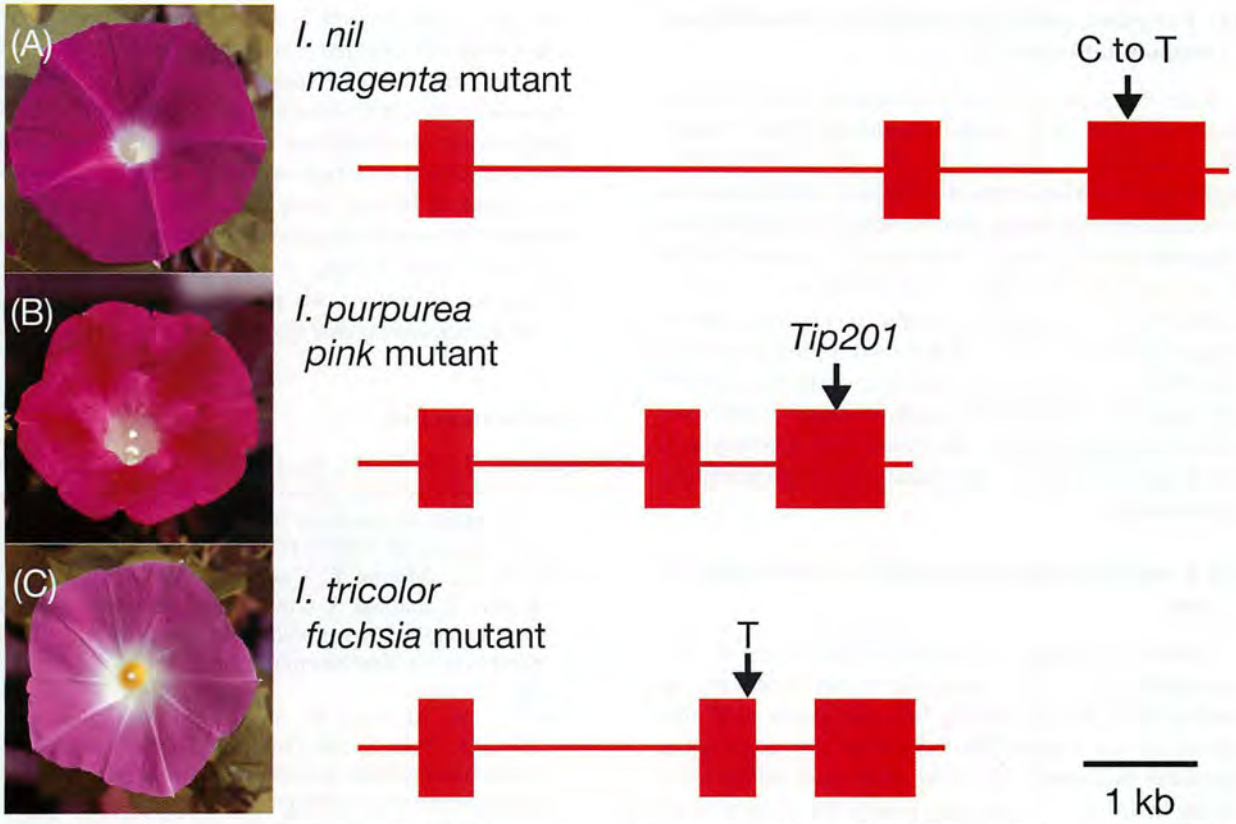

Fig.1. Flower phenotypes and structures of the *F3'H* genes in *I. nil*, *I. purpurea*, and *I. tricolor* exhibiting reddish flowers. **A.** *I. nil* carrying the *magenta* allele. **B.** *I. purpurea* carrying the *pink* allele. **C.** *I. tricolor* carrying the *fuchsia* allele.

containing a bHLH DNA-binding motif. Each of the tandem repeats is flanked by a 3-bp sequence AAT, indicating that the 3-bp microhomology is used to generate the tandem duplication. The transcripts in the pale-blue flower buds of the mutant contain an internal 583-bp tandem duplication that results in the production of a truncated polypeptide lacking the bHLH domain. The mRNA accumulation of most of the structural genes encoding enzymes for anthocyanin biosynthesis in the flower buds of the mutant was significantly reduced. The transcripts identical to the wild-type mRNAs for the transcriptional activator were present abundantly in blue spots of the variegated flowers, whereas the transcripts containing the 583-bp tandem duplication were predominant in the pale-blue background of the same flowers. The flower and seed variegations are caused by somatic homologous recombination between an intragenic tandem duplication in the *bHLH* gene, whereas various flower variegations in *Ipomoea* are known to be caused by excision of DNA transposons inserted into pigmentation genes.

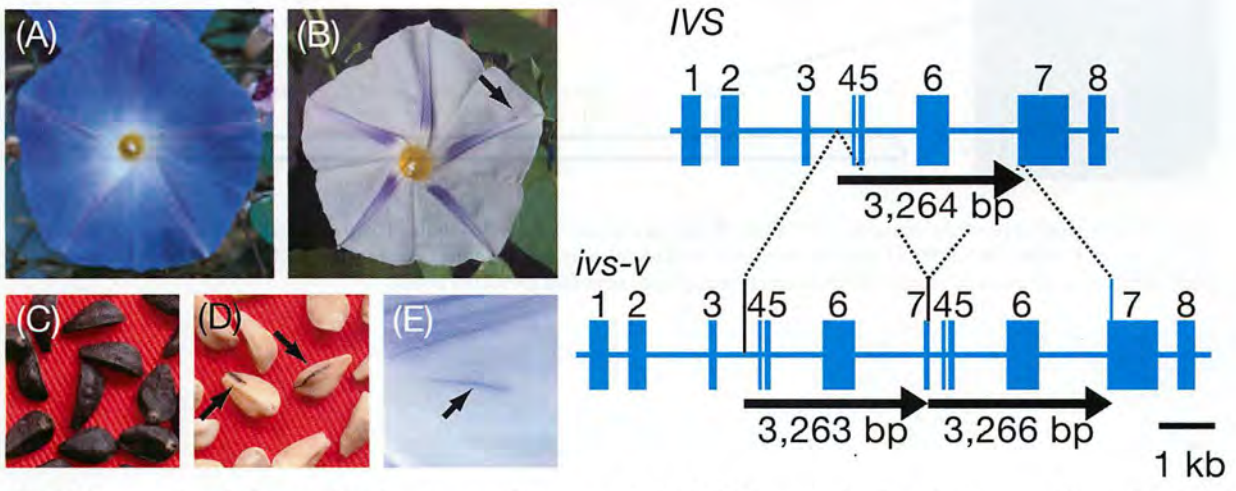

Fig.2. Flower and seed pigmentation phenotypes and structures of the bHLH transcriptional regulatortor gene for anthocyanin pigmentation in the *I. tricolor* wild-type cultivar, Heavenly Blue (**A** and **C**), and its mutant, Blue Star (**B**, **D** and **E**).

II. Targeted gene disruption by homologous recombination in rice.

Rice (*Oryza sativa* L.) is an important staple food for more than half of the world's population and a model plant for other cereal species. We have developed a large-scale *Agrobacterium*-mediated transformation procedure with a strong positive-negative selection and succeeded in efficient and reproducible targeting of the *Waxy* gene by homologous recombination without concomitant occurrence of ectopic events, which must be an important first step for developing a precise modification system of the genomic sequences in rice. By improving our transformation procedure further, we are attempting to modify *Adh* genes, which belong to a small multigene family and reside adjacent to repetitive retroelements.

III. Characterization of mutable *virescent* allele in rice.

Leaves of seedlings in the *virescent* mutant of rice are initially pale yellow green due to partial deficient in chlorophyll and gradually become green with the growth of the mutant. We have been characterizing a spontaneous mutable *virescent* allele *pale yellow leaf-variegated (pyl-v)*, conferring pale yellow leaves with dark green sectors in its seedlings (Figure 3). The *pyl-v* mutant was isolated among progeny of a hybrid between *indica* and *japonica* rice plants. The leaf variegation is regarded as a recurrent somatic mutation from the recessive pale yellow allele to the dark green revertant allele. The availability of the genomic sequences of both *japonica* and *indica* subspecies facilitates map-based cloning of the *pyl-v* allele. Mapping data indicate the mutation resides in the short arm of the chromosome 3, and excision of a new DNA transposon from the *pyl* gene appears to be responsible for conferring the leaf variegation. It is important to emphasize here that tissue culture is necessary in all of the currently available rice reverse genetic approaches. No somaclonal variation is likely to occur in mutant lines induced by our newly characterizing endogenous element, because no tissue culture has been involved in its activation. Using a newly identified DNA transposon, therefore, we are attempting to develop a novel transposon tagging system in rice.

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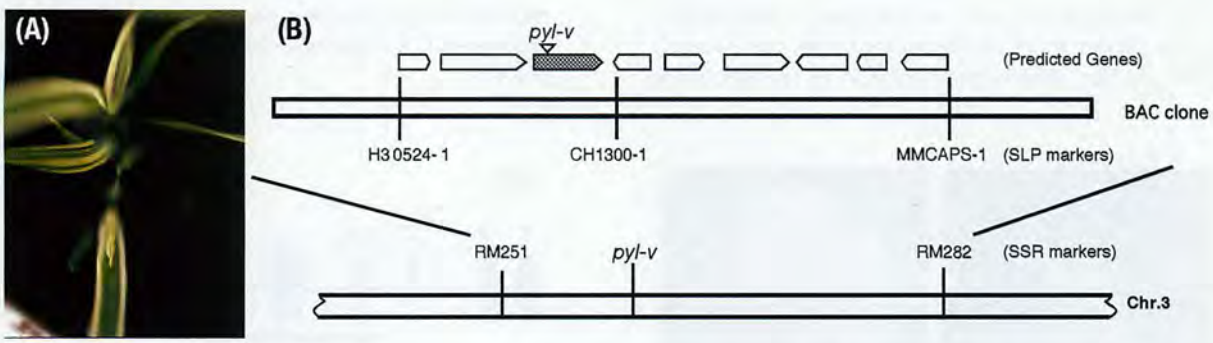

Fig.3. **A.** Leaf phenotype of the mutable *pyl-v* allele. **B.** Identification of the *pyl-v* allele. The *pyl-v* allele was mapped between the SSR markers RM251 and RM282 on chromosome 3 and subsequently located between the SLP markers CH30524-1 and MMCAPS-1 on a single BAC clone. The horizontal pentagonals represent predicted genes.

DIVISION OF GENE EXPRESSION AND REGULATION II

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** *from Sep. 1, 2003*
*** *from Nov. 30, 2003*

The genomes of higher organisms contain significant amounts of repetitive sequences, which in general, are unstable. At present, neither the physiological function(s) of repeated sequences nor the mechanism controlling the instability is fully understood. To clarify these aspects, we are pursuing the following themes using *E. coli* and *S. cerevisiae*: (1) the amplification mechanism of repeated sequences or genes, especially rRNA repeated genes, (2) the mechanism of replication fork block-dependent recombination, a key reaction that increases or decreases the number of repeats, and (3) development of in vivo artificial gene amplification systems. Structural and functional analyses of the *E. coli* genome are also being carried out. In 2003, work on the following three subjects has advanced our knowledge of the dynamics and structure of the genome.

I. The replication fork barrier site forms a unique structure with Fob1p and inhibits the replication fork.

The DNA replication fork blocking sites are found in genomes of various organisms. In *E. coli*, the fork block site, called *Ter*, has the fork blocking activity in a polar fashion. For the blocking activity, two components are required, one is a cis-element, *Ter*, the other is the Ter-binding protein, called Tus protein. In eucaryotes, the similar blocking site, called RFB (replication fork blocking barrier), has been identified in the ribosome RNA gene (rDNA) cluster from yeast to human. In *S. cerevisiae*, about 150 copies of rDNA are clustered in a specific region on the chromosome XII. The RFB is an approximately 100-bp DNA sequence located near the 3' end of the rRNA genes in the yeast *Saccharomyces cerevisiae*. This site inhibits the progression of the DNA replication fork coming from the direction opposite to 35S rDNA transcription. However, the RFB-binding protein has not been identified so far. The most likely candidate for the RFB-binding protein is Fob1p, because it had shown to be required for the replication fork blocking activity at the RFB site. Furthermore, it was found later that it is essential for recombination in the ribosomal DNA (rDNA), including increase and decrease of rDNA repeat copy number, production of extra-chromosomal rDNA circles, and possibly homogenization of the repeats. Despite the central role that Fob1p plays in both replication fork blocking and rDNA recombination, not only the molecular mechanism by which Fob1p mediates these activities, but also its RFB-binding activity had not been determined. We have shown by using chromatin immuno-precipitation, gel shift, foot-printing, and atomic force microscopy assays that Fob1p directly binds to the RFB (see Figure A and B). Fob1p binds to two separated sequences in the RFB (Figure D). A predicted zinc finger motif in Fob1p was shown to be essential for the RFB binding, replication fork blocking, and rDNA recombination activities. The RFB seems to wrap around Fob1p, and this wrapping structure may be important for function in the rDNA repeats (Figure B, C and D).

II. Transcription-mediated hyper-recombination in *HOT1*

Recombination hot-spots are DNA sequences which enhance recombination around the region. *HOT1* is one of the well-studied recombination hot-spot in mitotic yeast cells. Because *HOT1* includes a transcription promoter sequence of RNA polymerase I (PolI) which is responsible for the 35S ribosomal RNA gene (rDNA) transcription, and the *HOT1* activity is abolished in a PolI defective mutant, transcription of *HOT1* was thought to be an important factor for the recombination stimulation. However, it is not clear whether the transcription itself or other phenotypes shown in the PolI mutant activates the recombination. To understand the role of transcription, we used a highly-activated PolI transcription system for *HOT1* in a strain whose rDNA were deleted (*rdnΔΔ*). In the *rdnΔΔ* cells, it is known that the nucleolus disappears and PolI, which is normally localized to the nucleolus, diffuses into the cytoplasm. In the *rdnΔΔ* strain, the *HOT1* transcription was increased by about 25 times as compared with the wild type. Recombination activity stimulated by *HOT1* was also elevated by about 15 times as compared with that of wild type. These results indicate that frequency of PolI transcription in *HOT1* determines efficiency of the recombination. Moreover, Fob1p, which is essential for both *HOT1* recombination and transcription activities, was dispensable in the *rdnΔΔ* strains, thereby suggesting that the protein may be functioning as a PolI transcription activator in the wild type strain.

III. Gene amplification by designed double rolling circle replication utilizing break induced replication in *Saccharomyces cerevisiae*

Gene amplification is a phenomenon widely found in the genomes of various organisms and probably has played an important role in the processes of gene evolution. For example, rDNA must have amplified during evolution and in some amphibians, further amplification occurs during their development. Oncogene amplification is frequently observed when cancer is in progress. Gene amplification also occurs when cultured cells acquire drug-resistance. However, human cultured cells lack the ability to amplify drug-resistance genes at a detectable level. Spontaneous amplification also occurs in insects and plants, but the molecular mechanism remain uncertain. Thus, if a novel artificial amplification could be realized using normal organisms, it would be helpful for better understanding the basic mechanism of the various amplifications. Thus, we have tried to develop a novel designed amplification system using a normal *S. cerevisiae* strain. We assumed that a mode of replication, named double rolling-circle replication (DRCR), could amplify a gene between two directed replication forks, and tested it on plasmid-derived mini-chromosomes or on a resident chromosome utilizing break-induced replication (BIR). The latter system produced three kinds of amplification products; type-1 containing 5~7 copies of an amplification marker, *leu2d*, with the expected structure, type-2 containing several dozen copies of *leu2d* with a similar structure to type-1, but the sequences flanked by the two inverted *leu2ds* are oriented randomly, and type-3 having an acentric multi-copy mini-chromosome with *leu2ds*. The type-2 and -3 products seem to correspond to HSR (homogeneously staining region) and DM (double minute) in higher eukaryotes. Surprisingly, the two latter types were also generated without HO cleavage, though at low frequency. This system may provide insights into the molecular mechanism of gene amplification.

VI. Complete genomic sequence of the *E. coli* K12 W3110 strain

Escherichia coli is one of the organisms that has been most extensively analyzed physiologically,

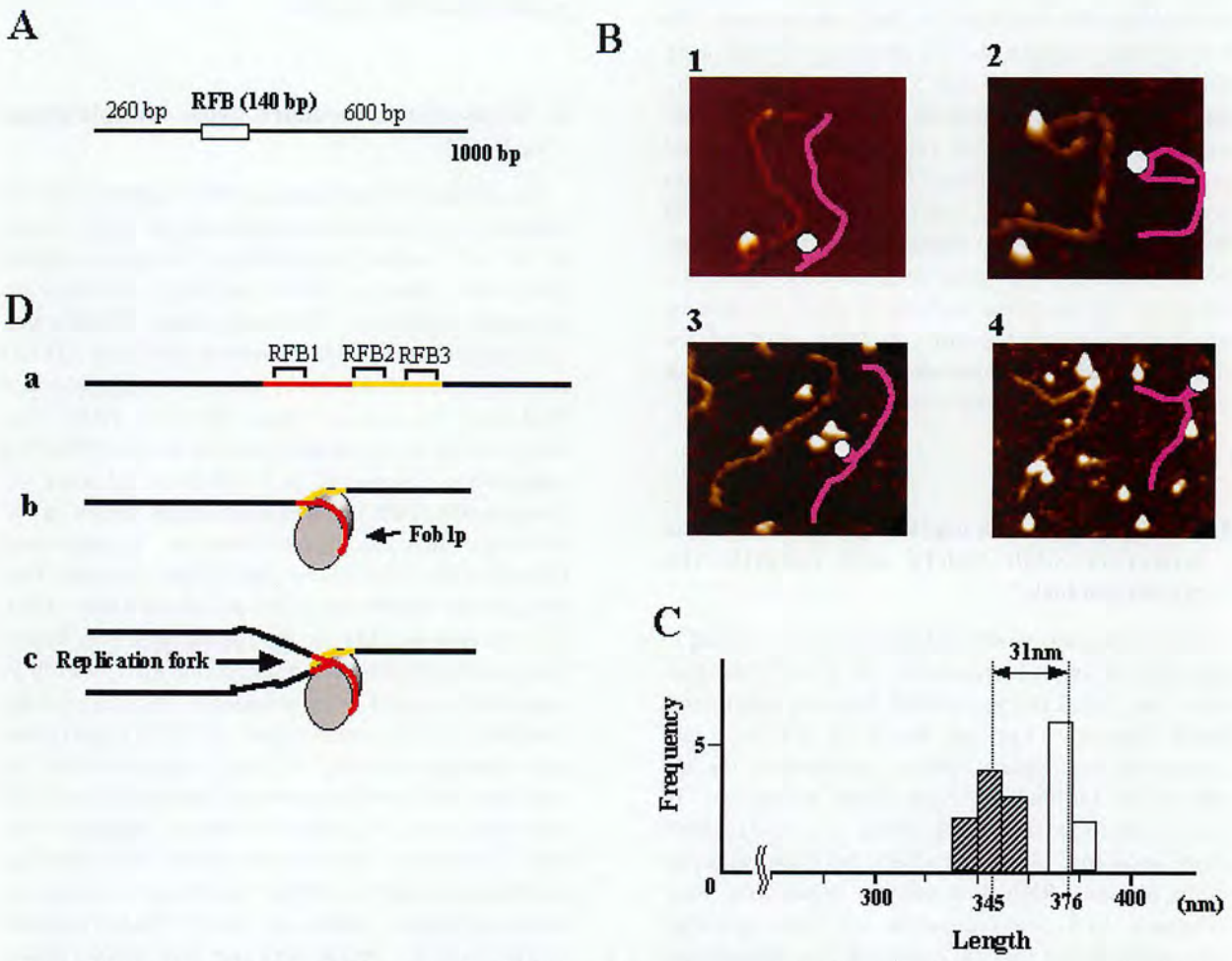

Figure Observation of the Fob1p-RFB complex by AFM (atomic force microscopy). (A) Structure of RFB fragments used in AFM analysis. (B) AFM images of the Fob1p-RFB complex. (C) Frequency plot (histogram) of the length of the RFB fragment measured from AFM images. White and hatched boxes indicate the number of free DNA molecules and Fob1p-RFB complexes, respectively. The mean fragment lengths are indicated below the graph by clotted lines. (D) Models of the Fob1p-RFB complex.

biochemically and genetically. Of all *E. coli* strains, *E. coli* K12 W3110 has probably been used most frequently as the wild-type strain in these experiments. Recently, we determined the complete nucleotide sequence of the genome of strain W3110, mainly by sing lambda phages from Kohara's bank. Previously a US group determined the genomic sequence from another K12 wild-type derivative, MG1655. Both strains were derived from a common ancestor strain, W1485, probably about 50 years ago. Comparing the two sequences, we obtained the following results.

The total number of nucleotides in the W3110 genome is 4660170 bp. There were 349 bp conflicts between the sequences of W3110 and MG1655. Re-sequencing of the conflict sites by a PCR method using genomic DNA as templates revealed that only eight sites (9 bp) were true conflicts. Seven of them are base-change type conflicts and one is a two base frame-shift. All of these differences reside within genes, seven in ORFs and one in a 23S rRNA gene.

We attended the *E. coli* K12 annotation workshop held at Woods Hole Marine Biology Research Institute, Mass., USA, 2003, Nov. 13-18, which was organized by Dr. Monica Riley. In this workshop, we discussed (1) the definition of the starting point of the *E. coli* genome sequence, (2) boundaries of ORFs, that is start and end points of ORFs, and (3) the descriptions of gene names and their function. We agreed there to make an effort to complete a new annotation version using our revised sequence by the coming February. We can safely say that the revised sequence and the annotation will be among the most accurate of all the genomes whose sequences have been determined.

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Higuchi K, Katayama T, Iwai S, Hidaka M, Horiuchi T, Maki H.(2003). Fate of DNA replication fork encountering a single DNA lesion during oriC plasmid DNA replication in vitro. *Genes Cells* **8**, 437-49

Kobayashi, T. (2003) The replication fork barrier site forms a unique structure with Fob1p and inhibits the replication fork. *Mol. Cell. Biol.* **23**, 9178-88.

DIVISION OF SPECIATION MECHANISMS I

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Our research is focusing to understand mechanisms underlying memory, formation and evolution of the brain. For one approach to understand these questions, we are studying the genes that are expressed in specific areas of the primate neocortex. Using differential display method, we obtained three genes that showed marked differences within areas of the primate neocortex. Our second approach is to understand informational processing in the brain underlying learning behaviors by examining gene expression. The third approach is to study voles of CNTF in the nervous system.

I. Genes expressed in specific areas of the neocortex

The neocortex, most remarkably evolved in primates, plays the major role in higher functions of the brain. It is known to be divided into distinct functional and anatomical areas and has been a matter of debate what extent the area of the neocortex are genetically and environmentally deter-mined. It is also puzzling why, during the evolution of mammals, the neocortex was most markedly expanded while the number of the genes in the mammal was little changed. To access these questions, we studied gene expression within different areas of the neocortex.

1) In collaboration with Professor Hiroyuki Nawa (Nigata university), we used the DNA macroarray technique to examine gene expression in the areas of human prefrontal, motor and visual cortexes. We found almost all the genes among 1088 genes examined showed only less than a factor of two in the difference of their expressions. Only one gene showed more than three fold difference and another one was between two and three fold difference within the three areas. These results suggest that the genes that are expressed among the different areas of the human neocortex are very similar. However, the question remained whether there are any genes that show marked difference within areas of neo-cortex.

2) In order to answer this question, we employed differential display methods and found at least two 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. Furthermore, the expression of occ1 turned out to be activity dependent, because, in the monocularly deprived monkeys injected with TTX into one of the eyes, the expression of occ1 is markedly decreased in the ocular dominance columns of the primary visual cortex (V1). We also demonstrated that occ1 expression was markedly increased postnataly in V1 (Fig 1, Tochitani et al., 2003).

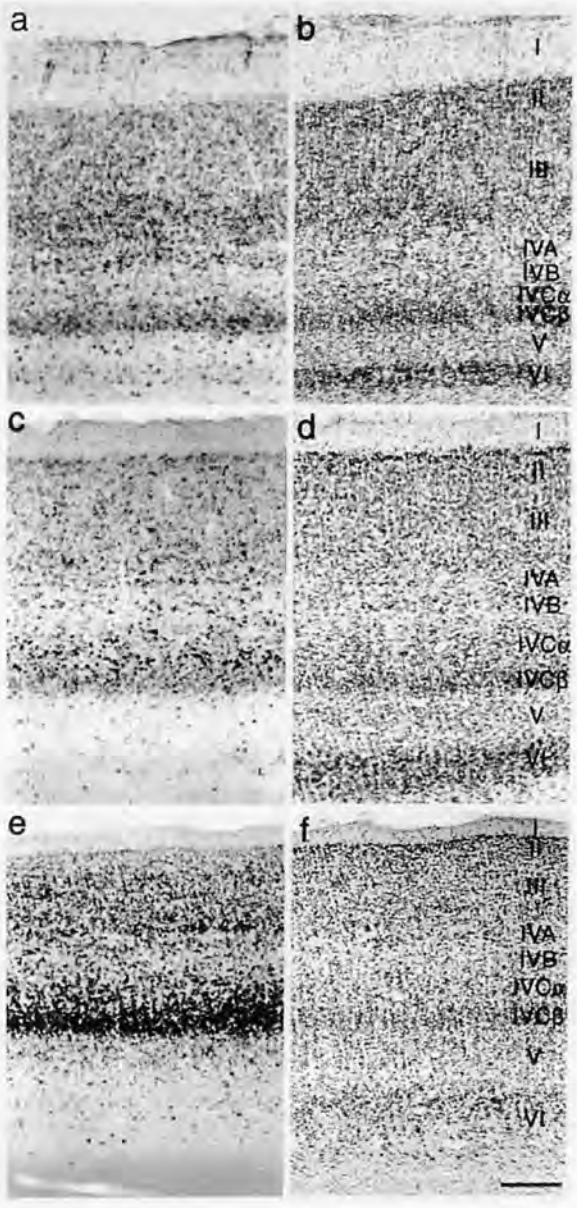

Fig. 1 Developmental Expression pattern of occ1 in the visual cortex.
In situ hybridization pattern of occ1 in the primate visual cortex. occ1 is markedly expressed in the layer IVcb and moderately in the layers of II, III and IVa in area V1 (e). This adult expression pattern is regulated by postnatal development. (a, b) newborn; (c, d) three month; (e, f) adult monkey. (a, c, d) with occ1 antisense probe and (b, d, f) with thionin for cell bodies. (See. Tochitani et al., Eur. J. Neurosci:, 13, 297-307, 2001)

ii) The other gene that showed marked difference within the neocortex, is gdf7, a member of BMP/TGF-b family, which is specifically expressed in the motor cortex of the African green monkey. We are currently examining the detailed expression pattern of the gene.

iii) The third gene that we designated tentatively as 134G which was preferentially expressed in association and higher areas in the neocortex (Komatsu et al., Society for Neuroscience 32nd Annual Meeting, 2002, being submitted).

3) We have also further isolated several area specific genes with RLCS (Restriction Landmark cDNA Scanning). We are now characterizing these genes to reveal the mechanisms that form neocortical areas.

In summary, our studies thus far revealed the following points.

(1) Genes that are specifically expressed within neocortical areas in the primate neocotex are not different overall.
(2) We have identified several genes that are distinctively different among neocortical areas.
(3) These genes are specific in visual, motor and association areas.
(4) A gene specific in the visual cortex (occ1) is activity dependent and also postnatally regulated.
(5) An association area specific gene is expressed in a complimentary manner to the expression of occ1.
(6) These results suggest that these genes may be useful markers to study the mechanisms underlying neocortical formation.

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 employed c-Fos mapping techniques, for which we used gene expression of c-Fos. There have been an increasing number of studies using c-Fos as markers to examine neuronal activities ever since c-Fos induction by electrical stimulation was found by Morgan and Curran. However, many sensory stimuli per se are now known to cause c-Fos induction. So, we should be very careful to distinguish the c-Fos expression that is caused by learning process from that caused by sensory stimuli. For this purpose, it is necessary to use behavioral systems that are able to distinguish the two. Although a few behavioral systems in rodents have been successfully used for physiology, animal behavior and recently for analyses of knockout mice, little behavioral systems in fact distinguish it. Therefore, we prepared ourselves for using two behavioral systems, which represent declarative and non-declarative memory, respectively.

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

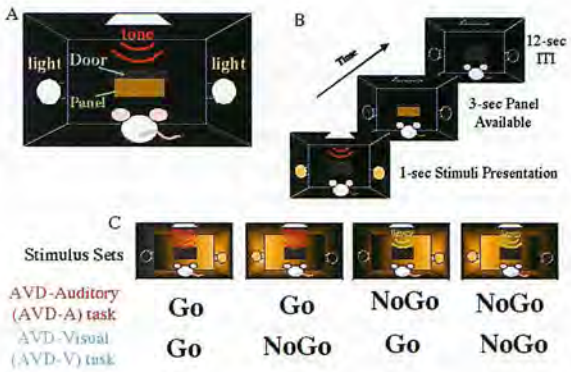

Fig.2. AVD tasks.
A) A rat face to a panel with a visual cue (a light from right or left) or an auditory cue (a high or low tone) was shown to push the panel with the time sequence shown in (B). The combination of possible cues by auditory and visual tasks to obtain a food pellet is shown in (C). (See Sakata et al., Eur. J. Neurosci., 15, 735-743, 2002, for the detail of the tasks)

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

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

The other task we developed is a wheel running system in which a water-deprived mouse is asked to run to obtain water in front because the wheel with the pegs is turning to the other direction (Kitsukawa et al.,

Society for Neuroscience 32nd Annual Meeting, 2002). The pegs can be changed with various patterns as desired. The task required for the mouse thus can be regarded to represent a non-procedural learning. We examined a various areas of brains following to the change of the peg pattern. Among the areas examined, we found marked c-Fos expression in the striatum. The striatum, which is composed of projection neurons and several distinguished types of interneurons, is known to play an important role in a reward-based learning. The characterization of these subtypes of interneurons has been progressed. However, their roles in behavioral tasks have been little known. We hope our system combined with c-Fos mapping technique reveals it. We are currently doing collaborative work with Professor Ann Graybiel (MIT) to record with tetrode from the striatum under the task (supported by US-Japan exchange program). We hope our wheel running system combined with c-Fos mapping technique and electrophysiology reveals molecular and cellular mechanisms under the Wheel Running task.

III CNTF is specifically expressed in the developing pineal gland

CNTF, a member of the IL-6 family, attracts quite attentions of developmental neuroscientists because it shows various effects on neurons and glial cells. CNTF knockout mice, however, only indicate moderate motor neuron deficiency in the adult, but no apparent phenotype in the development. In order to explore the function of the IL-6 family, we extensively examined the expression of members of the family and their receptors and found the specific expression of CNTF in the embryonic pineal glands and eyes. This was to our knowledge for the first time to show a clear expression

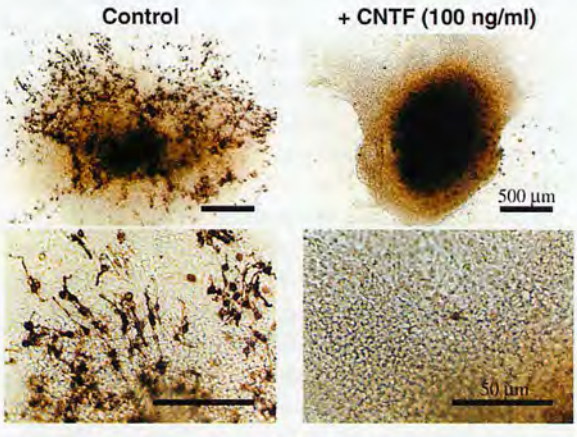

Fig. 3. CNTF suppress Rhodopsin expression in pineal glands in vitro. CNTF was added to the medium of rat pineal organ culture. (Right) Control culture without CNTF on day 10. Rhodopsin-positive photoreceptor-like cells with many processes are seen at a higher magnification. (Left) CNTF (100 ng/ml) was added for 10 days. Photoreceptor-like cells of rhodopsin –positive cells are very rare. If any, they do not show long processes (lower, a higher magnification). These results were published in Hata et al., Dev. Brain Res., 143, 179-187, 2003.

of CNTF in the development. Next question to be asked is the functional role of CNTF in the pineal development. Because of no reported phenotype of CNTF knockout mice in development, it has been difficult to know the developmental roles of CNTF if any. In fact, we also found there seemed no apparent difference in the pineal development of CNTF knockout mice compared to that in wild type mice. This is presumably because the CNTF gene knockout is largely compensated by other CNTF-like factors.

We therefore studied cultured pineal organs in rodents to ask if there were any effects of CNTF on it following to a previous study that shows that cultured neonatal pineal organs develop photoreceptor-like cells (Araki, 1992). We found that CNTF inhibits photoreceptor-like cells (Fig. 3, Hata et al., Dev Brain Res., 2003). Our observation raises an interesting possibility that CNTF plays a critical role in the pineal development to suppress a certain phenotype such as photoreceptors. It also raises a question what roles CNTF play in developing pineal glands if it play any roles in the development. CNTF has been studied for nearly thirty years and still the role in vivo remains to be studied. We hope that our study gives a clue to solve this long standing question.

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DIVISION OF SPECIATION MECHANISMS II

Professor:	*HASEBE, Mitsuyasu*
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NIBB Research Fellows:	*HIWATASHI, Yuji (~Sept. 30)*
	NISHIYAMA, Tomoaki (Sept. 16~)
JSPS Research Fellow:	*SATO, Yoshikatsu*
Postdoctoral Fellows:	*NISHIYAMA, Tomoaki (~Sept. 15)*
	AONO, Naoki (April 1~)
	MIYAZAKI, Saori (May 19~)
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	SAKAKIBARA, Keiko(~March 31)
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	BITOH, Yoshimi
	NARUSE, Mayumi
	AOKI, Etsuko
	OONO, Chikako (Feb. 1~)
	SUZUKI, Yoriko (Feb. 1~)
Visiting Scientists:	*Paul G. Wolf [1] (Sept. 22~Dec. 22)*
	Carol A. Rowe [1] (Sept. 22~Dec. 22)
Secretary:	*KABEYA, Kazuko*

[1] *from Utah State University, USA*

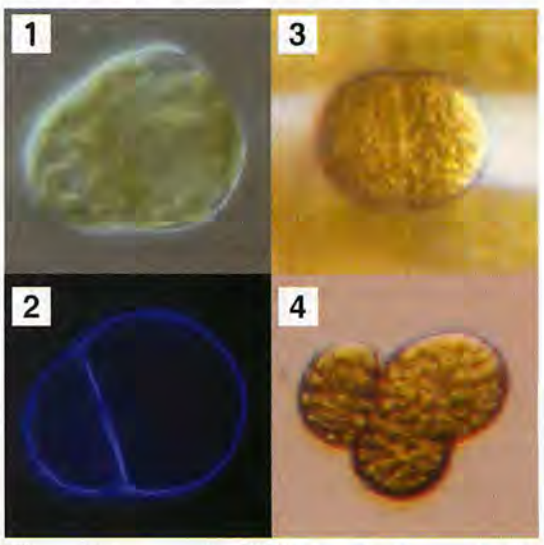
All living organisms evolved from a common ancestor that lived more than 3.5 billion years ago, and the accumulation of mutations in their genomes has resulted in the present biodiversity. Traces of the evolutionary process are found in the genomes of extant organisms. By comparing the gene sequences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

I. Origin of the Plant Cell

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

II. Evolution from unicellular to multicellular organisms

The first evolutionary step from unicellular to multicellular organisms is to form two different cells from a single cell via asymmetric cell division. The first cell division of a protoplast isolated from the protonemata of the moss *Physcomitrella patens* is asymmetric regarding to its shape and nature, and gives rise to an apical meristematic cell and a differentiated non-meristematic cell. A systematic overexpression screening for genes involved in asymmetric cell division of protoplasts in *P. patens* is in progress by a team directed by T. Fujita. We constructed three full-length cDNA libraries from non-treated, auxin-treated, and cytokinin-treated protonemal cells of *P. patens*, then determined the sequences of more than 40,000 cDNAs from the both ends (Nishiyama, Fujita et al. 2003). We used these clones as materials for the overexpression screening. Individual cDNAs were selected based on their sequence, subcloned under a constitutive promoter and introduced into the protoplasts of *P. patens* for transient expression. We observed and categorized phenotypes of the regenerating protoplasts. Thus far we identified many cDNAs, whose overexpression resulted in symmetric cell division rather than asymmetric cell division, isotropic outgrowth with no polarity or curved cells showing incorrect direction of growth. These preliminary results indicate that some of these genes likely function for polarity formation and/or asymmetric cell division of the protoplasts. Functional analyses of these genes should help us to understand molecular mechanisms of how plants generate distinct cell lineages to build their multicellular bodies.



(Figure 1) Asymmetric cell division of a Physcomitrella protoplast. Regular asymmetric cell division (1, 2) and defective cell division with overexpression (3, 4). Cell wall of the cell in (1) is visualized with calcoflor in (2).

III. Evolution from cells to tissues

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

Organization of γ-tubulin

γ-tubulin is a protein that is essential for the formation of microtubules in animal cells. We found that γ-tubulin is located at the end of cortical microtubules, and a loss of γ-tubulin due to gene silencing causes a malformed organ with irregularly shaped cells. *In vitro* experiments using isolated plasma membrane/microtubule complexes suggested that γ-tubulin attaches onto the side of existing cortical microtubules, and initiates a new cortical microtubule from it. The nucleus is known to initiate microtubules after cell division. We hypothesize that microtubules formed around the nucleus elongate to cell surface, and trigger initiation of cortical microtubules via attachment of γ-tubulin. Once cortical microtubules are formed, they can turnover without microtubules from the nucleus. Factor(s) responsible for attachment of γ-tubulin onto the side of microtubules is a key element responsible for the difference between plant and animal cells. Isolation of the factor(s) responsible for attachment of γ-tubulin onto the side of microtubules by biochemical and other approaches is inprogress by a team directed by T. Murata.

Dynamics of actin filaments and microtubules in the moss *P. patens*

Cells of the moss *P. patens* gametophytes are an excellent model to study dynamics of cytoskeleton because of the easiness for observation and the feasibility of gene-targeting. Transformants with reporter constructs by fusing a GFP with *P. patens* α-tubulin or an actin binding domain of mouse talin were established by Y. Sato and collaborators to visualize microtubules and actin filaments. These transformants will be useful to investigate the dynamics of microtubules and actin filaments in the processes of cell division, cell elongation, and chloroplast movement.

IV. Evolution of molecular mechanisms in the development of vegetative organs

Meristem initiation and maintenance

Postembryonic growth of land plants occurs from the meristem, a localized region that gives rise to all adult structures. Meristems control the continuous development of plant organs by balancing the maintenance and proliferation of stem cells, and directing their differentiation. Meristem initiation and maintenance is a fundamental question in plant development research. However, the molecular mechanisms involved in meristem initiation and maintenance have not been studied in detail because most loss-of-function mutants are lethal. In the moss *Physcomitrella patens*, the developmental process of meristem is well defined at the cellular level, and gene targeting based on homologous recombination is feasible. Thus, meristem development in *P. patens* is used as a model system for studies of meristem development in land plants. We established approximately 20,000 gene- or enhancer-trap lines in *P. patens* to clone genes involved in meristem development. Seven lines, exhibiting reporter gene (*uidA*) expression preferentially in the apical cells, were isolated. Corresponding genes in three of these trap lines were identified as encoding kinesin- and ubiquitin-like proteins, and an unknown protein. Disruption of the gene encoding ubiquitin-like protein suggests that the gene be involved in cell division and elongation through microtubule organization. The functions of other genes in the meristem are currently under investigation by a team directed by Y. Hiwatashi.

The morphology of the shoot meristem in land plants varies. To investigate whether the molecular mechanisms of shoot development in angiosperms are conserved in other land plants, the functions of the KNOX homeobox, and the ZWILLE, NAC, and PIN genes, which are indispensable for shoot meristem development in angiosperms, are being studied in the fern Ceratopteris and the moss Physcomitrella.

Rhizoid differentiation

Rhizoids are multicellular filaments with similar functions to root hairs, and are observed in a wide range of plants. A team directed by K. Sakakibara, who was a graduate student in our lab, examined mechanisms underlying rhizoid development in the moss, *Physcomitrella patens* (Sakakinara et al. 2003).

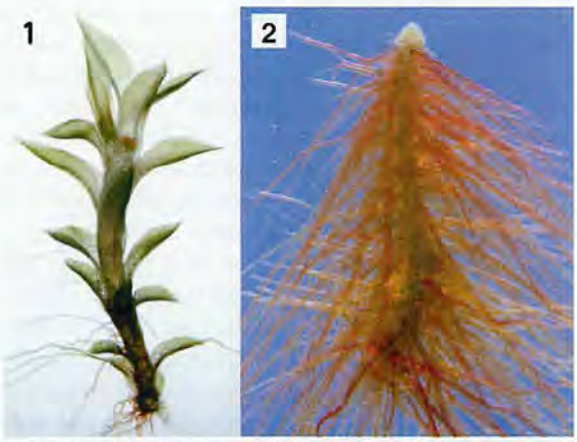


Figure. 2 (1) a gametophyte of *P. patens*. (2) an auxin-treated gametophyte with plenty of rhizoids.

V. Origin and evolution of floral homeotic genes

The flower is the reproductive organ in angiosperms, and floral homeotic genes, such as MADS-box genes and FLO/LEAFY genes, regulate floral organ identity. To investigate the origin of floral homeotic genes, the functions of MADS-box genes and the FLO/LEAFY genes in gymnosperms (*Gnetum, Ginkgo*, and cycads), a fern (*Ceratopteris*), a moss (*Physcomitrella*), and three green algae (*Chara, Coleochaete*, and *Closterium*) are

being analyzed.

Land plants are believed to have evolved from a gametophyte (haploid)-dominant ancestor without a multicellular sporophyte (diploid plant body); most genes expressed in the sporophyte probably originated from those used in the gametophyte during the evolution of land plants. To analyze the evolution and diversification of MADS-box genes in land plants, gametophytic MADS-box genes were screened using macroarray analyses for 105 MADS-box genes found in the Arabidopsis genome (Kofuji et al. 2003). Eight MADS-box genes were predominantly expressed in pollen, male gametophyte, and analyses of their function are in progress by a team directed by N. Aono.

VI. Evolution of life cycles

The mosses and flowering plants diverged more than 400 million years ago. The mosses have haploid-dominant life cycles, while the flowering plants are diploid-dominant. The common ancestors of land plants are inferred to have been haploid-dominant, suggesting that genes used in the diploid body of flowering plants were recruited from the genes used in the haploid body of their ancestors during the evolution of land plants.

To assess the evolutionary hypothesis that genes used in diploid body of flowering plants were recruited from the genes used in haploid body of the ancestors, a team directed by T. Fujita constructed an expressed sequence tag (EST) library of Physcomitrella, and T. Nishiyama mainly worked on the comparison of the moss transcriptome to the genome of Arabidopsis. We constructed full-length enriched cDNA libraries from auxin-treated, cytokinin-treated, and untreated gametophytes of Physcomitrella, and sequenced both ends of more than 40,000 clones. These data, together with the mRNA sequences in the public databases, were assembled into 15,883 putative transcripts. Sequence comparisons of Arabidopsis and Physcomitrella showed that the haploid transcriptome of Physcomitrella appears to be quite similar to the Arabidopsis genome, supporting the evolutionary hypothesis. Our study also revealed that a number of genes are moss specific and were lost in flowering plant lineage. Our full-length cDNA library will be a good resource for functional genomic studies in plants. Our EST data together with some information on the moss is open to public at PHYSCObase (http://moss.nibb.ac.jp).

VII. Molecular mechanisms of speciation

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

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

VIII. Phylogenetic analysis of land plants

We determined the complete nucleotide sequence of the chloroplast genome of the leptosporangiate fern, Adiantum *capillus-veneris* L. (Pteridaceae) as a collaboration work with P. Wolf and C. Rowe from Utah State University (Wolf et al. 2003). Phylogenetic analysis of basal land plants using the sequence is in progress by T. Nishiyama. Many unusual start codons and internal stop codons were found, suggesting that extensive number of RNA editing exists in the genome.

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RESEARCH SUPPORT

CENTER FOR TRANSGENIC ANIMALS AND PLANTS
RESEARCH CENTER FOR INTEGRATIVE AND COMPUTATIONAL BIOLOGY
RESEARCH SUPPORT FACILITY
THE CENTER FOR ANALYTICAL INSTRUMENTS (managed by NIBB)
TECHNOLOGY DEPARTMENT

CENTER FOR TRANSGENIC ANIMALS AND PLANTS

Head:	*NAGAHAMA, Yoshitaka*
Associate Professors:	*WATANABE, Eiji*
	SASAOKA, Toshikuni (Aug. 03~)
	TANAKA, Minoru (Feb. 04~)
Technical Staffs:	*IINUMA, Hideko (~Oct. 03)*
	HAYASHI, Kohji (Spt. 03~)
Supporting Staffs:	*YASUDA, Mie*
	NOGUCHI, Yuji
	SHIMIZU, Naoki
	TAKEUCHI, Kazumi
	YAMAGUCHI, Kyoko

I. Research supporting activity

NIBB Center for Transgenic Animals and Plants was established in April 1998 to support researches using transgenic and gene targeting techniques in NIBB. The center building for transgenic animals has been completed in the end of 2003 (Figure 1). The building has a floor space of 2,300m^2, in which we can breed, develop, store and analyze transgenic, gene targeting and mutant mice under condition of "specific pathogen free". It is also equipped with breeding areas for transgenic aquatic animals, birds and insects.

Figure 1. A new center facility for transgenic animals built at the area E approximately 1 km away from the area A.

The expected activities of the Center are as follows:

1. Provision of information, materials, experimental techniques and breeding space of trausgenic animals and plants to researchers.
2. Equipment of various kinds of instruments to analyze mutant, trangenic, and gene-targeted animals and plants.
3. Development of novel techniques related to transgenic and gene targeting technology.

II-I. Academic activity (Watanabe Lab, *Physiological role of Nax sodium channel*)

We are studying the functional role of Nax (also called Nav2 or NaG) sodium channel in collaboration with Division of Molecular Neurobiology. Nax sodium channel was long classified as a subfamily of voltage-gated sodium channels (NaChs) that serve to generate action potentials in electrically excitable cells such as neuronal and muscle cells. Comparing with the other NaChs, however, Nax has unique amino acid sequences in the regions, which are known to be involved in ion selectivity and voltage-dependent activation and inactivation, suggesting that it must have specific functional properties.

To clarify the functional role of Nax, Nax-deficient mice were generated by gene-targeting technique and the physiological phenotypes have been examined. Behavioral studies suggested that the Nax channel plays an important role in the central sensing of body-fluid sodium level and regulation of salt intake behavior. Nax-deficient mice ingest hypertonic sodium chloride solution in excess in comparison with wild type-mice. LacZ reporter gene knocked into Nax-gene locus revealed that Nax gene is expressed in the circumventricular organs (CVOs), which is the specialized central organs involved in sensing of sodium concentration and osmosity in body fluids. Sodium ion imaging and electrophysiological studies using cultured CVO neurons also suggested that Nax is an extracellular sodium-level sensitive sodium channel. Recently, we found that Nax is expressed in non-myelinating Schwann cells and alveolar type II cells in addition to the neurons and ependymal cells in the CVOs. Nax is thus likely to be involved in reception of sodium-level in body fluids at the CVOs and sodium absorption in the visceral nervous system and in the lung (Figure 2).

More recently, we found in collaboration with Prof. Yamamoto's group of Osaka University that the peripheral nervous system has only subtle effects on the higher preference for sodium chloride as ovserved in the mutant mice. The results suggest that the mutant phenotype is mainly due to the lack of Nax channels in the central nervous system.

We are now trying to construct functional expression systems of Nax sodium channel using various heterologous cell lines. The heterologous expression system will provide us useful information on the channel charactors of Nax.

II-II. Academic activity (Sasaoka Lab)

(1) *Studying the function of dopaminergic transmission*

The dopamine receptors are divided into two subgroups, referred to as D1-like (D1, D5) and D2-like (D2, D3 and D4) receptors on the basis of their gene structure and their pharmacological and transductional properties. The dopaminergic system is implicated in the regulation of the several peptide hormones in the pituitary, the modulation of motor activity, the modulation of synaptic plasticity, and the neural development. The dopaminergic system is also implicated in motivated behaviors, several neurological and psychiatric disorders, such as Parkinson's disease and schizophrenia. Since D1-like and D2-like receptors often work synergistically, it is necessary to delete both D1-like and D2-like receptors in order to understand the role of dopaminergic transmission. We have generated multiple dopamine receptor-deficient mice by combination of single mutant mice, and are studying phenotype of the multiple dopamine receptor mutant mice.

(2) *Developing a novel conditional mutagenesis technique in mice*

The aim of the study is to overcome the limitations of the conventional mouse molecular genetic approach in the functional analysis of target genes. We substituted one critical amino acid residue of N-methyl-D-aspartate receptor (NMDAR), leading to NMDAR activation. By our technique, we accomplished conditional substitution of the amino acid in mice and our mutant mice exhibited NMDAR activation and a neurological phenotype, similar to that of mouse models for neurological disorders. The development of our mutant mice should contribute to understanding the function of the critical amino acid residue and the mechanism of neurological disorders. Our new technique is vastly applicable to functional analysis of any desired gene and should contribute to studies on the structural and functional relationships of relevant genes.

(3) *Studying of the function of the sarcoglycan complex (SGC)*

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

To understand a physiological role of the SGC, we generated the β-SG-deficient (BSG-/-) and γ-SG-deficient (GSG-/-) mice. The dystrophin complex isolated from the skeletal muscles of BSG-/- mice was unstable in the absence of the SGC and sarcospan. This indicates that SGC and sarcospan play an important role in stabilizing the dystrophin axis connecting the basement membrane and the cytoskeleton. The BSG-/- and GSG-/- mice developed progressive muscular dystrophy with notable muscle hypertrophy. We found that the number of muscle fibers increased with age and most of the fibers were regenerating fibers in their hypertrophic muscle. Therefore, muscle hypertrophy is a consequence of excess regenerating process but not due to fibrous and fat tissue replacement, which has been assumed to be present in so-called pseudohypertrophy in human diseased muscle.

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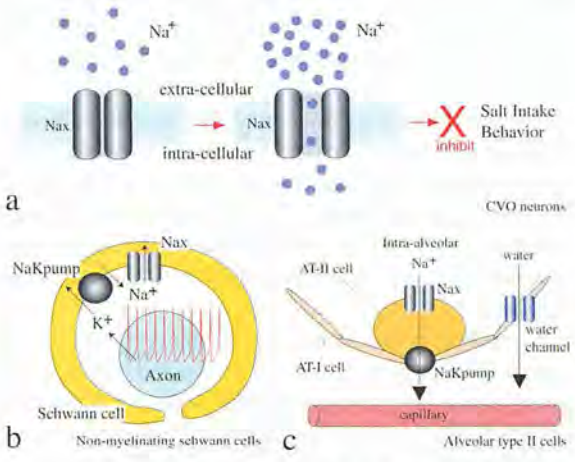

Figure 2. A hypothetical scheme for the physiological role of Nax sodium channel. (a) Nax channel is activated by increase of extracellular sodium concentration. The activated Nax may promote the excitability of GABAergic neurons in SFO. The activated GABAergic neurons in SFO will inhibit the salt-intake-promoting neurons. (b) Repetitive action potentials cause extracellular increase of potassium concentration. Nax may contribute to the potassium uptake by Schwann cells. (c) The osmotic gradient between intra-alveolar space and capillary is a major driving force for the removal of water through water channel expressed in ATI cells. Nax may be one of the molecules generating the osmotic gradient.

RESEARCH CENTER FOR INTEGRATIVE AND COMPUTATIONAL BIOLOGY

Head: *YAMAMORI, Tetsuo*
Professo (Adjunct) : *MIYATA, Takashi*
(Kyoto University)

The neutral theory of molecular evolution, the basic theory for explaining evolutionary changes at the molecular level, claims that most evolutionary changes at the molecular level are caused by random drift of neutral mutants. This is sharply contrasted to the evolution at the organismal level, where it is generally thought that natural selection is the major driving force by which evolutionary changes occur. How can we explain the evolutionary changes at the two levels in a unified way. This is the major problem that remains unsolved for molecular evolutionists. As a first step for understanding the final problem, we are investigating relationships of evolutionary diversifications between molecular and organismal levels, based on molecular phylogenetic approach.

I. Divergence pattern of animal gene families and relationship with the Cambrian explosion

In multicellular animals, a variety of gene families involved in cell-cell communication and developmental control have evolved through gene duplication and gene shuffling, basic mechanisms for generating diverse genes with novel functions. Each of these animal gene families is thought to have originated either from a few ancestral genes which are shared with plants and fungi or from an ancestral gene created uniquely in animal lineage. The major groups of bilateral animals are thought to have diverged explo-sively at or prior to the Vendian - Cambrian boundary. No direct molecular evidence has been provided to date as to whether the Cambrian explosion was triggered by a dramatical increase in the number of genes involved in cell-cell commu-nication and developmental control either immediately prior to or in concert with the Cambrian explosion.

A molecular phylogeny-based analysis of several animal-specific gene families has revealed that the gene diversification by gene duplication occurred during two active periods interrupted by a long intervening quiescent period. Intriguingly, the Cambrian explosion is situated in the silent period, indicating that there is no direct link between the first burst of gene diversification and the Cambrian explosion itself. The above result also suggests the importance of gene recruitment as a possible molecular mechanism for morphological diversity.

To understand a possible origin of animal-specific gene families, we have carried out cloning and sequencing of genes related to the animal-specific protein tyrosine kinase (PTK) genes from choanoflagellates, a group of unicellular plotists known to be the closest relatives of multicellular animals. Many PTK related genes including both the receptor and non-receptor type genes have been identified, some of which are likely to be orthologous to animal PTKs. A molecular phylogenetic tree of the PTK family members including those from animals and choanoflagellates showed an unexpected pattern of the PTK gene family: Most gene duplications that gave rise to different PTK subfamilies recognized in all animal groups occurred at very ancient times before the divergence of animals and choanoflagellates.

II. The origin of land vertebrates and evolutionary relationships among the coelacanth, lungfishes, and tetrapods based on nuclear DNA-coded genes

The phylogenetic relationships among tetrapods and two living groups of lobe-finned fishes, the coelacanth and the lungfishes are still unsolved and debated, despite many studies based on morphological and molecular data. Recent analyses based on complete mitochondrial sequences resulted in confusing phylogenetic trees, which are obviously inconsistent with generally accepted trees. To resolve this difficult phylogenetic question at statistically solid bases, we have cloned and sequenced ten nuclear DNA-coded genes from fourteen major groups of vertebrates, including the coelacanth and the three lungfish species. These sequences, together with sequences available from databases, have been subjected to phylogenetic analyses based on the maximum likelihood (ML) method, using a cyclostome and a lancelet as an outgroup. The obtained ML tree supports the close association of the coelacanth and the lungfish, suggesting that the coelacanth and the lungfish equally closely related as sister groups of tetrapods.

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Associate Professor: *MOCHIZUKI, Atsushi*
NIBB Research Fellow: *TOHYA, Shusaku*
Postdoctoral Fellow: *FUJITA, Hironori*
Graduate Students: *AYABE, Yoshiko*
YAMAGAMI, Ayumi

I. Mathematical method for biological phenomena

We are studying biological phenomena of higher orders by using mathematical models. Mathematical models give us integrative understanding for complex behavior of biological systems including a lot of factors.

Study of the mechanisms responsible for morphological difference between species is an important research focus of the current developmental biology. One way to answer this question is to model the pattern formation process and to show how the species-specific pattern can be formed by the same model with different parameter values. Such a theoretical study would be useful in identifying candidates of cell-cell interaction that are likely to be responsible, to which future experimental study can be focused.

II. Pattern formation of the cone mosaic in the zebrafish retina

In teleost fish, there are several subtypes of cone cells, which are sensitive to different wave-lengths of light. In retinas of some species of teleost fish, regular arrangements of cone cells are observed, where each subtype of cone cells appears periodically in the two-dimensional retinal sheet. These patterns are called "cone mosaics." The biological mechanism of the pattern formation is still under examination. Some species show quite different mosaic patterns between peripheral region and more central region in the retina, which suggests mobility of cone cells in retinal space.

Different patterns are observed in different species. For example, in the zebrafish retina, there are four subtypes of cones, maximally sensitive to blue, red, green and ultra-violet. A green-sensitive cone cell and a red-sensitive cone cell are in tight contact and form a double cone. The cells of the other two subtypes are called single cones. The blue-, ultra violet-, red-, and green-sensitive cones are also called long single, short single, long double, and short double cones, respectively. In an adult zebrafish retina, rows of single cones and those of double cones appear alternatively. This pattern is called a "row mosaic".

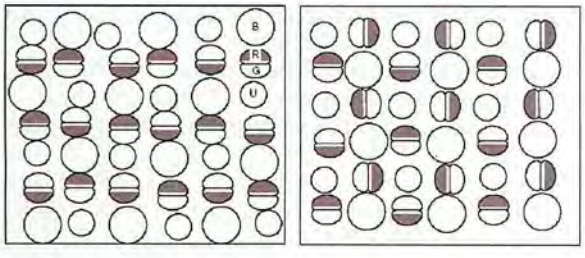


Figure 1. Cone mosaic of fish retina, (Left) in zebrafish, and (Right) in medaka. In fish retina, there are four kinds of differentiated cells. A pair of red-sensitive and green-sensitive cones is in close contact, constituting a double cone. Here four kinds of cones are indicated by capitals (R, G, B, U). In the regular mosaic pattern of zebrafish (Left), the rows of double cones and those of single cones (blue- and UV-sensitive cones) are in parallel and alternately. Two small cones of a red-light sensitive and a green-light sensitive cones form a "double cone".

In contrast, in the medaka retina, four types of cones are arranged in a different manner as illustrated in Fig. 1. In the medaka pattern, each blue-sensitive cone is surrounded by four double cones, and the red-sensitive part of a double cone is close to the green-sensitive part of another. This is called a "square mosaic".

We have studied the mechanism of the pattern formation by using mathematical models. In this study, we examined a process of cell-cell interaction to generate the regular mosaic pattern -- namely cell rearrangement. In the model, cells have already been determined as to their final subtypes, but they change their locations in the pattern formation process and the cell movement is affected by their neighbours.

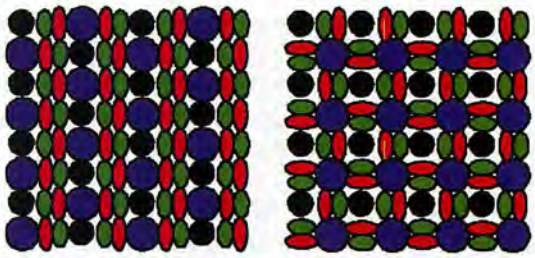


Figure 2. The examples of obtained patterns by cell rearrangement model. The obtained patterns were completely the same as the actual zebrafish retina, when the used adhesion was appropriate.

We show that the same model can produce both row and square mosaic pattern. If the cell-cell interaction is restricted to nearest neighbors only, the square mosaic pattern cannot be generated. We studied the model considering the adhesion working between nearest neighbors and next nearest neighbors, with different weighting between them. Two "shape factors" specifying how to combine adhesion in different geometric are very important in determining whether row mosaic (zebrafish pattern) or square mosaic (medaka pattern) is to be formed.

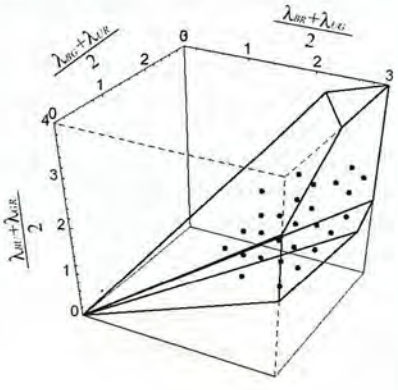


Figure 3. The region in which zebrafish pattern is formed. Dots indicate the parameters that generated the zebrafish pattern obtained by computer simulations. Planes specified the borders separating the parameter region by comparing the zebrafish pattern with four other patterns. A region determined by theoretical analysis was consistent with the numerical results.

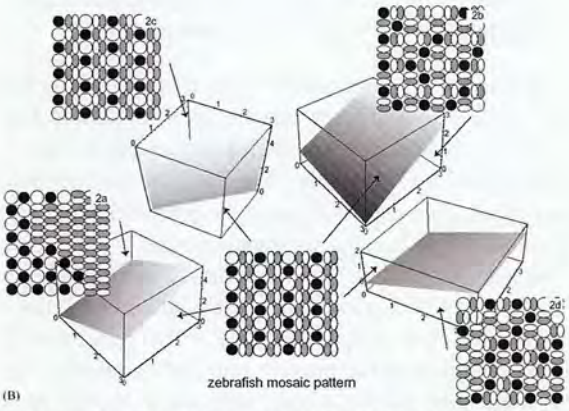


Figure 4. Alternative irregular patterns obtained the parameter regions around the area shown in (A).

We study the conditions for generating mosaic pattern. The condition for generating zebrafish mosaic are shown in Fig. 3, 4. They are the necessary condition and that should be satisfied even in actual organisms if they make the arrangement based on cell adhesion and cell movement.

III. Directionality of Stripes Formed by Anisotropic Reaction-Diffusion Models.

The pattern formation of animal coating has been studied mathematically by a pair of partial differential equations, named a reaction-diffusion (RD) model. By the model, starting from an initial distribution very close to uniformity, a spatial heterogeneity emerges and a stable periodic pattern is formed spontaneously. This simple mechanism suggests that the reaction of a small number of chemicals and their diffusion might create stable non-uniform patterns. When we analyze the model in a two dimensional plane, striped patterns in addition to spotted patterns often emerge. This was considered as the basic mechanism explaining the stripe patterns observed among animal coating.

We focused on the directionality of the stripes. Most of the stripes observed in the fish skins are either parallel or perpendicular to their anterior-posterior (AP) axis. The direction of stripes is considered of importance in the behavioral and ecological viewpoints. However, very little is known about the mechanisms that makes the strong directionality either in the actual fish skin or in the theoretical models. The standard RD model doesn't determine the direction of stripes. To explain the directionality of stripes on fish skin in closely related species, we have studied the effect of anisotropic diffusion of the two substances on the direction of stripes, in the cases in which both substances have the high diffusivity in the same direction.

We also studied the direction of stripes in more general situations in which the diffusive direction may differ between the two substances. We derived a formula for the direction of stripes, based on a heuristic argument of unstable modes of deviation from the uniform steady state. We confirm the accuracy of the formula by computer simulations. When the diffusive direction is different between two substances, the directions of stripes in the spatial pattern change smoothly with the magnitude of anisotropy of two substances. When the diffusive direction of the two substances is the same, the stripes are formed either parallel to or perpendicular to the common diffusive direction, depending on the relative magnitude of the anisotropy. The transition between these two phases occurs sharply.

Figure 5. The stripe formation in the skin of Genicanthus. These two species are closely related.

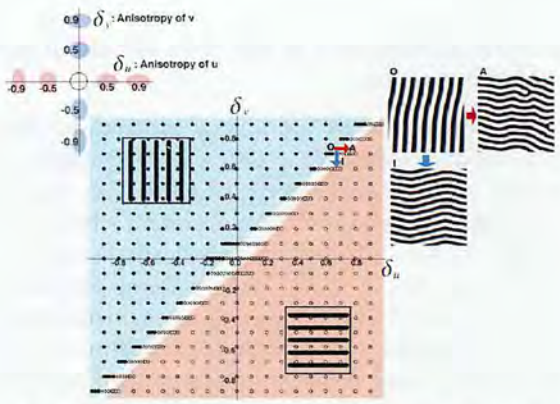


Figure 6. Summary of the direction of stripe patterns obtained by the anisotropic diffusion model. Horizontal and vertical axes indicate anisotropy of activator and that of inhibitor, respectively. The left-upper corner indicates the distortion of diffusion range. Each point indicates the direction of the observed stripe: horizontal; vertical; or not-determined. The direction is determined only by the difference between anisotropies. A small difference in diffusion anisotropy can alter the final pattern to one with opposite directionality. Note the transition of the stripes by changing the anisotropies from O to A (or I).

Publication List

Tohya, S., Mochizuki, A. and Iwasa, Y. (2003) Random cell sorting can form cone mosaic patterns in fish retina and explain the difference between zebrafish and medaka. *J. Theor. Biol.* **221**, 289-300.

Shoji, H., Mochizuki, A., Iwasa, Y., Hirata, M., Watanabe, T., Hioki, S. & Kondo, S. (2003) Origine of directionality in the fish stripe pattern. *Dev. Dyn.* **226**, 627-633.

Ryohji, T., Mochizuki, A. & Iwasa, Y. (2003) Possibility of Tissue Separation Caused by Cell Adhesion. *J. Theor. Biol.* **221**, 459-474.

RESEARCH SUPPORT FACILITY

Head of Facility: *MURATA, Norio (~Mar. 2003), NODA,Masaharu (Apr.~Nov. 2003), NISHIMURA, Mikio (Dec. 2003~)*
Associate Professor: *WATANABE, Masakatsu*
Research Associates: *HAMADA, Yoshio (Tissue and Cell Culture)*
UCHIYAMA, Ikuo (Computer)
Technical Staffs: *HIGASHI, Sho-ichi (Large Spectrograph)*
NAKAMURA,Takanori (Large Spectrograph;Oct. 2003~)
MIWA, Tomoki (Computer)
NANBA, Chieko (Plant Culture, Farm, Plant Cell)
NISHIDE, Hiroyo (Computer)
ICHIKAWA. Chiaki (Large Spectrograph)
TAKESHITA, Miyako (Tissue and Cell Culture)
MAKIHARA,Nobuko (Computer)
SUZUKI, Keiko (Plant Culture, Farm, Plant Cell)

I. Facilities

1. The Large Spectrograph Laboratory

This laboratory provides, for cooperative use, the Okazaki Large Spectrograph (OLS), which is the largest spectrograph in the world, 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.* 1982, *Photochem. Photobiol.*, **36**, 491-498).

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) *etc.* was also introduced.

2. Tissue and Cell Culture Laboratory

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

3. Computer Laboratory

Computer laboratory maintains several computers to provide computation resources and means of electronic communication in this Institute. Currently, the main system consists of three servers and two terminal workstations: biological information analysis server (SGI Ori-gin 2000), database server (Sun Enterprise 450), file server (Sun Enterprise 3000), data visualization terminal and molecular simulation terminal (both are SGI Octanes). Some personal computers and color/monochrome printers are also equipped. On this system, we provide various biological databases and data retrieval/analysis programs, and support large-scale data analysis and database con-struction for the Institute members.

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

4. Plant Culture Laboratory

There are 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.

5. Experimental Farm

This laboratory consists of two 20 m^2 glass-houses with precise temperature and humidity control, three green houses (each 6 m^2) at the P1 physical containment level, a small farm, 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

Autotrophic and heterotrophic culture devices and equipment for experimental cultures of plant and microbial cells in this laboratory. A facility for preparation of plant cell cultures including an aseptic room with clean benches, is also provided.

7. Laboratory of Stress-Resistant Plants

This laboratory was found to study transgenic plants with respect to tolerance toward various environmental stresses. It is located in the Agricultural Experimental Station of Nagoya University (30 km from National Institute for Basic Biology). The laboratory provides a vari-ety of growth chambers that precisely control the condi-tions of plant growth and dacilities for molecular biologi-cal and physiological enaluations of transgenic plants.

The laboratory is also a base of domestic and international collaborations devoted to the topic of stress resistant transgenic plants.

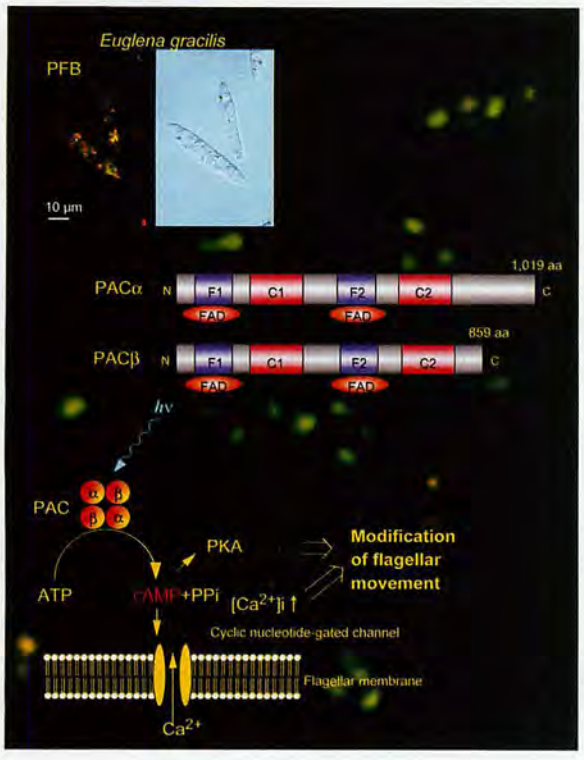
II. Research Activities

1. Faculty

The faculty of the Research Support Facility conducts its own research as well as scientific and administrative public services.

(1) Photobiology: Photoreceptive and signal transduction mechanisms of phototaxis of unicellular algae are studied action spectroscopically (Watanabe 2004, *In* "CRC Handbook of Organic Photochemistry and Photobiology, 2nd ed.") by measuring computerized-videomiceographs of the motile behavior of the cells at the cellular and subcellular levels. Photoreceptive and signal transduction mechanisms of algal gene expression were also studied by action spectroscopy.

A novel blue-light receptor with an effector role was found from *Euglena gracilis* (Fig. ; Iseki *et al.* 2002, *Nature* **415**, 1047-1051): *Euglena gracilis*, a unicellular flagellate, shows blue-light type photomovements. The action spectra indicate the involvement of flavoproteins as the photoreceptors mediating them. The paraflagellar body (PFB), a swelling near the base of the flagellum has been considered as a photosensing organelle for the photomovements. To identify the photoreceptors in the PFB, we isolated PFBs and purified the flavoproteins therein. The purified flavoprotein (ca. 400 kDa), with noncovalently bound FAD, seemed to be a heterotetramer of a- and b-subunits. Predicted amino acid sequences of each of the subunits were similar to each other and contained two FAD-binding domains 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.



The involvement of PAC in positive and negative phototaxis (steering response with respect to stimulus light direction) was also demonstrated by its knock-down using RNAi (Ntefidou *et al.* 2003). Orthologues of PAC α and β were detected in several relatives of *Euglena* and their phylogenetic analysis indicated that they were trtansfered to euglenoids on the occasion of secondary endosymbiosis (Koumura *et al.* submitted).

(2) Developmental Biology: Replacement of the ankyrin repeats of mouse Notch2 gene with *E. coli* b-gactosidase gene induces early embryonic lethality around E10.5. The lethality was suggested due to defects in extraembryonic tissues, because the mutant embryo grew and differentiated further in vitro. Histological examination and in situ hybridization analysis with trophoblast subtype-specific probes revealed that the development of giant and spongiotrophoblast cell layers are normal in the mutant placenta, while vasculogenesis in the labyrinth layer apperaed compromised at E9.5. Since the lethality was circumvented by production of chimeric mice with tetraploidy wild type embryos, we concluded that the embryonic lethality is due to defect in growth and/or differentiation of labyrinthine trophoblast cells. The mutant embryo, however, could not be rescued in the tetraploid chimeras beyond E12.5 because of insurfficient development of umbilical cord, indicating another role of Notch2 signaling in the mouse development. Chimeric analysis with diploid wild type, however, revealed contribution of mutant cells to these affected tissues by E13.5. Thus, Notch2 are not cell autonomously required for the early cell fate determination of labyrinthine trophoblast cells and allantoic mesodermal cells, but plays an indispensable role in the further formation of functional labyrinth layer and umbilical cord.

(3) Computational Biology: Comparative genomics is a useful approach to find clues to understanding complex and diverse biological systems. Our research aim is to develop new methods as well as practical analysis systems to compare a large number of genomic sequences, and to apply them to real data analyses.

Our main developing system is a workbench for microbial genome comparison named MBGD, which now contains more than a hundred of microbial genome sequences. We have continued to enhance the efficiency of the database to treat such large number of genomes. As a key component of MBGD, we have also continued to develop an automated method for orthologous grouping among multiple genomes. In addition to splitting fusion genes into orthologous domains, we are also trying to enhance the algorithm to incorporate some other information such as gene arrangement and species phylogeny.

In addition to these developments, we are also trying to apply the computational methods to real data analyses, especially to the comparative genomics with some closely related organisms. In collabolation with Dr. Takami's group (JAMSTEC), we are comparing genomic sequences of some Bacillus-related organisms that live in several different environmental conditions.

2. Cooperative Research Program for the Okazaki 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 scientists as well as those in the Institute.

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

Publication List:

I. Faculty

Mikami, K. and Murata, N. (2003) Membrane fluidity and the perception of environmental signals in cyanobacteria and plants. *Progress Lipid Res.* **42**, 527-543.

Mikami, K., Suzuki, I. and Murata, N. (2003) Sensors of abiotic stress in *Synechocystis*. *In* "Topics in Current Genetics, vol. 4, Plant Stress Response" (H. Hirt and K. Shinozaki eds.), pp 103-119.

Nishiyama T, Fujita T, Shin-I T, Seki M, Nishide H, Uchiyama I, Kamiya A, Carninci P, Hayashizaki Y, Shinozaki K, Kohara Y, Hasebe M. (2003) Comparative genomics of *Physcomitrella patens* gametophytic transcriptome and *Arabidopsis thaliana*: implication for land plant evolution. *Proc. Natl. Acad. Sci. USA.* **100**, 8007-8012.

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Suzuki, T., Yamasaki, K., Fujita, S., Oda, K., Iseki, M., Yoshida, K., Watanabe, M., Daiyasu, H., Toh, H., Asamizu, E., Tabata, s., Miura, K., Fukuzawa, H., Nakamura, S., Takahashi, T. (2003) Archaeal-type rhodopsins in *Chlamydomonas*: model structure and intracellular localization. *Biochem. Biophys. Res. Commun.* **301**, 711-717.

Tamura, K., Shimada, T., Ono, Y., Nagatani, A., Higashi, S-I, Watanabe, M., Nishimura, M., Hara-Nishimura, I. (2003) Why green fluorescencent fusion proteins have not been observed in the vacuoles of higher plants. *Plant J.* **35**, 545-555.

Uchiyama, I. (2003) MBGD: microbial genome database for comparative analysis. *Nucleic Acids Res.* **31**, 58-62.

Watanabe, M. (2003) Action spectroscopy for photosensory processes. *In* "CRC Handbook of Organic Photochemistry and Photobiology, 2nd ed." (W. Horspool and F. Lenci eds.) pp. 115-1~115-16, CRC Press, Boca Raton

II. Cooperative Research Program for the Okazaki Large Spectrograph

Andrady, A.L., Halis, S. H., and Torikai, A. (2003) Effect of climate change and UV-B on materials. *Photochem. Photobiol. Sci.* **2**, 68-72.

Andrady, A.L., Hamid, S. H., and Torikai, A., (2003) Effect of climate change and UV-B on materials. *In* " Environmental Effect of Ozone Depletion and Interactions with Climate Change:2002 Assessment" .

Arimoto-Kobayashi, S., Ando, Y., Horai, Y., Okamoto, K., Hayatsu, H and Michael H. L. G. (2002) Mutation, DNA strand cleavage and nitric oxide formation caused by N-nitroproline with UVA & UVB. *J. Photosci.* **9**, 49-50.

Sasaki, M., Takeshita, S., Oyanagi, T., Miyake, Y. and Sakata, T. (2002). Increasing trend of biologically active solar ultraviolet-B irradiance in mid-latitude Japan in the 1990s. *Optical Engineering* **41**, 3062-3069.

Tamura, K., Shimada, T., Ono, E., Tanaka, Y., Nagatani, A., Higashi, S-i., Watanabe, M., Nishimura, M. and Hara-Nishimura, I. (2003) Why green fluorescent fusion proteins have not been observed in the vacuoles of higher plants. *Plant J.* **35**, 545-555.

THE CENTER FOR ANALYTICAL INSTRUMENTS

(managed by NIBB)

Head of Facility: *KOBAYASHI, Satoru*
Technical Staffs: *MORI, Tomoko*
MAKINO, Yumiko
TAKAMI, Shigemi
Technical Assistant: *MORIBE, Hatsumi*

The Center serves for amino acid sequence analysis, and chemical syntheses of peptides and nucleotids to support researchers in NIBB and NIPS. Newly installed instruments in 2003 are Protein Sequencer (ABI Procise 492cLC), Color Laser 3D Profile Microscope (KEYENCE VK-8500) and so on. Instruments of the Center can be used by researchers outside the Institute upon proposal.

Figure 1. Protein Sequencers.

Figure 2. MALDI/TOF-MS

Representative instruments are listed below.

Protein Sequencers (ABI Procise 494HT,492cLC)

Amino Acid Analyzer (Hitachi L8500A)

Peptide Synthesizers (ABI 433A, 432A)

Plasmid Isolation Systems (Kurabo PI-100Σ, PI-50, PI-200)

Automatic Nucleic Acid Isolation System (Kurabo NA-2000)

DNA Sequencers (ABI 377, 373S, 310, 3100)

Thermal Cyclers (Perkin Elmer PJ-9600, Takara TP-300, Biometra TGRADIENT)

Integrated Thermal Cyclers (ABI CATALYST Turbo 800)

Particle Delivery System (Bio-Rad BiolisticPDS-1000/He)

Gas Chromatograph (Shimadzu GC-14APF-SC)

Glycoprotein Analysis System (Takara Glyco-Tag)

High Performance Liquid Chromatographs (Shimadzu LC-10AD, LC-6AD, Waters 600E, Alliance UV system)

Integrated Micropurification System (Pharmacia SMART)

Flow Cytometer (Coulter EPICS XL)

Biomolecular Interaction Analysis Systems (Pharmacia BIACORE 2000, Affinity Sensors IAsys)

Laboratory Automation System (Beckman Coulter Biomek 2000)

NMR Spectrometer (Bruker AMX-360wb)

EPR Spectrometer (Bruker ER-200D)

GC/Mass Spectrometer (JEOL DX-300)

MALDI/TOF-MS (Bruker Daltonics REFLEX III)

Inductively Coupled Plasma Atomic Emission Spectrometer (Seiko SPS1200A)

Spectrofluorometers (Hitachi 850, F-4500, Simadzu RF-5000)

Spectrophotometers (Hitachi 330, 557, U-2001,Varian Cary 5G,Perkin Elmer Lambda-Bio)

Microplate Luminometer (Berthold MicroLumat LB 96P)

Microplate Readers (Corona MTP-120, MTP-100F)

Spectropolarimeter (JASCO J-40S)

FT-IR Spectrophotometer (Horiba FT-730)

Laser Raman Spectrophotometer (JASCO R-800)

Bio Imaging Analyzers (Fujifilm BAS 1500, 2000, 5000)

Fluorescence Bio Imaging Analyzer (Takara FMBIO)

Electrophoresis Imaging System (BIOIMAGE)

Microscopes (Carl Zeiss Axiophot, Axiovert)

Environmental Scanning Electron Microscope (PHILIPS XL30 ESEM)

Confocal Laser Scanning Microscope (Leica TCS SP2)

Color Laser 3D Profile Microscope (KEYENCE VK-8500)

High-Resolution Quick Microscope (KEYENCE VH-5000)

TECHNOLOGY DEPARTMENT

Head: HATTORI, Hiroyuki

Common Facility Group

Chief : FURUKAWA, Kazuhiko

Reseach Support Facilities
HIGASHI, Sho-Ichi (Unit Chief) <Large Spectrograph>
NANBA, Chieko (Subunit Chief) <Plant Culture>
NISHIDE, Hiroyo <Computer>
NAKAMURA, Takanori <Large Spectrograph>
SUZUKI, Keiko (Technical Assistant)
MAKIHARA, Nobuko (Technical Assistant)
ICHIKAWA, Chiaki (Technical Assistant)
HARADA, Miyuki (Technical Assistant)

Center for Analytical Instruments
MORI, Tomoko (Unit Chief)
MAKINO, Yumiko
TAKAMI, Shigemi
MORIBE, Hatsumi (Technical Assistant)

Transgenic Animal Facility
HAYASHI, Koji (Subunit Chief)
YAMAMOTO, Mamiko (Technical Assistant)

Disposal of Waste Matter Facility
MORI, Tomoko (Unit Chief)
FURUKAWA, Kazuhiko

Radioisotope Facility
MATSUDA, Yoshimi (Unit Chief)
SAWADA, Kaoru (Subunit Chief)
IINUMA, Hideko
ITOH, Takayo (Technical Assistant)

Research Support Group

Chief : KOBAYASHI, Hiroko

Cell Biology Group
KONDO, Maki (Unit Chief)
KABEYA, Yukiko (Subunit Chief)

Developmental Biology Group
TAKAGI, Chiyo
OKA, Sanae

Regulation Biology Group
YAMAGUCHI, Katsushi
TAKEUCHI, Yasushi

Gene Expression and Regulation Group I
TANAKA, Sachiko (Unit Chief)
MOROOKA, Naoki

Gene Expression and Regulation Group II
OHSAWA, Sonoko (Unit Chief)
SUMIKAWA, Naomi

Integrated Bioscience Group
MIWA, Tomoki (Unit Chief)
MIZUTANI, Takeshi
UTSUMI, Hideko
NODA, Chiyo
KONDO, Makiko (Technical Assistant)

The Technology Department is a supporting organization for researchers and research organization within the NIBB. The Department develops and promotes the institute's research activities and at the same time, maintains the research functions of the institute.

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

Technical staffs participate, through the department, in mutual enlightenment and education increase their capability in technical area. Each technical staff is proceeded to the fixed division usually and they support the various research with their special biological and biophysical techniques.

The Department 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 soon after the meeting.

CENTER FOR INTEGRATIVE BIOSCIENCE (jointly managed by NIBB)

Chairperson: KITAGAWA Teizo

DEPARTMENT OF DEVELOPMENT, DIFFERENTIATION AND REGENERATION
- DIVISION OF DEVELOPMENTAL GENETICS
- DIVISION OF MOLECULAR & DEVELOPMENTAL BIOLOGY

DEPARTMENT OF BIO-ENVIRONMENTAL SCIENCE
- DIVISION OF BIO-ENVIRONMENTAL SCIENCE
- DIVISION OF PLANT DEVELOPMENTAL GENETICS
- DIVISION OF BIOINFORMATICS

The center is jointly managed by NIBB and two other institutes in Okazaki, IMS (Institute for Molecular Science) and NIPS (National Institute for Physiological Sciences). The following projects will be the main focus of the center: 1) Development, Differentiation and Regeneration. 2) Strategic Methodology. 3) Bio-Environmental Science.

DIVISION OF DEVELOPMENTAL GENETICS

Professor:	*KOBAYASHI, Satoru*
Research Associate:	*MUKAI, Masanori*
Technical Staff:	*NODA, Chiyo*
NIBB Research Fellow:	*SATO, Kimihiro*
JSPS Postdoctral Fellow:	*SHIGENOBU, Shuji*
Postdoctoral Fellow:	*AMIKURA, Reiko*
Graduate Students:	*HAYASHI, Yoshiki*
	KITADATE, Yu
	HAYASHI, Makoto [1]
Technical Assistants:	*ARITA Kayo*
	UEDA, Yoshiko
	TSUCHIYA, Naomi
	SATO, Kaori
Secretary:	*SUZUKI, Masako*

[1] *Graduate School of Biological Sciences, University of Tsukuba*

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

I. Role of Mitochondrial Ribosomal RNAs in Pole Cell Formation

Ultrastructural studies have shown that the germ plasm is basically composed of polar granules and mitochondria. While the primary roles of the mitochondria are oxidative phosphorylation and biosynthesis of many metabolites, it has now become evident that they are also involved in germline formation.

In *Drosophila*, pole cell formation requires the function of mitochondrial ribosomal RNA in germ plasm. We have previously reported that mitochondrial large rRNA (mtlrRNA) and small rRNA (mtsrRNA) are both transported from mitochondria to polar granules. This transportation occurs during early embryogenesis, when mitochondria are tightly associated with polar granules in germ plasm, and it depends on the function of the maternally-acting gene, *tudor*, that is known to be required for pole cell formation. Mitochondrial rRNAs remain on the polar granules until pole cell formation and are no longer discernible on the granules within pole cells. Reduction of the extra-mitochondrial mtlrRNA amount results in the failure to form pole cells and injection of mtlrRNA is able to induce pole cells in embryos whose ability to form these cells has been abolished by uv-irradiation. These observations clearly show that the extra-mitochondrial mtlrRNA on polar granules has an essential role in pole cell formation, presumably cooperating with mtsrRNA.

Recently, we found that mitochondrial rRNAs form mitochondrial-type of ribosomes on polar granules, cooperating with mitochondrial ribosomal proteins. This suggests the possibility that the protein (s) essential for pole cell formation is produced by the mitochondrial-type of ribosomes. To address this issue, we examined the effect of *Chloramphenicol* and *Kasugamycin* on pole cell formation. *Chloramphenicol* and *Kasugamycin* are known to inhibit mitochondrial (prokaryotic)-type of translation. When these antibiotics were injected into the posterior pole region of early embryos, pole cell formation was severely affected. In contrast, *Chloramphenicol* and *Kasugamycin* treatment did not affect somatic cell formation at a dose we used. These observations strongly suggest that the mitochondrial-type of translation system must be intact for the embryos to form pole cells. The project to identify a targer mRNA for the mitochondrial-type of translation machinery is now on going.

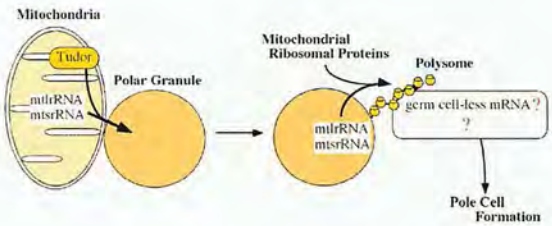

Fig. 1 Role of mitochondrial rRNAs in pole cell formation

II. Role of Nanos protein in pole cell differentiation

Pole cells differ from the soma in regulation of mitosis and transcriptional activity. Pole cells cease mitosis at gastrulation and remain quiescent in the G2 phase of the cell cycle throughout their migration to the gonads, while somatic cells continue to proliferate during the rest of embryogenesis. Furthermore, pole cells are transcriptionally quiescent until the onset of gastrulation, although transcription is initiated in the soma during the syncytial blastoderm stage. Consistent with this, RNA polymerase II (RNAP II), but not RNA polymerase I, remains inactive in early pole cells. Thus, the ability to express zygotic mRNA-encoding genes is

suppressed only in pole cells in early embryos.

Among the maternal components of germ plasm, Nanos (Nos) is essential for the germline-specific events occurring in pole cells. nos mRNA is localized in the germ plasm during oogenesis, and is translated in situ to produce Nos protein after fertilization. Nos is only transiently present in the posterior half of embryos during the preblastoderm stage, and is required there for posterior somatic patterning. Nos in the germ plasm is more stably inherited into the pole cells at the blastoderm stage, remaining detectable in these cells throughout embryogenesis. Pole cells that lack Nos (*nos*⁻ pole cells) are unable to follow normal germline development; they fail to migrate properly into the embryonic gonads, and consequently do not become functional germ cells. In *nos*⁻ pole cells, mitotic arrest at G2 phase is impaired, and they undergo premature mitosis. Furthermore, *nos*⁻ pole cells fail to establish and/or maintain transcriptional quiescence, and ectopically express somatically-transcribed genes, including *fushi tarazu (ftz)*, *even-skipped (eve)* and *Sex-lethal (Sxl)*.

Nos represses translation of mRNAs with discrete RNA sequences called Nos response elements (NREs). In the pathway leading to posterior somatic patterning, Nos acts together with unlocalized Pumilio (Pum) protein to repress translation of maternal *hunchback* (*hb*) mRNA. This translational repression is mediated by binding of Pum to NREs in the 3'-untranslated region (UTR) of hb mRNA. In pole cells, Nos also acts with Pum to regulate germline-specific events. Pum, like Nos, is required in pole cells for their migration to the gonads.

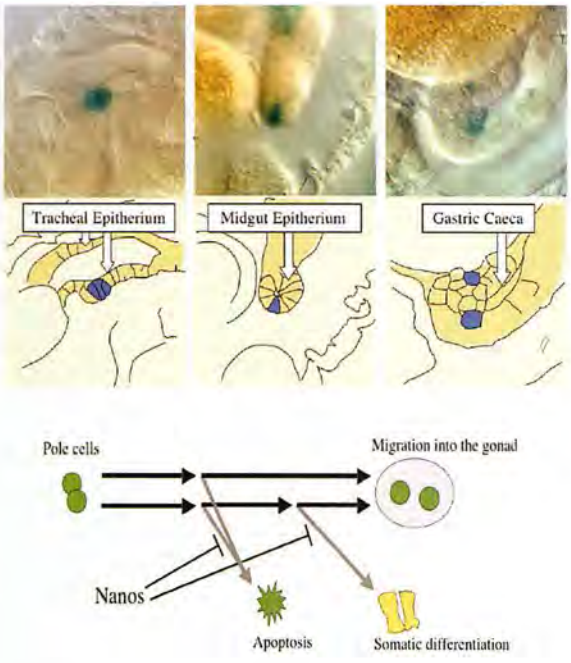

Fig. 2 Somatic differentiation of pole cells lacking Nos

We found that Nos, along with Pum, represses translation of importin α2 (impα2) mRNA in early pole cells. The impα2 mRNA contains an NRE-like sequence in its 3'-UTR and encodes a *Drosophila* importin a homologue that plays a role in nuclear import of karyophilic proteins. We found that Nos inhibits expression of a somatically-transcribed gene, *ftz*, in pole cells by repressing Impα2-dependent nuclear import of a transcriptional activator for ftz, Ftz-F1. Furthermore, the expression of another somatic gene, *eve*, and RNA Polymerase II activity are also repressed by Nos in pole cells through its effects on Impα2-dependent nuclear import.

The above results raise the question whether the pole cells lacking Nos (*nos*⁻ pole cells) are able to differentiate into somatic cells. However, it is difficult to study their developmental fate, since Nos also represses apoptosis of pole cells, and almost all of *nos*⁻ pole cells are eliminated until at least the end of embryogenesis. To overcome this problem, we used Df (3L) H99, a deletion for three genes required for apoptosis. Introduction of the H99 deficiency results in nos- pole cells being escaped from apoptosis. We transplanted the *nos*⁻ H99⁻ pole cells into normal embryos and observed their behavior, and found that some of *nos*⁻ H99⁻ pole cells were able to differentiate as somatic cells. This suggests that pole cells have the ability to differentiate as somatic cells, but its ability is inhibited by Nanos activity. Recently, we have found that somatic differentiation of *nos*⁻ H99⁻ pole cells requires Impα-2 activity, suggesting that Nos inhibits somatic differentiation by repressing Impα-2 production.

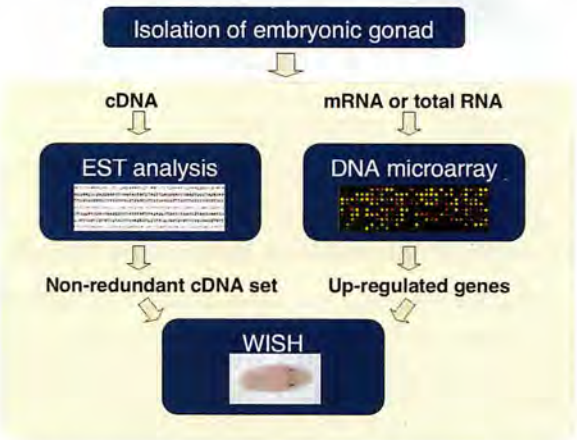

Fig. 3 Transcriptome analysis of the embryonic gonads

III. Comprehensive analysis of genes expressed in *Drosophila* gonad

Pole cells migrating into the gonads are specified to be the primordial germ cells (PGCs). It has been believed that zygotic genes expressed in pole cells within the gonads are required for their fate specification. To explore the regulatory mechanism of germline specification, we attempted to identify genes

expressed in pole cells and/or in somatic cells within the gonad by a comprehensive approach. From the embryos carrying EGFP-vasa transgene that express GFP only in pole cells, we isolated the gonads by using fluorescence-activated cell sorting (FACS), and costructed a gonad cDNA library. Each cDNA clone was sequenced from both 5' and 3' ends, and these Expression Sequence Tags (ESTs) were computationally condensed into sequence clusters, which were then subjected to whole-mount *in situ* hybridization (WISH). Approximately 20,000 of ESTs were generated, and were clustered into 2900 distinct genes. The WISH analysis identified more than 130 genes that were expressed predominantly in the gonads. In addition, we found gonad-specific splicing form in some transcripts. These transcriptome data will allow us to illustrate genetic networks governing the germline specification.

We also started to identify genes expressed predominantly in the embryonic gonads, using DNA microarray technique.

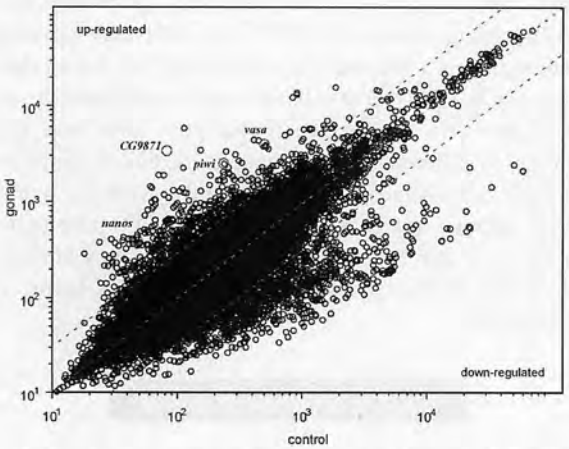

Fig. 4 Scatter plot of expressin levels in control (whole embryos) and the embryonic gonads.

IV. *sva53*, a Maternal Gene Required for Meiosis

It has been believed that maternal factors localized in germ plasm may ultimately trigger germline-specific events, such as meiosis. We have isolated an X-linked maternal mutation, *sva53* that affects meiosis. Pole cells that were formed in the embryos derived from *sva53* homozygous germline clone (*sva53* pole cells) were able to develop into the oocytes, but they failed to execute meiosis. We also found that the germline-specific expression of *vasa* gene was severely affected in *sva53* pole cells. These results suggest that the maternal factor encoded by *sva53* gene may activate gene expression, which is essential for meiosis. In order to identify *sva53* gene, we mapped *sva53* mutation to 200 kb-genomic region of 11C by using duplications and deficiencies. Within this region, we found a gene encoding a Zn-finger transcription factor, of which mRNA is maternally supplied into embryos.

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DIVISION OF MOLECULAR & DEVELOPMENTAL BIOLOGY

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One of the research interests of this laboratory is to understand molecular mechanism how a cell signaling molecule, including members of Wnt, BMP and FGF families, regulates different developmental events. A number of evidence indicated that each signal is involved in many aspects of the vertebrate development. For instance, we have revealed that Wnt-3a, a members of Wnt family, plays essential roles in a number of aspects of the mouse development, including somite development, neural crest formation and neural development. However, cellular and molecular mechanisms how a cell signaling molecule regulates these different events. Thus, we are focusing on precise functional analysis of cell-to-cell signals and identification of target genes induced by these signals.

Another interest is to understand molecular mechanism of development of the vertebrate trunk, especially somite. One of interesting features of the vertebrate trunk development is that it proceeds gradually. To understand how metameric structures of somites are gradually generated in an anterior to posterior order along the both sides of embryonic body axis and how each somite are characterized differently along the anterio-to-posterior axis, we are also trying genetical approach with the zebrafish.

I. Roles of Wnt signals during neural development

The Wnt family of genes that encode cysteine rich secreted proteins consists of at least 17 members in the vertabrate. It has already been shown that some of them are expressed and play important roles during neural development. For instance, we showed that Wnt-1 and Wnt-3a, which are expressed in the most dorsal region within the developing central nervous system, direct specification of the dorsal interneurons. Analysis of mouse embryos lacking both Wnt1 and Wnt3a and culture of explants from the neural plate indicated that these Wnt signals promote generation of the most dorsal subclass of the interneuron, called D1 and D2, at the expense of that of more ventral subclass, called D3.

Wnt signaling is also implicated in the control of cell growth and differentiation during CNS development from studies of mouse and chick models, but its action at the cellular level has been poorly understand. In vitro stem cell culture is a powerful tool for examining the effect of an external signal on neural stem cells. In vitro clonal analysis has shown that, in a serum-free defined medium supplemented with FGF-2, single cells derived from the embryonic or adult brain proliferate and form floating spherical colonies, called neurospheres. Single cells derived from neurospheres self-renew to generate a new neurosphere or differentiate into neurons or glia depending on the culture condition, indicating that they have characteristics of stem cells.

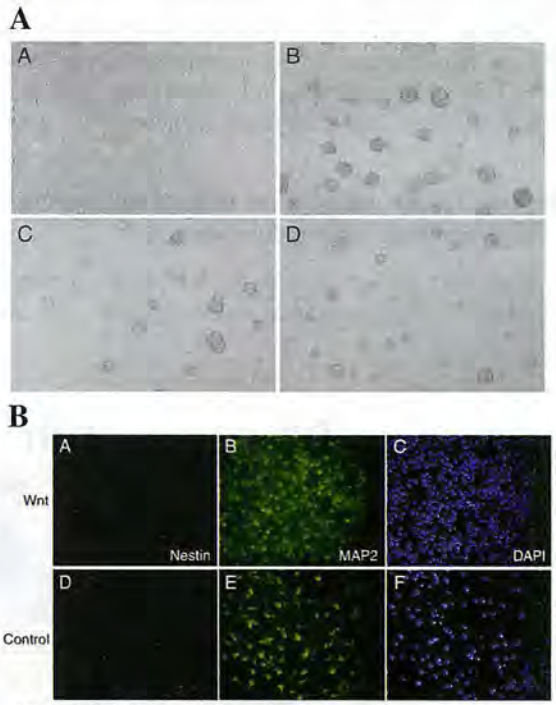

Fig.1. Inhibition of neurosphere formation and promotion of neural cell differentiation in the presence of Wnt3a CM.

A: Cells dissociated from the E11.5 mouse forebrain were cultured for 2 days in low attachment multiwell plates under various conditions: with Wnt3a conditioned medium (Wnt-3a CM) (**A**), with immunoabsorbed Wnt-3a CM (**B**), with control conditioned medium (control CM) (**C**), and without conditioned medium (**D**). Many large spheres were generated in the culture with control CM (**D**). In contrast, treatment with Wnt-3a CM significantly increased the number of differentiated cells and almost wiped out neurospheres (**A**). This effect was dependent on Wnt-3a concentration (data not shown). These effects of Wnt-3a CM were almost eliminated by immunoabsorption, indicating that these effects are due to the Wnt-3a protein (**B**). Total 10 fields were counted under phase-contrast microscope using 100x magnification.

B: Dissociated cells from the primary neurospheres obtained from the E11.5 telencephalon were cultured for two days on poly D-lysine coated coverslips in the presence of Wnt3a CM and FGF2. Then, these cells were continuously cultured for an additional four days with Wnt3a CM (**A-C**) or control CM (**D-F**), but without FGF2. After 4 days in culture, cells were immunostained for Nestin or MAP2. Immunofluorescence microscopy for Nestin (red) (**A**, **D**), MAP2 (green) (**B**, **E**) and DAPI (blue) (**C**, **F**).

Thus, to understand function of Wnt signaling on the neural stem cells, we examined the in vitro function of Wnt signaling in embryonic neural stem cells, dissociated from neurospheres derived from E11.5 mouse telencephalon. Conditioned media containing active Wnt-3a proteins were added to the neural stem cells and its effect on regeneration of neurospheres and differentiation into neuronal and glial cells was examined. Wnt-3a proteins inhibited regeneration of neurospheres, but promoted differentiation into MAP2-positive neuronal cells (FIG. 1). Wnt-3a proteins also increased the number of GFAP- positive astrocytes but suppress the number of oligodendroglial lineage cells expressing PDGFR or O4. These results indicate that Wnt-3a signaling can inhibit the maintenance of neural stem cells, but rather promote the differentiation of neural stem cells into several cell lineages.

II. Functional Analysis of molecular targets of Wnt signaling during development

Wnt-3a is also expressed in the primitive streak ectoderm during gastrulation and in the tailbud in later development of the mouse. For dissecion of the complex developmental events regulated by Wnt-3a signaling in these regions, it is important to identify genes regulated by this signal. It has already been demonstrated that T (Brachyury) is a direct target of Wnt-3a in the anterior primitive ectoderm, which is fated to give rise to the paraxial mesoderm, suggesting that Wnt-3a modurates a balance between mesodermal and neural cell fates via T.

To gain more insight into roles of Wnt signaling during embryogenesis, we searched for potential target genes of this signaling by an induction gene trap screening in mouse ES cells. In at least three ES cell clones among 794 clones screened, expression of beta-geo reporter genes was dramatically changed in response to the conditioned medium of Wnt-3a expressing cells. The expression analysis of the reporter genes in embryos generated from these ES cell clones revealed that the spatiotemporal expression patterns of these reporter genes were well correlated to those of several Wnt genes. These results suggested that an induction gene trap approach is effective for screening of target genes of Wnt signaling during embryogenesis.

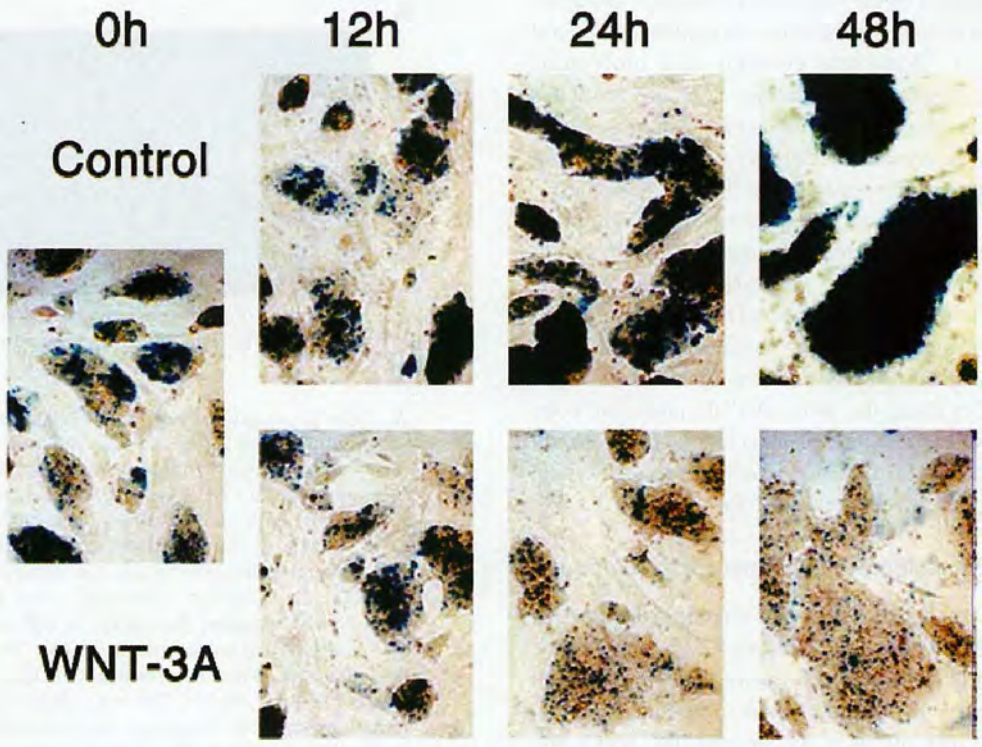

Fig. 2. An example of the expression of trapped genes which are repressed by Wnt signal in ES cells. In vivo expression and *in vivo* function of this gene have been examined by generating mice carrying this trapped allele.

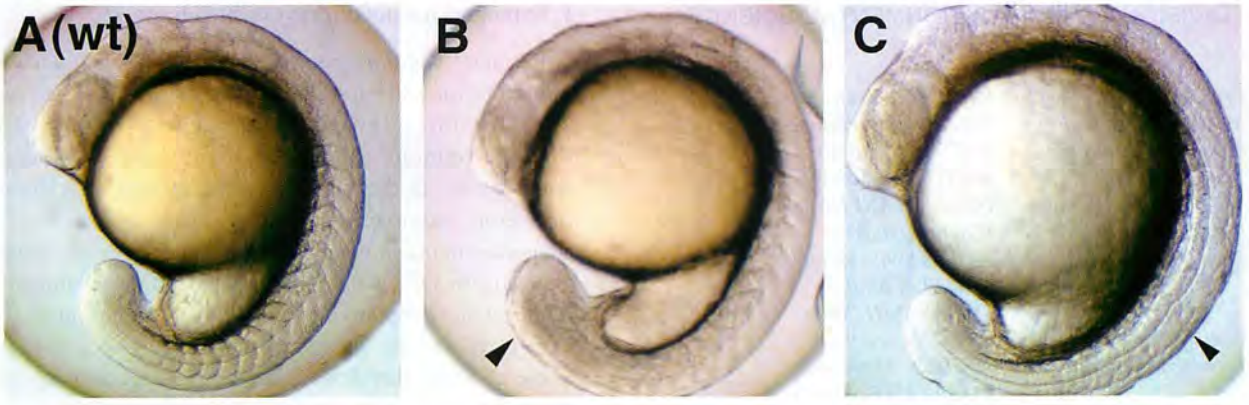

Fig. 3. Zebrafish mutant embryos which display abnormal shape of the tailbud (B) and a segmentation defect of somites (C).

III. Genetical approaches for revealing molecular mechanism of trunk development in zebrafish

To identify genes involved in several aspects during early embryogenesis of the vertebrate, we have performed screening of zebrafish mutants induced by ENU, a chemical mutagen. Until now, we have screened 630 of F2 families and found a number of mutants whose phenotypes are different from those already reported. For instance, some of these mutants displayed defects in the somite and tailbud development. Cloning of genes that are responsible for these defects is in progress.

To complement a forward genetical approach, we have also screened genes expressed in the tailbud and presomitic mesoderm, in which somite progenitors exist. Until now, we have identified 50 genes that are expressed preferentially in these regions. To examine developmental roles of these genes, functional analysis of these genes has been performed by injecting morpholino anti-sense oligonucleotides.

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Synthetic chemicals found in the environment have the capacity to disrupt endocrine system development and function in both wildlife and humans. This has drawn public concern since many of these chemicals may bind to estrogen receptors (ER) and evoke estrogenic effects. Early evidence that estrogenic chemicals could pose a threat to human health during development came from studies of diethylstilbestrol (DES), which was used to prevent premature birth and spontaneous abortion. Laboratory experiments have demonstrated that exposure of animals to sex hormones during perinatal life can cause permanent and irreversible alterations of the endocrine and reproductive systems as well as the immune system, nervous system, bone, muscle, and liver in both sexes. Although many of these chemicals may bind to ER and evoke estrogenic effects in wildlife and humans, the effects of estrogen are not well understood even now. Thus, understanding the effects of sex hormones at the molecular level, especially during development, is very important to resolve these problems.

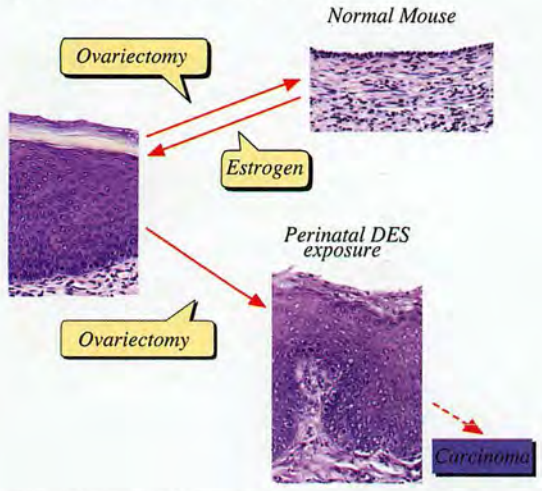

FIG. 1 Scheme of estrogen-dependent and -independent vaginal epithelial cells in mice induced by neonatal estrogenization.

I. Estrogen-induced irreversible changes

Perinatal sex-hormone exposure has been found to induce lesions in reproductive tracts in female mice. The possible relevance of the mouse findings to thedevelopment of cancer in humans has been emphasized. In the early seventies, a close correlation between occurrence of vaginal clear cell adenocarcinoma in young women and early intrauterine exposure to DES was demonstrated. Many chemicals released into the environment have the potential to disrupt endocrine function in wildlife and humans. Some of these chemicals induce estrogenic activity by binding to ER. The neonatal mouse model has been utilized especially to demonstrate the long-term effects of early sex hormone exposure on the female reproductive tract. Neonatal treatment of female mice with estrogens induces various abnormalities of the reproductive tract: ovary-independent cervicovaginal keratinization, adenosis, uterine hypoplasia, epithelial metaplasia, oviductal tumors, polyovular follicles (PF) and polyfollicular ovaries. Female reproductive tracts in mice exposed prenatally to estrogen show altered expression of Hoxa genes and Wnt genes and the analysis of knockout mice lacking Hoxa-10 or Wnt7a show uterine hypoplasia. The growth response of neonatally DES-exposed reproductive organs to estrogen is reduced, as are ER levels and epidermal growth factor (EGF) receptor levels, in addition to other hormone receptor levels.

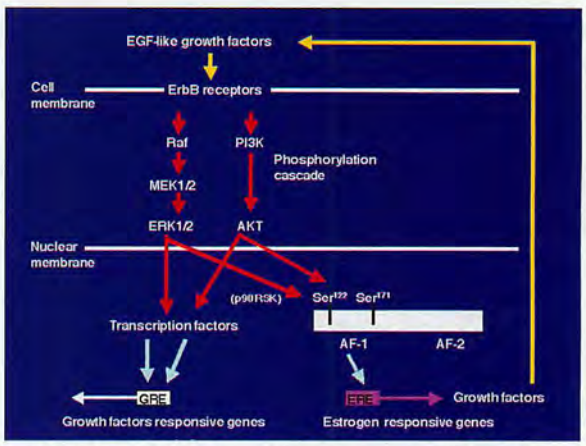

FIG.2 A hypothetical model for the estrogen-independent ER activation pathway in mouse vagina. EGF-like growth factors activate the protein-phosphorylation cascade via erbB receptors. In nuclear, estrogen receptor is phosphorylated on serine 122 and 171 in AF-1 domain. Furthermore, transcription factors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

Growth factors and ER signaling cooperate to play essential roles in cell proliferation, differentiation and tumor progression in mouse reproductive organs. The mechanisms of the estrogen-dependent and -independent pathways remain unknown. EGFR and erbB2 were activated in the vaginal epithelium of mice by estrogen treatment. This activation was also

encountered in vaginae from neonatally DES-exposed mice, along with the expression of EGF, TGF-α, HB-EGF, amphiregulin and neuregulin. Immunochitochemical analysis indicated that erbB2 was primarily expressed in vaginal epithelium. Serine 118 and 167 located in the AF-1 domain of ERa were phosphorylated in these vaginae. AG825, AG1478 or ICI 182,780, that are erbB2, EGFR and ER antagonists, respectively, administration blocked proliferation of vaginal epithelium induced by neonatal DES exposure. Signal transduction via EGFR and erbB2 could be related to the estrogen-induced vaginal changes and persistent erbBs phosphorylation and sustained expression of EGF-like growth factors, leading to ERα activation that may result in cancerous lesions in vaginae from neonatally DES- exposed mice later in life.

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

Estrogenic compounds such as bisphenol A (BPA) and nonylphenol as well as dioxins and PCBs were found in the human umbilical cord. BPA can easily cross the placenta and enter the fetus in Japanese monkey and mice. BPA can be found in fetal brain, testis and uterus when given to pregnant mice and monkeys. Neonatal exposure to a high BPA dose induced ovary-independent vaginal changes, polyovular follciles and infertility lacking corpora lutera. Prenatal exposure to a low BPA dose induced acceleration of vaginal opening in the offspring. Thus, the developing mammal is sensitive to exposure to estrogenic agents.

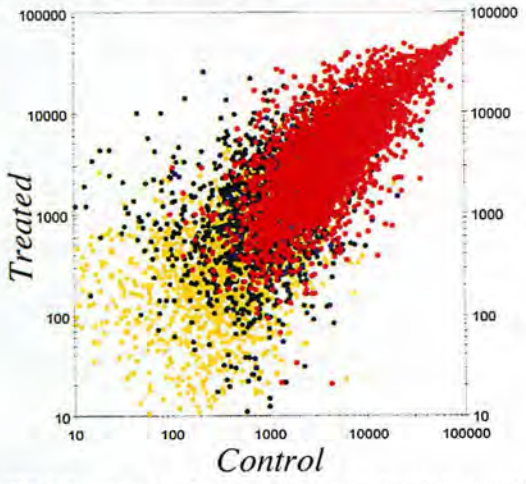

FIG.3 Scatter plot of average expression levels in control and chemical-treated uterus

II. MicroArray analysis

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

III. Effect of estrogen on reptile, amphibian and fishes

During embryogenesis, exogenous estrogen exposure induces abnormal sex differentiation and the abnormal bone formation in African clawed frog, *Xenopus laevis*, the cyprinodont fish, mummichog (*Fundulus heteroclitus*), mosquitofish (*Gambusia affinis*). To analyze the function of estrogen, we have isolated cDNA clones of ERα and β from *F. heteroclitus, G. affinis*. In UK rivers exposure of roach (*Rutilus rutilus* – a common cyprinid fish) to effluents from sewage treatment works, containing complex mitures of endocrine disrupting chemicals (EDCs) has been shown to alter sexual development and impact negatively on their reproductive capabilities. To unravel the mechanisms of disruption of sexual development in roach exposed to EDCs, we have isolated the cDNAs related to sex determination and sexdifferentiation containing estrogen receptor, aromatase, StAR, Sox9, vasa etc. We are examining the gene expression during gonadal differentiation with or without EDCs exposure. Furthermore, we have isolated cDNAs of steroid hormone receptors from American alligators (*Alligator mississippiensis*). As the estrogen-responsive genes must play important roles, we are isolating the estrogen-responsive genes to understand the molecular physiology of estrogen action. Japanese tree frog (*Hyla japonica*) takes water through ventral skin. We found that sex steroids and endocrine disruptors interfere with water absorption through ventral skin in frogs. Further, using the amphibian and fish as model animals we aim to analyze the effects of numerous chemicals released into the environment on endocrine system function in wildlife.

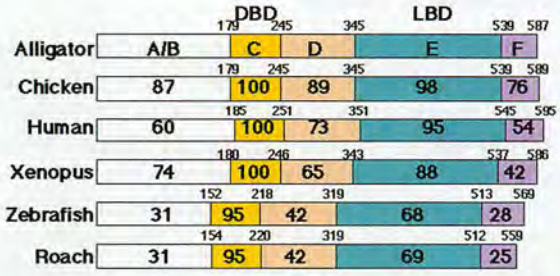

FIG. 4 Comparison of alligator and roach ERα proteins with estrogen receptors of several species. The functional A/B to F domains are schematically represented with the numbers of amino acid residues indicated.

IV. Molecular Target Search

Abnormalities caused by endocrine disrupting chemicals are reported but the molecular mechanisms of the effects are not well studied. Although estrogen receptor is one of the strongest candidates possibly responsible for the endocrine disrupting function of many chemicals, it alone cannot explain the variety of phenomena induced by endocrine disrupting chemicals. Thus, we are also looking for new target molecules that may be responsible for endocrine disruption. In parallel, we also are studying the ligand-binding mechanisms of nuclear receptors to hormones and other chemicals.

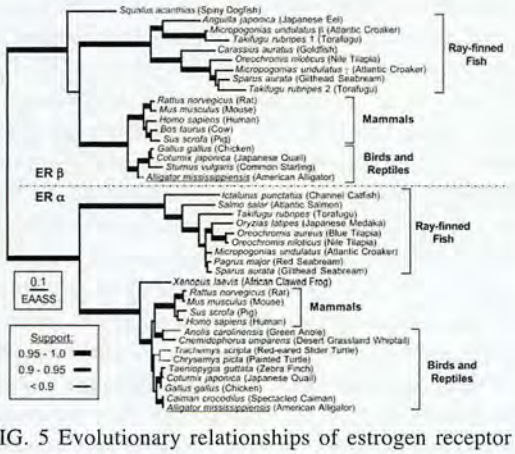

FIG. 5 Evolutionary relationships of estrogen receptor sequences.

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DIVISION OF PLANT DEVELOPMENTAL GENETICS

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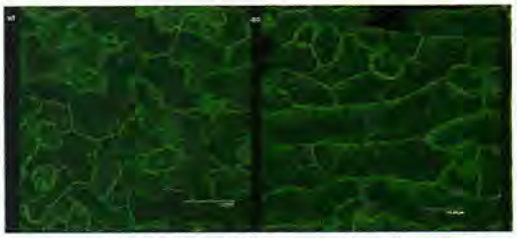
Diversity of plant form is mostly attributable to variation of leaf and floral organs, which are modified leaves. The leaf is the fundamental unit of the shoot system, which is composed with leaf and stem. So the leaf is the key organ for a full understanding of plant morphogenesis. However, the genetic control of development of these shapes had remained unclear. Recently, studies of leaf morphogenesis has been in a turning point, after our successful application of the techniques of developmental and molecular genetics to it, using model plants, *Arabidopsis thaliana* (L.) Heynh. (reviewed in Tsukaya 2003).

In a determinate organ, a leaf, number of leaf cells is not necessarily reflected on leaf shape or, in particular, leaf size. Genetic analyses of leaf development in *Arabidopsis* shows that a compensatory system(s) act in leaf morphogenesis and an increase of cell volume might be triggered by a decrease in cell number. Thus, leaf size is, at least to some extent, uncoupled from the size and number of cells by the compensatory system(s). Based on these facts, a new perspective on understanding of mechanisms for leaf morphogenesis, Neo-Cell theory, is proposed (Tsukaya, 2003). Neo-Cell theory is the Cell theory which stipulated positional cooperation of the behavior of cells. In short, Neo-Cell theory hypothesizes as follows: cells are the unit of morphogenesis; however, each cell is also controlled by factors that govern the morphogenesis of the organ of which the cells are a part.

Focusing on mechanisms that govern polarized growth of leaves in a model plant, *Arabidopsis thaliana*, we found that the two genes act independently to each other on the processes of polar growth of leaves: the *ANGUSTIFOLIA* (*AN*) gene regulates width of leaves and the *ROTUNDIFOLIA3* (*ROT3*) gene regulates length of leaves. The *AN* gene controls the width of leaf blades and the *ROT3* gene controls length. Cloning of the *AN* gene (Kim et al., 2002) revealed that the gene is a member of CtBP (C-terminal binding protein) gene family which had been found from animal kingdom. In the animal kingdom, CtBPs self-associate and act as a co-repressor of transcription. We found that also the AN protein can self-associate in yeast two-hybrid system (Kim et al. 2002). Furthermore, microarray analysis suggested that the *AN* gene might regulate the expression of certain genes, for example, the gene involved in formation of cell walls, *MERI5*. We also found that the abnormal arrangement of cortical MTs in *an-1* mutant leaf cells appeared to account entirely for the abnormal shape of the cells. It suggested that the *AN* gene might regulate the polarity of cell growth by controlling the arrangement of cortical MTs (below).



Arrangement of cortical MTs in leaf epidermis of wild type (left) and the *an-1* mutant (right).

The *ROT3* gene encodes a cytochrome P450. Transgenic experiments proved that the *ROT3* gene regulates leaf-length without affect on leaf-width via biosynthesis of steroids. In relation to it, we recently revealed that a steroid hormone, brassinosteroid, controls both proliferation and expansion of leaf cells (Nakaya et al., 2002). To understand the role of the ROT3/CYP90C1 enzyme in biosynthesis of brassinosteroid, biochemical analysis of the *rot3* mutants has been carried out. As a result, we found that CYP90C1 and its closely related homolog, CYP90D1, are involved in the critical steps of biosynthesis of brassinolide (Kim et al., submitted).

On the other hand, by studying role of light condition in leaf development, we found that the length of leaf petioles was related exclusively to genetic control of the length of individual cells in all leaf morphological mutants examined. In contrast, both the size and the number of cells were affected by the mutations examined in leaf blades (Tsukaya et al., 2002).

Leaf expansion is controlled not only by polar cell expansion but also by polar cell proliferation. We have revealed that *ANGUSTIFOLIA3* (*AN3*) gene is involved in maintenance/establishment of activity of cell proliferation in leaf primordia. AN3 encodes a co-activator, and is speculated to control cell cycling in leaf primordia (Horiguchi et al., in prep.). Interestingly, the *an3* shows clear "compensation", namely, accelerated cell expansion in relation to decrease of number of leaf cells. Using various mutants with altered number and/or size of leaf cells, we are currently analyzing genetic system of the compensation.

We also identified a novel gene that regulates number of leaf cells: *ROT4*. ROT4 is a member of novel peptide family which is specific to seed plants. Overexpression of the *ROT4* results in stunted leaves with normal width (Narita et al., submitted), suggesting importance of peptides in control of leaf shape.

Apart from the aspects of leaf expansion, we also analyzed genes for identification of leaf primordia. The *AS1*, *AS2* and *BOP* genes are needed for determinate growth of leaf. We isolated and analyzed a novel mutant, *bop* (blade-on-petiole) mutant, which strongly

enhances the *as2* phenotype, in collaboration with a reaseach teams of Prof. Nam, POSTECH, Korea and Prof. Machida, Nagoya Univ. (Ha et al. 2003). Three class I *knox* genes, namely, *KNAT1*, *KNAT2* and *KNAT6*, were misexpressed in the leaves of the *bop1-1* mutant as in those of the *as2* mutant. The *bop1* single mutant results in ectopic, lobed blades along the adaxial side of petioles of the cotyledon and foliage leaves. The *bop* mutation strongly enhances the morphological abnormality of leaves when combined with the *as1* or *as2* mutation, resulting in repeated compound leaves. We, thus, suggest that *BOP1* promotes or maintains a developmentally determinate state of leaf cells through regulation of class I *knox1* genes (Ha et al. 2003).

On the other hand, we are trying to identify molecular mechanisms which distinguish developmental pathway of leaves from that of shoots by studying tropical plants with 'fuzzy' morphology. For such purposes, we introduced tropical plants having queer developmental program for leaf morphogenesis, namely, *Chisocheton*, *Guarea* and *Monophyllaea*, as materials for the studies. The indeterminate compound leaves of members of the genus *Chisocheton* in Southeast Asia and of the genus *Guarea* in the New World and Africa, in the family Meliaceae, are unique and can develop indeterminately as a result of the activity of the leaf apical meristem, which can function very similarly to a shoot apical meristem. We performed a molecular phylogenetic study of these genera with a research team of Dr. Yokoyama, Tohoku Univ., and the result suggested that indeterminate program in the leaves of members of these two genera might have evolved only once in Meliaceae (Fukuda et al. 2003). Cloning of apical-meristem-specific genes from these species are now underway. Similarly, we are trying to clone key gene(s) which may support the indeterminate leaf growth in *Monophyllaea* spp. (Gesneriaceae).

On the other hand, leaf shape variation is sometimes linked to variation of ploidy level. In *Cayratia japonica*, Vitaceae, trifoliate leaves are rarely observed in specific strains. Detailed analysis of morphological features including fertility of pollen grains and chromosome numbers in collaboration with Prof. okada, Osaka City Univ., revealed that there are two types of *Cayratia japonica* in Japan: fertile diploid type that sometimes develops trifoliates and sterile triploid type that develops only quiquefoliates (Okada et al., 2003). In the course of this study, we have found several curious features in diploid type of *Cayratia japonica*, *e.g.*, low fertility. To understand why, analyses of closely related species are underway (Okada and Tsukaya, 2003).

In addition, we are interested in environmental adaptation of leaves. Adaptive responses of leaves against light, gravity and other environmental factors are also analyzed in a model plant, *Arabidopsis thaliana* and anatomical analyses of various types of morphological adaptation of leaves against certain kinds of environments were also performed. To obtain clues for understanding of natural variation in plant form, molecular systematic analyses of wild plants are also carried out (Yokoyama et al., 2003; Tsukaya et al., 2003). So called "Evo/Devo" study of leaf morphogenesis is also one of our research project.

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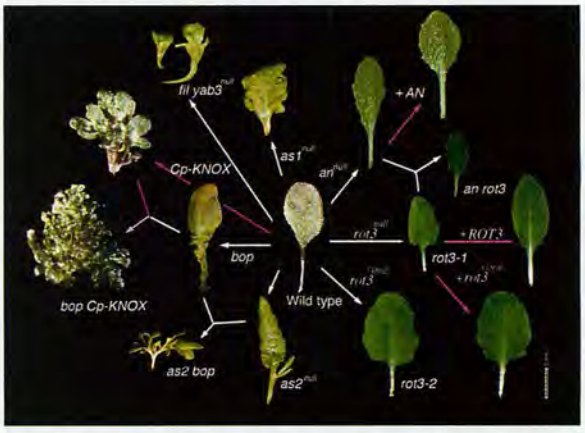

Leaf shape control by various genes in *Arabidopsis thaliana*.

DIVISION OF BIOINFORMATICS

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(Osaka University)

The aim of this laboratory is structural bioinformatics covering molecular modeling and design of proteins and other biological macromolecules: Development of a new database, eF-site, for protein surface geometry with the physicochemical properties, and identification of protein functions using the database, and Development of new algorithms and softwares for large scale simulation calculations by parallel computers to examine free energy landscapes of biomolecular systems. A new hybrid method of Quantum Mechanics and Molecular Mechanics has also been developed for analysis of the electronic state in biological macromolecular systems.

Publication list:

Kinoshita, K., Nakamura, H. (2003) Protein informatics towards function identification. Curr. Opin. Struct. Biol. **13**, 396-400.

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CENTER FOR RADIOISOTOPE FACILITIES (CRF)

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	(from April 12)
	IINUMA, Hideko
	(from October 16)
Supporting Staff	*ITO, Takayo*
	IIDA, Yumi
	KATAGIRI, Izumi

I. Research supporting activity

Technical and supporting staffs of the CRF maintain five controlled areas being adaptable for the law. The purchase of radioisotopes from JRA (Japan Radioisotope Association) and the transfer of radioisotope wastes to JRA is under our surveillance.

In this year, we discontinued two controlled areas; NIBB (National Institute for Basic Biology)-branch and NIPS (National Institute for Physiological Science)-branch. The name of Center located in Common Building I of the area A has been changed to Common Building I (COBI)-branch. Matsuda, Iinuma, Ito, and Iida are maintaining COBI-branch and LGER (Laboratory of Gene Expression and Regulation)-branch in the area A, and Ogawa, Sawada, and Katagiri working in the area E.

Users going in and out the controlled areas counted by the monitoring system are 6,965 persons in 2003. The items in each area is shown in Figure 1.

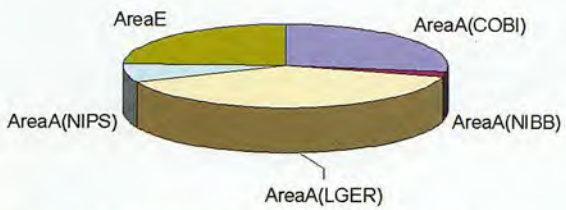


Figure 1. Percentage of users going in and out the controlled areas in 2003.

II. Academic activity

Dyneins are a group of microtubule-activated ATPases that serve to convert chemical energy into mechanical energy and divided into axonemal and cytoplasmic dyneins. Figure 2 shows the localization of two isoforms of dynein in the outer arms of sperm axonemes and the mitotic apparatus of cleaving egg visualized by anti-axonemal dynein (Fragment A) antibodies (Fig. 2).

Figure 2. Dynein family. Axoneme (left) and Cleaving egg (right).

Dyneins are very large and range in molecular mass up to 1 to 2 mega Da. They are complex proteins containing heavy, intermediate, and light chains defined by the molecular mass. The project is the molecular cloning of polypeptides contained in outer arm dynein of sea urchin sperm flagella to understand the mechanism how dynein interacts with microtubules, resulting in producing the force.

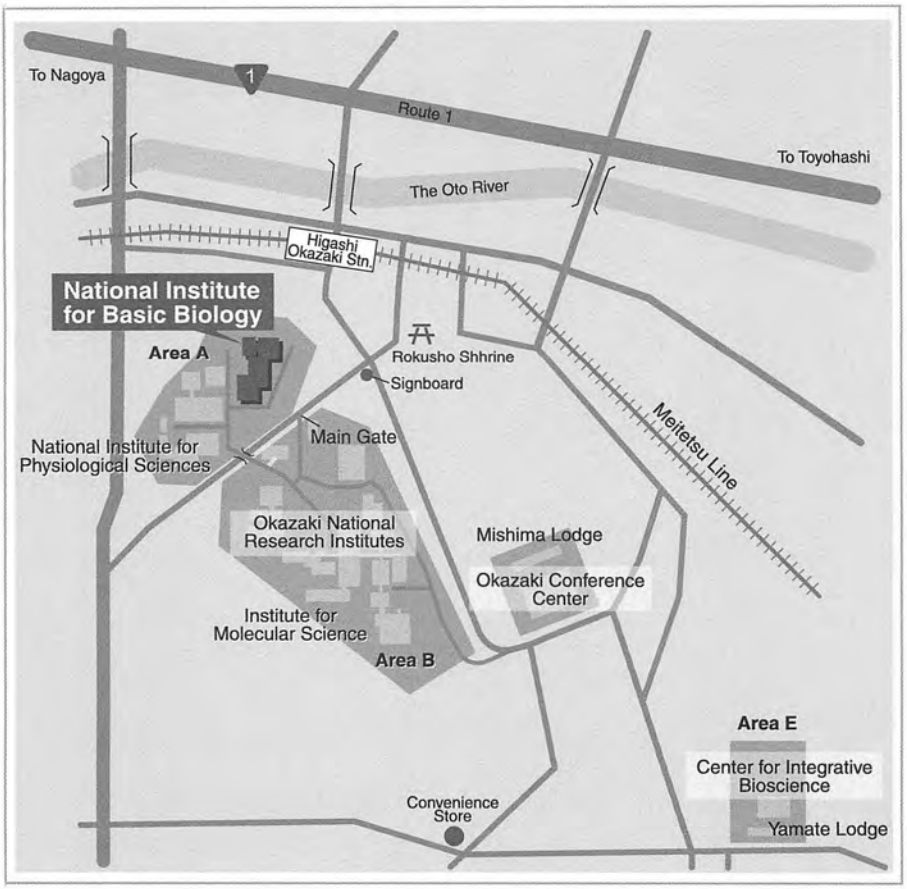
Outer arm dynein consists of two heavy chains with ATPase activity. The motor activity is closely related to this polypeptide. The first successful molecular cloning of this huge polypeptide (520 kDa) was performed in 1991. Since then cDNA clones for axonemal and cytoplasmic dyneins have been isolated in a variety of organisms. The sequences of heavy chains, without exception, contain four P-loop motives referred to as ATP-binding sites in the midregion of the molecules. Outer arm dynein contains three intermediate chains (IC1, IC2, and IC3) that range in molecular mass from 70 to 120 kDa. IC2 and IC3 belong to the WD-family. By contrast, IC1 is a hybrid protein such that the N-terminal part is homologous to the sequence of thioredoxin and the middle part consists of three repetitive sequences homologous to the sequence of NDP kinase. The IC1-related proteins are found in distantly related species.

Six light chains with molecular masses of 23.2, 20.8, 12.3, 11.5, 10.4, and 9.3 kDa are associating with outer arm dynein. We have already isolated cDNA clones of five LCs. LC1 (23.2 kDa) and LC3 (12.3 kDa) are highly homologous to mouse Tctex2 and Tctex1, respectively, which are encoded by the t complex region that is involved in transmission ratio distortion (TRD), male sterility and the development of germ cells. In this year, we found that LC1 and LC3 are able to making sperm motility activating complex (Smoac). Our finding raises the possibility that axonemal dynein proteins are involved in this phenomenon. TRD may be caused by the dysfunction of multiple axonemal dynein proteins.

Publication

K. Ogawa and K. Inaba. Biochem. Biophys. Res. Commun. 310 (2003) 1155-1159. Smoac: Sperm motility activating complex formed by t-complex distortors

Access to NIBB



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