National Institute for Basic Biology

2004 ANNUAL REPORT

CONTENTS

The cover photograph is the panel of whole mount in situ hybridization of african clawed frog (Xenopus laevis) neurula embryos. The blue staining represents where each gene is expressed. These data are available in XDB3, the database of X. laevis EST of NIBB. See also page 24.

INTRODUCTION

On April 1st, 2004, eighteen Inter-University Research Institutes, to which the National Institute for Basic Biology belongs, were realigned to 4 new organizations.

Our National Institute for Basic Biology (NIBB) became a member of the National Institutes of Natural Sciences that consists of five National Institutes, National Astronomical Observatory, National Institute for Fusion Science, National Institute for Physiological Sciences, Institute for Molecular Science, and our-selves (NIBB). Each Institute managed by the Director-General, who is assisted by the Advisory Committee in each Institute, is independent from each other. Therefore, the NINS is a United Research Institutes of Natural Sciences. A selection of the Director-General is performed through the recommendation from the Advisory Committee to the Director-General Selection Committee. Then the Director-General is appointed by the president with an approval of the Management Council and Education and Research Council.

The NIBB established in 1977. 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 basic biology but also the modern biological sciences by conducting first-rate research on site as well as in cooperation with national, public and private universities and research organizations. Researchers at the NIBB investigate Cell Biology, Developmental Biology, Neurobiology, Evolutionary Biology and Biodiversity, Environmental Biology and Theoretical Biology to elucidate the general and fundamental mechanisms underlying various biological phenomena.

In 2004 after the establishment of NINS, NIBB changed the research unit system from 3 departments to 30 independent divisions and laboratories. The purpose of this change is to make researchers move easily to the new field if necessary.

On the higher education, NIBB became a member of the Graduate University for Advanced Studies when it was established in 1988, as a department of Molecular Mechanics in School of Life Science. Three students for five year Doctoral course and six for three year senior Doctoral course are open to applicants for entrance.

On March 31st, 2004, adjunct professors Dr. MIYATA Takashi, Dr. YANAGIDA Mitsuhiro, and Dr. NAKAMURA Haruki left NIBB just after the completion of their appointments. Research associates Dr. ISHIHARA Satoru, Dr. YAMAMOTO Hiroshi, Dr. MIZUSHIMA Noboru and Dr. KOBAYASHI Tohru were promoted to chief investigators of the Research Institutes and on December 31st, 2004, Dr. SUZUKI Iwane to Tsukuba University as an assistant professor.

In congratulation, associate professor Dr. WATANABE Masakatsu left NIBB on August 31st as he was promoted to a professor of the Graduate University for Advanced Studies.

Conversely five researchers were newly appointed in 2004, Dr. TANAKA Minoru from Hokkaido University as an associate professor, Dr. TSUKAMURA Hiroko from Nagoya University as an adjunct associate professor, Dr. YAMAUCHI Daisuke from Himeji Institute of Technology as an adjunct associate professor, Dr. IMAMURA Takuya from Institut Jacques Monod (JSPS Post-Doctoral fellow for abroad) as a research associate and Dr. SHIGENOBU Shuji from JSPS Post-Doctoral fellow as a research associate.

In addition, a number of postdoctoral fellows, technical assistants, secretaries, and graduate students after awarded Ph.D. 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.

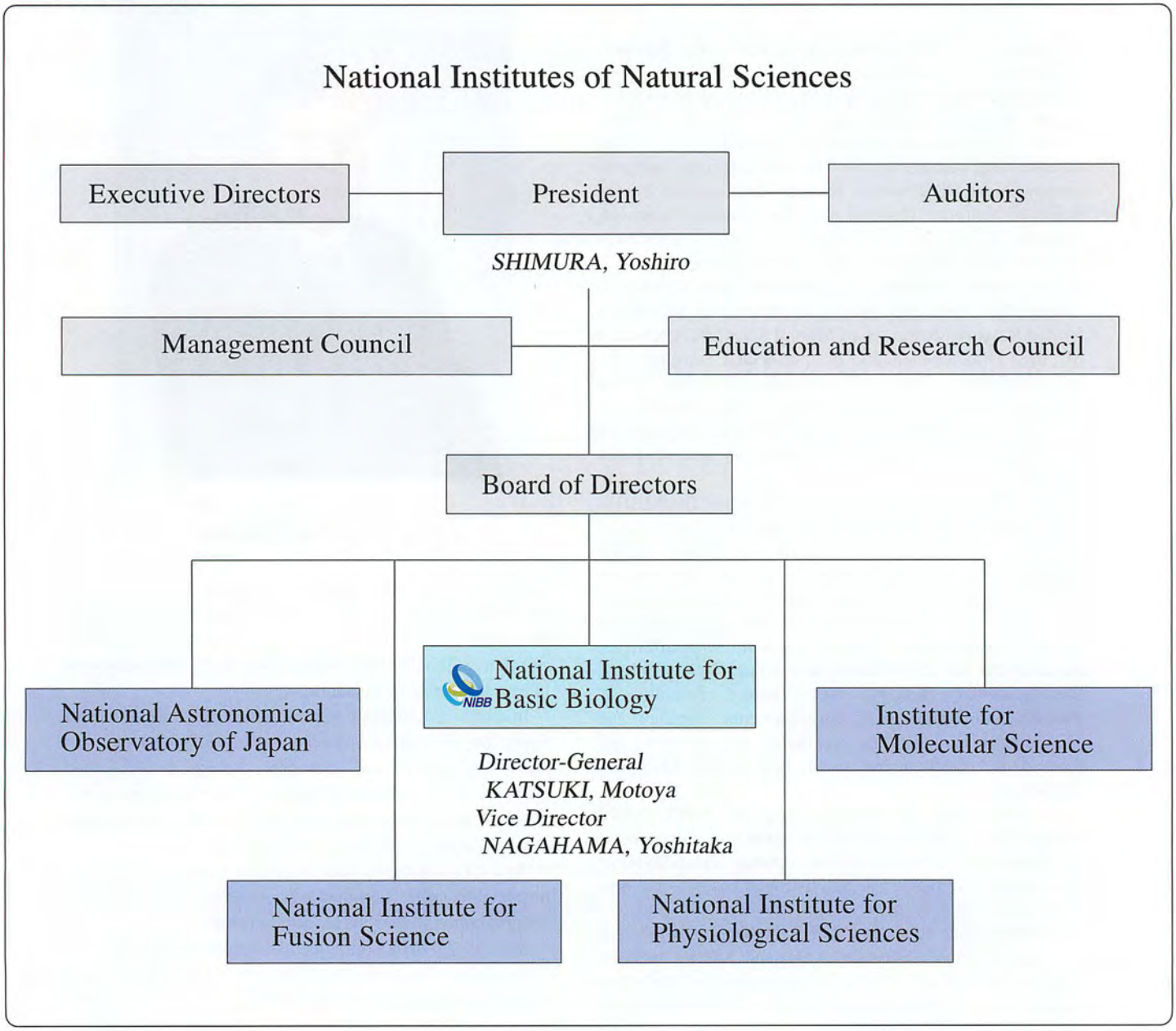
Moreover, NIBB continues to sponsor interdisciplinary symposia and study meetings on current topics by inviting leading scientists from around the world to the NIBB. Based on this concept, NIBB started to support a series of Okazaki Biology Conference (OBC) through intimate cooperation with a variety of biological scientific societies. The first OBC was held on January 25-30, 2004, entitled "The Biology of Extinction" and the second on September 26-30, entitled "Terra Microbiology." Both meetings were very successful. Thirty to forty scientists from abroad and equivalent number of them from Japan had an exciting discussion on the new field of future biology. NIBB also provides a training course in biological sciences for young investigators.

Finally, I would like to celebrate the retirement of Mr. HATTORI Hiroyuki with appreciation of his dedicated work as the Head Technical Staff. Toward his respectful contribution, the NIBB awarded him a title for NIBB Technical Staff Emeritus on March 31st, 2004.

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

KATSUKI, Motoya, D.Sc.
Director-General

ORGANIZATION



Research Unit

Cell Biology
- Division of Cell Mechanisms
- Division of Molecular Cell Biology
- Laboratory of Cytoskeleton
- Laboratory of Cell Sociology

Developmental Biology
- Division of Reproductive Biology
- Division for Sex Differentiation
- Division for Morphogenesis
- Division of Developmental Genetics
- Division of Molecular and Developmental Biology
- Laboratory of Developmental Regulation
- Laboratory of Molecular Genetics for Reproduction

Neurobiology
- Division of Molecular Neurobiology
- Division of Brain Biology
- Division of Behavioral Biology (adjunct)
- Laboratory of Neurophysiology
- Laboratory of Neurochemistry

Evolutionary Biology and Biodiversity
- Division of Molecular Genetics
- Division of Genome Dynamics
- Division of Evolutionary Biology
- Division of Speciation Mechanism (adjunct)
- Laboratory of Morphodiversity

Environmental Biology
- Division of Cellular Regulation
- Division of Molecular Environmental Endocrinology
- Division of Plant Developmental Genetics
- Division of Photobiology (adjunct)
- Laboratory of Photoenvironmental Biology
- Laboratory of Stress Response

Theoretical Biology
- Division of Theoretical Biology
- Laboratory of Genome Informatics

- Laboratory of Director General

Research Support

Research Support Facility
- The Large Spectrograph Laboratory
- Tissue and Cell Culture Laboratory
- Computer Laboratory
- Plant Culture Laboratory
- Experimental Farm
- Plant Cell Laboratory

Research Center for Integrative and Computational Biology

Center for Transgenic Animals and Plants

Technical Division

Okazaki Institute for Integrative Bioscience
- Department of Development, Differentiation and Regeneration
  - Division of Developmental Genetics
  - Division of Molecular and Developmental Biology
- Department of Bio-Environmental Science
  - Division of Bio-Environmental Science
  - Division of Plant Developmental Genetics

Center for Radioisotope Facilities

Center for Experimental Animal

Research Center for Computational Science

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

ORGANIZATION OF THE INSTITUTE

The National Institute for Basic Biology (NIBB) is an institute in the National Institutes of Natural Sciences (NINS) that are composed of five independent institutes. Among them, the NIBB, the Institute for Molecular Science (IMS) and the National Institute for Physiological Sciences (NIPS) 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 (CIB) was established as a common facility of the three Okazaki Institutes in 2000 and began in 2001. The CIB was changed its name to Okazaki Institute for Integrative Bioscience on April 1st, 2004, at the beginning of the new organization.

Policy and Decision Making

The Director-General oversees the operation of the NIBB assisted by an advisory body, the Advisory Committee for Programming and Management. The Advisory Committee, comprised of professors within the NIBB 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 NIBB. The Director-General selection committee makes a nomination of Director-General to the President after hearing the recommendation from the Advisory Committee. The Advisory Committee makes recommendations on the Director-General and on faculty appointments, the NIBB's annual budget and future prospects.

Administration

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

Research

The NIBB conducts its research programs through 30 research units, 4 research support facilities including Technology Department, and the Research Center for Integrative and Computational Biology.

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

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

Research Support Facilities

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

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 are common facilities of the three Okazaki Institutes.

Campus

The three Okazaki Institutes 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 one-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 built in the new campus at November, 2003.

MEMBERS OF THE ADVISORY COMMITTEE FOR PROGRAMMING AND MANAGEMENT

Chairman	
NAGAHAMA, Yoshitaka	Vice Director & Professor, National Institute for Basic Biology
Vice-Chairman	
MACHIDA, Yasunori	Professor, Nagoya University
AIZAWA, Shinichi	Deputy Director & Group Director, RIKEN Center for Developmental Biology
KOMEDA, Yoshifumi	Professor, The University of Tokyo
KONDO, Hisato	Professor, Osaka University
KUROSAWA, Yoshikazu	Professor, Fujita Health University
MURAKAMI, Fujio	Professor, Osaka University
OKADA, Kiyotaka	Professor, Kyoto University
SAGA, Yumiko	Professor, National Institute of Genetics
SEHARA, Atsuko	Professor, Kyoto University
WADA, Masamitsu	Professor, Tokyo Metropolitan University
HASEBE, Mitsuyasu	Professor, National Institute for Basic Biology
HORIUCHI, Takashi	Professor, National Institute for Basic Biology
IIDA, Shigeru	Professor, National Institute for Basic Biology
MOROHASHI, Ken-ichirou	Professor, National Institute for Basic Biology
NISHIMURA, Mikio	Professor, National Institute for Basic Biology
NODA, Masaharu	Professor, National Institute for Basic Biology
OHSUMI, Yoshinori	Professor, National Institute for Basic Biology
TAKADA, Shinji	Professor, Okazaki Institute for Integrative Bioscience
UENO, Naoto	Professor, National Institute for Basic Biology
YAMAMORI, Tetsuo	Professor, 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 five year Ph.D. course as well as a three year senior course those who have completed a master's course or equivalent at any university are eligible to apply.

The Department consists of the 30 Research Units appeared in the page 3.

2. Graduate Student Training Program

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

DIVISION OF CELL MECHANISMS

Professor:	*NISHIMURA, Mikio*
Associate Professor:	*HAYASHI, Makoto*
Research Associates:	*MANO, Shoji*
	YAMADA, Kenji
Technical Staff:	*KONDO, Maki*
JSPS Postdoctoral Fellows:	*NITO, Kazumasa*
	KUROYANAGI, Miwa
	ARAI, Yuko
Postdoctoral Fellows:	*KAMADA, Tomoe (Apr. 1 -)*
	MUTSUDA, Michinori (Apr. 1 -)
	HATSUGAI, Noriyuki (Oct. 1 -)
	KAMIGAKI, Akane (Oct. 1 -)
Graduate Students:	*KAMADA, Tomoe [1] (- Mar. 31)*
	HATSUGAI, Noriyuki [1] (- Sep. 30)
	OGASAWARA, Kimi [2] (Apr. 1 -)
Technical Assistants:	*NAKAMORI, Chihiro*
	YAGI, Mina
	YOSHINORI, Yumi
	SUZUKI, Iku
	FUKAZAWA, Mitsue (Nov. 1 -)
Secretaries	*UEDA, Chizuru*
	IYODA, Yuri (- Mar. 31)

[1] *Graduate University for Advanced Studies*
[2] *Tokyo University of Marine Science and Technology*

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 degradation. We now engage in proteomic and transcriptomic analyses of the reversible peroxisomal transition in *Arabidopsis* cotyledons.

II. Transcriptomics, proteomics and phenomics of plant peroxisomes

Enzymes localized in plant peroxisomes are synthesized in the cytosol, and function after their post-translational transport into peroxisomes. Almost all of the peroxisomal matrix proteins are known to contain one of two targeting signals (PTS1 and PTS2) within the molecules. PTS1 is a unique tripeptide sequence found in 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 a 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 another 30 genes of non-PTS-containing proteins from *Arabidopsis* genome. Custom-made DNA microarray covering all these genes was used to investigate expression profiles of the peroxisomal genes in various organs. Statistical analyses revealed that the peroxisomal genes could be divided into five groups. 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.

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.

Bioinfomatic analysis of *Arabidopsis* genome predicted the presence of 15 kinds of genes for peroxisomal biogenesis factors, called *PEX* genes. We comprehensively investigated whether these predicted *PEX* genes function in peroxisome biogenesis by generating knock-down mutants that suppress *PEX* gene expression by RNA-interference. Phenotypes of these mutants allowed us to identify the functional *PEX* genes, which can be classified into two groups, i.e. *PEX* genes regulating for peroxisomal morphology and peroxisomal protein import.

III. Identification of novel components essential for peroxisome biogenesis

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 isolated a number of *Arabidopsis* mutants having aberrant peroxisome morphology (*apm* mutants) based on the different pattern of GFP fluorescence (Fig. 1).

In one of these mutants, *apm1*, the peroxisomes are long and reduced in number, apparently as a result of inhibition of division. *APM1* gene encodes DRP3A (Dynamin-related protein 3A). Interestingly, *apm1* mutation also caused inhibition of mitochondrial division.

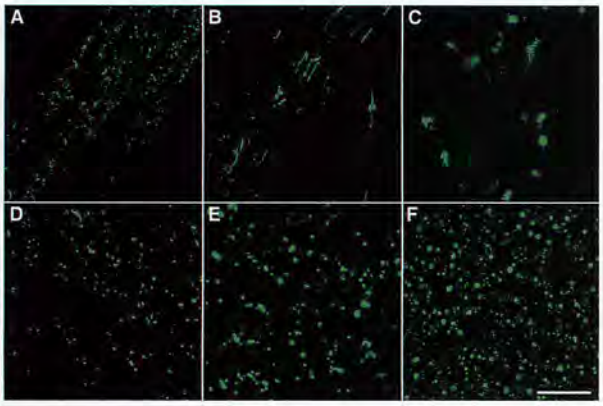


Fig. 1 Phenotype of *apm* mutants.

(A) to (C) root cells. (D) to (F) leaf cells. (A) and (D) show GFP-labelled peroxisomes in GFP-PTS1 as a parent plant. (B) *apm1* mutants have long peroxisomes. (C) Distribution of peroxisomes is altered in *apm5* mutants. (E) In *apm2* mutant, the decrease of efficiency of protein transport to peroxisomes results in the observation of GFP fluorescence in the cytosol and peroxisomes. (F) *apm3* mutants contain larger peroxisomes. Bar indicates 50 μm.

Actually APM1/DRP3A protein is localized at peroxisomes and mitochondria. These findings showed that APM1/DRP3A protein is involved in both peroxisomal and mitochondrial division.

With regard to another *apm* mutants, *apm2*, *apm4* and *apm7*, the GFP fluorescence is observed in not only peroxisomes but also the cytosol, suggesting that the efficiency of protein transport to peroxisomes is decreased in these mutants. *apm3* and *apm6* mutants have larger peroxisomes. These phenotypes indicate that both mutants are defective in peroxisomal division. In *apm5* mutants, the distribution of peroxisomes within cells changed to the one position in the cell and peroxisomes hardly move, whereas peroxisomes in GFP-PTS1 are dispersed and move frequently. Analyses of these *apm* mutants and identification of *APM* genes will identify components necessary for peroxisome biogenesis and address the regulation of its mechanism.

IV. ER-derived organelles for protein storing and defense strategy.

Plant cells develop various types of endoplasmic reticulum (ER)-derived structures with specific functions. ER bodies are ER-derived compartment specific to the *Brassicaceae*, including *Arabidopsis*. They 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* expressing GFP fused with an ER retention signal (GFP-HDEL). ER bodies were widely distributed in the epidermal cells of whole seedlings. Rosette leaves had no ER bodies, but accumulated ER bodies after wounding or jasmonic acid treatment. This suggests that ER bodies function in the defense against herbivores. We isolated an *Arabidopsis* mutant, *nai1*, which has no ER bodies in whole plants. The *nai1* mutant did not accumulate PYK10, a β-glucosidase with an ER-retention signal (KDEL), in seedlings. An electron microscopic analysis showed that PYK10 was accumulated in ER bodies of the wild-type plants. This indicates that PYK10 is the main component of ER body. We cloned *NAI1* gene using a positional cloning strategy. *NAI1* encodes a transcription factor that has a basic-helix-loop-helix (bHLH) domain. The *nai1* mutant had a single nucleotide change at an intron acceptor site of *NAI1* gene. Because of this mutation, aberrant splicing of *NAI1* mRNA occurs in the *nai1* mutant. Transient expression of *NAI1* induced ER bodies in the *nai1* mutant. Two-dimensional electrophoresis and RT-PCR analyses showed that the amounts of mRNA and protein of a putative lectin in the *nai1* mutant were decreased, as was the case of PYK10. These results provide direct evidence that this bHLH protein plays a role in the formation of ER bodies.

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

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 exhibits substrate specificity toward asparagine and aspartic acid residues, the amino acid well conserved at a position in the processing sites of various vacuolar/lysosomal proteins. VPE was originally identified as an enzyme responsible for the processing and maturation of seed storage proteins in plants. We have shown that mouse VPE is responsible for the processing

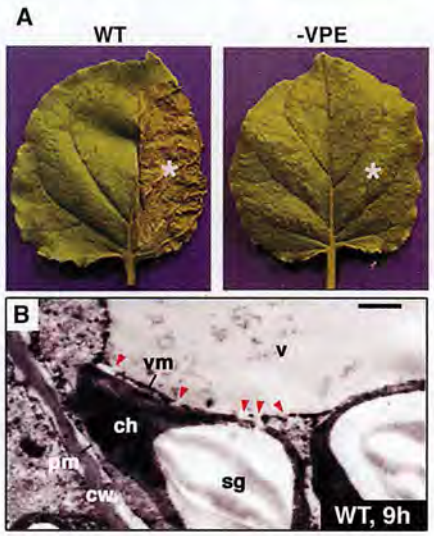


Fig. 2 *VPE* deficiency suppresses the vacuolar collapse leading to the TMV-induced hypersensitive cell death. (A) The non-silenced (WT) and *VPE*-silenced (-VPE) *Nicotiana benthamiana* plants were infected with TMV on halves of their leaves (indicated by asterisks). The photographs were taken after 24 hours. (B) Morphological changes of the TMV-infected regions of the non-silenced leaves (WT) at 9 hours under the electron microscope. Bar indicates 1μm. cw, cell wall; pm, plasma membrane; vm, vacuolar membrane; v, vacuole; ch, chloroplast; sg, starch granule. Red triangles indicate the disintegrated regions of vacuolar membranes.

of lysosomal proteases, cathepsins B, H, and L, from the single-chain forms into the two-chain forms, and VPE deficient mice accumulated macromolecules in the lysosomes.

Plant VPE homologues are separated to two subfamilies: one seed type and the other vegetative type. Seed-type VPE is responsible for the maturation of seed storage proteins. On the other hand, the function of vegetative-type VPE was obscure. Recently, we found a novel function of vegetative-type VPE in programmed cell death (PCD). The plant hypersensitive response (HR), a type of defense strategy, constitutes well-organized PCD. The evidences from extensive studies indicate that caspase activity is involved in plant PCD. VPE is identified as a proteinase that exhibits caspase activity. No visible lesions formed on the tobacco mosaic virus (TMV)-infected leaves of *VPE*-silenced tobacco plants (Fig. 2). *VPE* deficiency prevented the typical characteristics of PCD, such as cell shrinkage, cytoplasmic condensation, and nuclear DNA fragmentation. An ultrastructural analysis showed that the disintegration of the vacuolar membranes occurs in the leaves before visible lesions are formed. The disintegration of the vacuolar membranes continued, resulting in complete vacuolar collapse in association with plasmolysis. On the contrary, vacuoles and vacuolar membranes remained intact in the *VPE*-silenced plants. These results suggest that VPE is involved in vacuolar collapse, which triggers PCD. Plants evolve a death strategy mediated by a vacuolar system, which is not seen in animals. Interestingly, a vacuolar enzyme is this key player in a plant-specific cell death system.

VI. Role of molecular chaperones on plant cell differentiation.

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

Previously, we characterized a mitochondrial co-chaperonin (Cpn10), chloroplast co-chaperonins (Cpn20 and Cpn10) and a small heat shock protein from *Arabidopsis*. Recently, we started to characterize HSP90s. Their evolutional and functional characterization is now under experiments.

Publication List:

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Hatsugai, N., Kuroyanagi, M., Yamada, K. Meshi, T., Tsuda, S., Kondo, M., Nishimura, M. and Hara-Nishimura, I. (2004) A plant vacuolar protease, VPE, mediates virus-induced hypersensitive cell death. *Science* **305**, 855-858.

Hayashi, M., Yagi, M., Nito, K., Kamada, T. and Nishimura, M. (2004) Differential contribution of two peroxisomal receptors to the maintenance of peroxisomal functions in *Arabidopsis*. *J. Biol. Chem.* in press

Mano, S., Nakamori, C., Kondo, M., Hayashi, M. and Nishimura, M. (2004) An *Arabidopsis* dynamin-related protein, DRP3A, controls both peroxisomal and mitochondrial division. *Plant J.* **38**, 487-498.

Mano, S. and Nishimura, M. (2004) Plant peroxisomes. *Vitamins and Hormones* in press

Matsushima, R., Fukao, Y., Nishimura, M. and Hara-Nishimura, I. (2004) *NAI1* gene encodes a basic-helix-loop-type transcription factor that regulates the formation of a novel ER-derived structure, the ER body. *Plant Cell* **15**, 1536-1549.

Nakaune, S., Yamada, K., Kondo, M., Kato, T., Tabata, S., Nishimura, M., Hara-Nishimura, I. (2004) A novel vacuolar processing enzyme is involved in seed coat formation at the early stage of seed development. *Plant Cell* in press

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Yamada, K., Fuji, K., Shimada, T., Nishimura, M. and Hara-Nishimura, I. (2004) Endosomal proteases facilitate the fusion of endosomes with vacuoles at the final step of the endocytotic pathway. *Plant J.* in press

Yamada, K., Nishimura, M. and Hara-Nishimura, I. (2004) The slow wound-response of VPE is regulated by endogenous salicylic acid in *Arabidopsis*. *Planta* **218**, 599-605.

Yamada, K., Shimada, T., Nishimura, M. and Hara-Nishimura, I. (2004) A VPE family supporting various vacuolar functions in plants. *Physiol. Plant.* in press

Yoshida, K., Kawachi, M., Mori, M., Maeshima, M., Kondo, M., Nishimura, M. and Kondo, T. (2004) The involvement of tonoplast proton pumps and $Na^+(K^+)/H^+$ exchangers in the change of petal color during flower-opening of morning glory, *Ipomoea tricolor* cv. Heavenly Blue. *Plant Cell Physiol.* in press

DIVISION OF MOLECULAR CELL BIOLOGY

Professor:	*OHSUMI, Yoshinori*
Research Associates:	*KAMADA, Yoshiaki*
	NODA, Takeshi [1]
	SUZUKI, Kuninori
Technical Staff:	*KABEYA, Yukiko*
NIBB Research Fellow:	*FUJIKI, Yuki*
JSPS Postdoctoral Fellows:	*SEKITO, Takayuki*
	HANADA, Takao
	NAKATOGAWA, Hitoshi
Postdoctoral Fellows:	*ICHIMURA, Yoshinobu*
	YOSHIMOTO, Kohki
	BABA, Misuzu
	OITA, Eiko
	ONODERA, Jun
	OHNEDA, Mamoru
	OBARA, Keisuke
Graduate Students:	*KAWAMATA, Tomoko* [3]
	MATSUI, Makoto [2]
	KAGEYAMA, Takuya [2]
Technical Assistants:	*IMAMURA, Yuko*
	ICHIKAWA, Rie
	TSUKESHIBA, Kumi
Secretary:	*HARA, Yoko*

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

This division aims to understand the autophagy in respects to its molecular mechanism and its physiological roles in yeast and higher eukaryotes. Cells execute degradation processes of their constituents, which 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 membrane structures, autophagosomes, in the cytoplasm. The autophagosome sequesters cytosol and organelles nonselectively. Then it is delivered to the vacuole/lysosome, and the cytoplasmic materials inside are degraded by vacuolar/lysosomal hydrolases. We had discovered autophagy in a simple model organism, *S. cerevisiae* and morphologically and genetically defined the whole process.

II. Interrelationships among Atg proteins during autophagy in yeast, *Saccharomyces cerevisiae*

Macroautophagy is a bulk degradation process induced by nutrient starvation in eukaryotic cells. In *Saccharomyces cerevisiae*, 16 *ATG* genes are essential for autophagosome formation. Recently, we demonstrated that these *ATG* genes can be classified into three groups on the basis of localization of GFP-Atg8p and Atg5p-GFP under nutrient-rich conditions. In starving cells, Atg8p is targeted to the membrane of nascent autophagosomes, eventually sequestered into autophagosomes, and finally transported to the vacuolar lumen. Our previous classification was performed under nutrient-rich conditions. In order to obtain further insights for the mechanism of autophagosome formation, we analyzed the intracellular localization of GFP-Atg8p in every *atg* mutant under autophagy-inducing conditions. GFP-Atg8p showed several distinct localization patterns in *atg* mutants (Figure 1). Subsequent analyses revealed epistatic relationships between Atg proteins during the process of autophagosome formation.

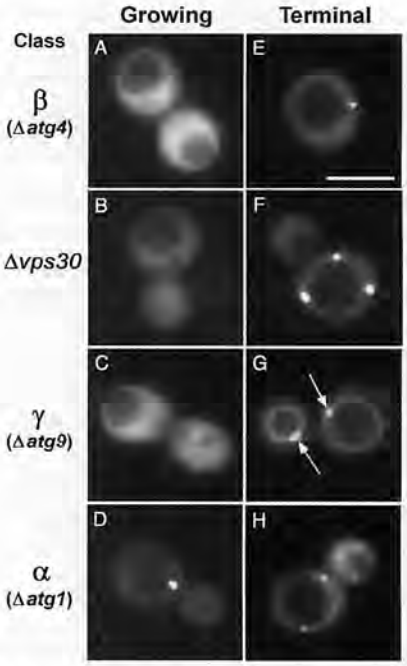

Figure 1. Localization of GFP-Atg8p in *atg* mutants before and after 24-hour rapamycin treatment. Cells expressing GFP-Atg8p were grown in SD + casamino acid medium and observed under the fluorescence microscope. Cells under growing conditions (Growing; A-D) and after 24-hour rapamycin treatment (Terminal; E-H). (A and E) Δ*atg4* cells showing the class β terminal phenotype. (B and F) Δ*vps30*/Δ*atg6* cells showing the Δ*vps30* terminal phenotype. (C and G) Δ*atg9* cells showing a typical class γ terminal phenotype. Arrows represent dots of GFP-Atg8p close to the vacuole often seen in the class γ mutants. (D and H) Δ*atg1* cells showing the class α terminal phenotype. Bar: 5 μm.

III. Starvation triggers the delivery of the endoplasmic reticulum to the vacuole via autophagy in yeast

In the course of studying mechanistic aspect of autophagy, we recently found that an active flow of COPII vesicular traffic from the ER is required for autophagosome formation. There are many studies to follow the changes in expression levels of proteins under the starvation, but it has not been studied how the protein transport pathway like early secretory pathway from the ER, the production site of cell surface and secretory proteins, is affected. Here we studied ER and Golgi resident proteins to see if and how the starvation stress

modulates the early secretory pathway, and found an autophagic degradation of ER under starvation conditions in addition to cytosolic protein degradation (Figure 2). Golgi membrane protein was not engulfed by the autophagosome under the same conditions, indicating that the uptake of ER by autophagosome is the specific event. Although the ER exists in a network structure that is mutually connected and resides predominantly around the nucleus and beneath the plasma membrane, most of autophagosomes engulfed ER. The extent of the ER uptake by autophagy was nearly identical to that of the soluble cytosolic proteins. This phenomenon was explained by the appearance of fragmented ER membrane structures in almost all autophagosomes. Furthermore, ER dynamism is required for this process: ER uptake by autophagosomes occurs in an actin dependent manner. Visualizing these proteins revealed dynamic changes in the early secretory pathway under the starvation condition. Significant amounts of both ER and Golgi proteins were transported to the vacuole during starvation.

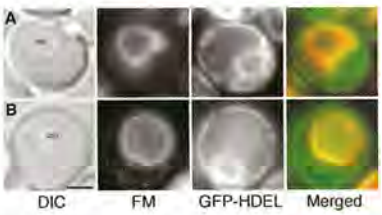

Figure 2. DsRed-HDEL is transported to the vacuole in an autophagy dependent manner with rapamycin treatment. The fluorescence and the Nomarski images of wild-type cells (JK9-3da) expressing DsRed-HDEL (A) under growing condition and (B) under rapamycin treated condition (10 hrs). The vacuolar limiting membrane was labeled with FM4-64. The vacuolar lumen (noted "vac") became fluorescent with starvation treatment.

IV. In vitro reconstitution of Atg8-PE conjugation system

In yeast, *S. cerevisiae*, Atg8 plays an important role during autophagosome formation. We have previously revealed that the Atg8 is covalently attached to phosphatidylethanolamine (PE) via a ubiquitin-like conjugation system (Figure 3). 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. Notably, Atg8 conjugation system, while similar to ubiquitination mechanically, utilizes a ubiquitous phospholipid, not a protein, as a target. We further developed *in vitro* Atg8-PE reconstitution system. The Atg8-PE was successfully reconstituted simply with $Atg8^{G116}$, Atg7 and Atg3 by using *in vitro* system. These results confirmed that $Atg8^{G116}$, Atg7, and Atg3 are the minimum components for 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 PE in a lipid bilayer is necessary for the Atg8 conjugation. These results should be very useful for future works to determine where Atg8-PE conjugation occurs during autophagy in yeast. Interestingly, we found that the PE-conjugated Atg8 has a higher affinity for an antiserum raised against the N-terminal region of the Atg8 protein. Further, Atg8 became highly sensitive to trypsin digestion after lipidation. Hence, we concluded that PE conjugation induces a conformational change of Atg8.

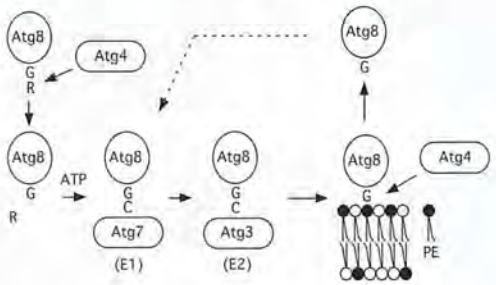

Figure 3. Atg8-PE conjugation system

Carboxy-terminal arginine (R) of newly synthesized Atg8 is removed by Atg4 protease, leaving a glycine residue (G) at the C terminus ($Atg8^{G116}$). The $Atg8^{G116}$ is activated by E1 enzyme, Atg7, and then transferred to E2 enzyme, Atg3. Finally, $Atg8^{G116}$ covalently conjugates with phosphatidylethanolamine (PE) through an amide bond between the C-terminal glycine and the amino group of PE. Liberation of Atg8 moiety from Atg8-PE by the action of Atg4 (deconjugation) is required for the normal progression of autophagy.

V. Autophagy-defective plant exhibit early senescence

So far, plant autophagy has been described in reports by morphological studies. In addition, a recent genome-wide search revealed significant conservation among *ATG* genes in yeasts and plants. It has not been proved, however, that Arabidopsis *ATG* genes are involved in plant autophagy. To evaluate this requirement, we examined the ubiquitination-like Atg8 lipidation system. We generated transgenic Arabidopsis expressing GFP-ATG8 fusion proteins and established a system monitoring autophagy in whole plants. In wild-type plants, GFP-ATG8s were observed as ring shapes in the cytoplasm and were delivered to vacuolar lumens under nitrogen-starved conditions. By contrast, in T-DNA-insertion double mutant of the *ATG4*s which encodes proteases required for C-terminal cleavage of ATG8 (*atg4a4b-1*), autophagosomes were not observed and the GFP-ATG8s were not delivered to the vacuole. In addition, we detected autophagic bodies in the vacuoles of wild-type roots (Figure 4 panel A) but not in those of *atg4a4b-1* (Figure 4 panel B) in the presence of concanamycin A, a V-ATPase inhibitor. The autophagy defective mutant exhibited early senescence of rosette

leaves even under nutrient-rich conditions (Figure 4 panel D) and fewer seeds under nutrient-limiting conditions; however, these plants were nevertheless able to undergo a complete life cycle. In addition, *atg* mutant displayed a reduction in the growth rate of roots under nutrient-limiting conditions. Autophagy is required for the maintenance of cellular viability regardless of the nutrient conditions and contributes to the development of a root system under conditions of nutrient limitation.

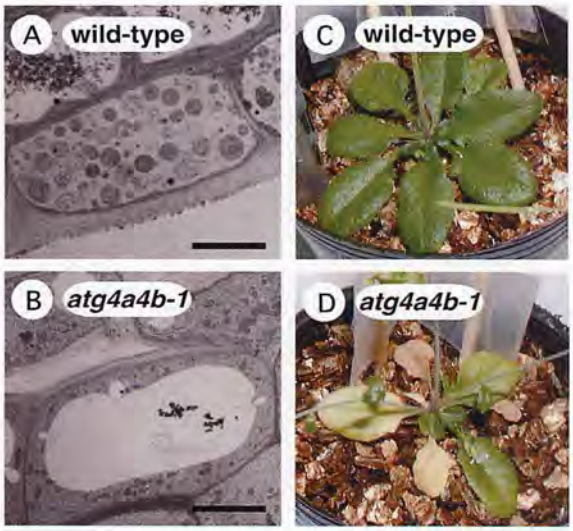

Figure 4. *atg* mutant is defective in accumulation of autophagic bodies and exhibited early senescence phenotype
(A) Electron micrographs of wild-type roots treated with concanamycin A.
(B) Electron micrographs of *atg4a4b-1* mutant roots treated with concanamycin A.
Roots of one-week-old wild-type and *atg4a4b-1* seedlings were treated with concanamycin A (1 μM) under nitrogen-starved conditions for 6 h. The same regions of the root tip were compared. Bars = 5 μm
(C) 7-week-old wild-type plant
(D) 7-week-old *atg4a4b-1* plant

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LABORATORY OF CYTOSKELETON

Associate Professor: *OGAWA, Kazuo*

I. Dynein family

Dynein family is a group of microtubule-activated axonemal and cytoplasmic ATPases that serve to convert chemical energy into mechanical energy. Axonemal dynein performs flagellar and ciliary movements. In contrast, cytoplasmic dynein transports organella precursor such as vesicular membranes in a cytoplasm. Dynein is a retrograde motor and transports the cargoes to cell center. Kinesin that is also microtubule-based motor protein is an ortho-grade motor and transports the cargoes to cell periphery. Dynein and kinesin are different proteins in terms of primary structure. However, they may be coupled partners to maintain the even distribution of motor proteins in a cell. Figure 1 shows the localization of axonemal dynein at the outer arms of sperm axonemes and cytoplasmic dynein at the mitotic apparatus of cleaving egg visualized by anti-axonemal dynein (Fragment A) antibodies.

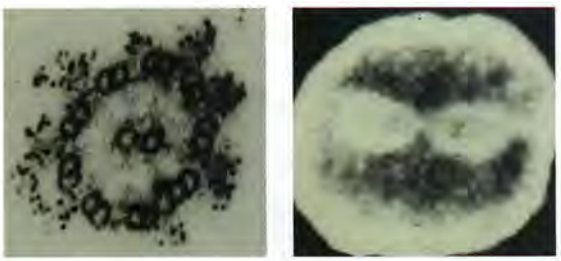

Figure 1. Dynein family. Axoneme (left) and Cleaving egg (right).

II. Complex structure of dynein

Dynein is a very large protein and has molecular mass up to 1 to 2 mega Da. It is complex protein consisted of heavy chains (HCs), intermediate chains (ICs), and light chains (LCs) classified by their molecular weights. Cytoplasmic dynein contains light-intermediate chains (LICs) in addition to these polypeptides.

III. HCs

Outer arm dynein consists of two heavy chains with ATPase activity. The motor activity is closely related to these polypeptides. Dynein is composed of three domains: stem, motor, and stalk. The first successful molecular cloning of this huge polypeptide (520 kDa) was performed in 1991. Since then cDNA clones for axonemal and cytoplasmic dyneins from a variety of organisms have been isolated and sequenced. The sequences of HCs contain, without exception, four P-loop motives referred to as ATP-binding sites in the midregion of the molecules. The NH_2-terminal one-thirds corresponding to the stem and may participate in the targeting of dynein to the cargo. The probable function of the COOH-terminal one-thirds has been unknown for a while, although our previous data had shown that the middle one-thirds and the COOH-terminal one-thirds make larger domain by their intra-molecular interaction corresponding to Fragment A or motor. Yeast genome project have proposed for the new family of AAA-proteins. Taking the novel idea of AAA-protein module into consideration, there may be two AAA-modules at the COOH-terminal one-thirds and four AAA-modules at the midregion. As a consequence, dynein HCs may be a hexamer of an AAA-module as shown in Figure 2 (Vale 2000).

There are two, interrupted hydrophobic heptapeptide repeats between the midregion and the COOH terminal region, forming extended flexible structure, stalk. Stalk may bind to microtubule in an ATP-dependent manner.

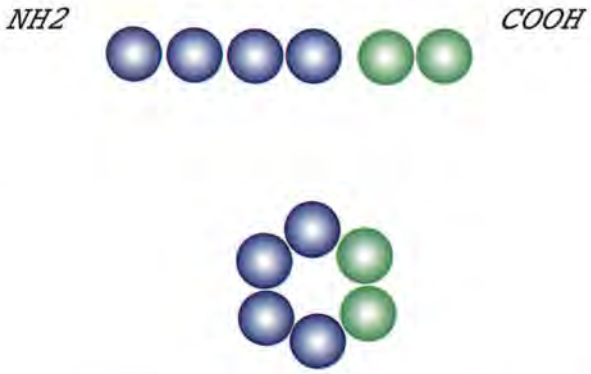

Figure 2. A hexamer model of an AAA-module for dynein HCs.

IV. ICs

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. WD-containing ICs have been found in cytoplasmic dynein. 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 have been found in distantly related species but cytoplasmic dynein may not contain it.

V. LCs

Six light chains with molecular masses of 23.2, 20.8, 12.3, 11.5, 10.4, and 9.3 kDa are closely associating with outer arm dynein. We have already isolated cDNA clones of five LCs.

Transmission ratio distortion is a dramatic example of non-Mendelian transmission. In mice, *t*-haplotype males produce dysfunctional +-sperm and normal *t*-sperm,

leading to transmission in favor of *t*-sperm. Genetic studies have indicated that the *t-complex responder locus*, *Tcr*, rescues *t*-sperm but not +-sperm from defective products of *t-complex distorter loci*, *Tcds*. LC1 and LC3 from sea urchin sperm outer arm dynein have sequence similarities to Tctex2 and Tectex1, respectively, both of which are wild-type products of *Tcds*. We showed that LC1 and LC3 are able to make a 1:1 complex. Since Tcr is a member of the Smok (sperm motility kinase) family (Herrmann et al., 1999) and LC1 is phosphorylated at the activation of sperm motility in a c-AMP-dependent manner, this complex in a dynein motor molecule might be a direct target of Smok/Tcr kinase in a signal cascade that regulates sperm motility. Thus, we designated it as Smoac (sperm motility complex).

Table I shows the polypeptide composition contained in outer arm dynein of sea urchin sperm flagella. Two polypeptides, α-HC and LC5 have not yet been cloned.

Table I. Molecular compositions of outer arm dynein from sea urchin sperm axonemes cloned in our laboratory.

Chains	Accession number	Calculated molecular weight
α-HC	Not cloned	
β-HC	D01021	511774.95
IC1	D63884	91622.50
IC2	D38538	79137.02
IC3	D28863	68223.32
LC1	BAA24185	20325.81
LC2	BAA24184	22201.21
LC3	AB004251	12535.95
LC4	BAA24152	12458.90
LC5	Not cloned	
LC6	AB004830	10325.67

VI. Targeting of dynein to the cargo

The mechanism how dynein targets the cargoes has been gradually made clear in terms of molecules participated in. In cytoplasmic dynein, less characterized IC and LIC that are closely associated with stem of dynein are thought to target the cargoes. They are linked with dynactin complex consisted of at least ten polypeptides. It is via dynactin complex that dynein targets a receptor of cargo membranes.

In flagellar and ciliary movement, outer and inner dynein arms are projected from the A-subfibers of peripheral doublet microtubules of axoneme corresponding to the cargo. They bind to the B-subfibers. They bind to the B-subfiber of neighbouring doublet microtubules in an ATP-dependent manner. Under the physiological condition, in contrast to cytoplasmic dynein, axonemal dynein is hardly detached from the A-subfibers being the cargo. In this point, axonemal dynein are different from cytoplasmic dynein in which case, the cargoes are thought to be detached from the motor after arrival to cell center to recruit the cargoes. Thus, there may be different targeting mechanism for dynein family to specific sites of the cargoes. Outer dynein arm is positioned at just 24 nm interval along axis of axonemes. The correct positioning of outer arm onto the A-subfibers is thought to be due to the outer dynein arm-docking complex (ODA-DC) that could link up end-to-end with some overlap, to form a filament with a 24 nm repeat structure (Kamiya, 2002).

When Triton-model sperm were exposed to hard condition such as a high salt solution, outer arm was detached from the A-subfibers. Extracted sperm without outer dynein arm swim with a beat frequency of control sperm. Rebinding of outer dynein arm onto the A-subfibers was possible by remixing the extracted Triton-sperm with the extract under a low salt condition. Recovered Triton-model sperm swam with a normal beat frequency (Gibbons and Gibbons, 1976). Thus, a high salt extract may containing some proteins necessary for correct positioning of outer dynein arm onto the A-subfibers. During the course of characterizing proteins containing in it, we found a novel protein with molecular mass of 58 kDa designated as ap58. Immuno-electron microscopy using antibodies raised against recombinant ap58 shows that gold-particles are found at 25 nm repeat along the length of axoneme coinciding with the repeat of outer dynein arm. Thus, we conclude that ap58 is binding to in situ outer dynein arm.

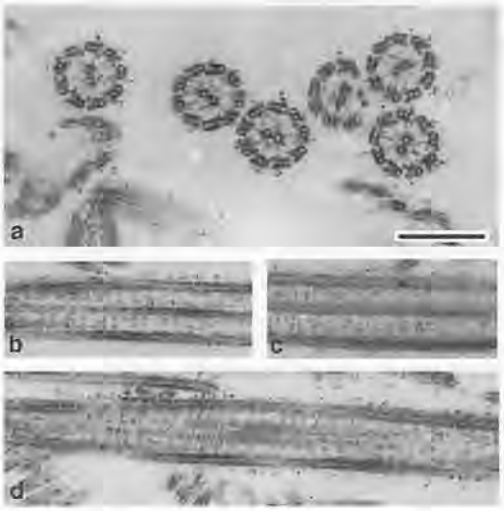

Figure 3. Localization of ap58 on outer dynein arm visualized by immuno-electron microscopy (Ogawa and Inaba)

LABORATORY OF CELL SOCIOLOGY

Research Associate: *HAMADA, Yoshio*

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

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

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

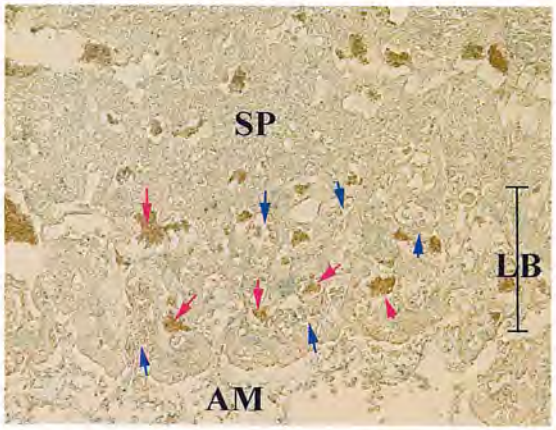

Figure 1. Expression of *Notch2* gene in developing mouse placenta. A cryo-section of $Notch2^{+/LacZ}$ placenta at E10.0 was incubated in X-gal staining solution. Trophoblast in maternal vascular bed side expresses the gene, but not in allantoic mesenchyme (AM) side in labyrinth layer (LB). The expression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

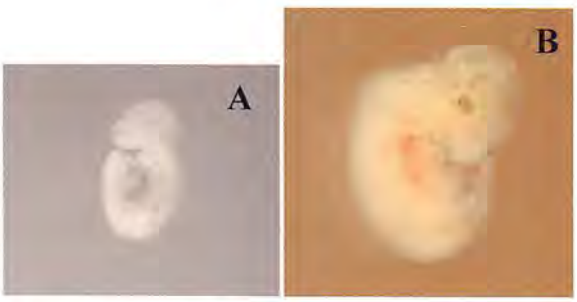

Figure. 2. Survival of *Notch2* mutant embryo in a chimera with teteraploid wild type embryo. The mutant obtained in the intercrosses by the heterozyotic mice shows developmental retardation at E10.5 (A) and die before E11.5. The mutant is able to survive in a chimera with wild type tetraploid embryo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

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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

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. Our 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 (Fig. 1). 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*.

DMY mRNA and protein are expressed specifically in the somatic cells surrounding primordial germ cells (PGCs) in the early gonadal primordium, before morphological sex differences are seen. However, somatic cells surrounding PGCs never express *DMY* during the early migratory period. Expression of DMY persists in Sertoli cells, from PGC-supporting cells to Sertoli cells, indicating that only *DMY* positive cells enclose PGCs during mitotic arrest after hatching.

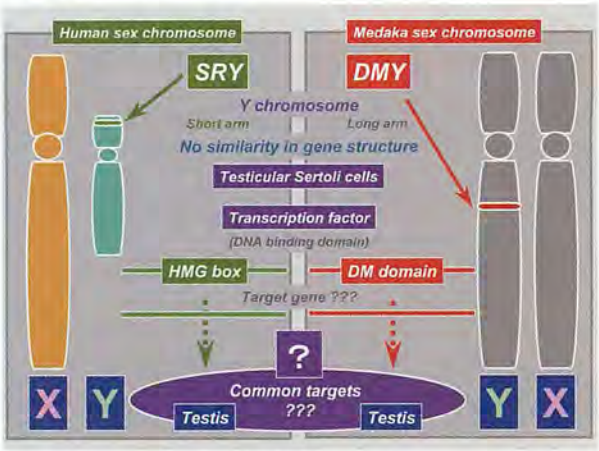

Fig. 1 Comparison of two known sex-determining genes in vertebrates, *SRY/Sry* in mammals and *DMY* in medaka.

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. In all genetic female tilapia, steroid-producing cells in ovaries prior to and during sex differentiation express all of the steroidogenic enzymes required for estrogen production. Estrogen receptors α and β first appear in XX gonads prior to sex differentiation. The treatment of XX fry with fadrozole (aromatase inhibitor) or tamoxifen (estrogen receptor antagonist) caused complete sex reversal to functional males. These results, together with evidence that the XX sex reversal induced by fadrozole was rescued completely with simultaneous estrogen treatment, suggest that endogenous estrogens are required for ovarian differentiation in tilapia. In contrast to XX fry, steroid-producing cells appear to differentiate after gonadal differentiation in XY fry. The *DMRT1* gene is expressed male-specifically in testicular Sertoli cells during sex differentiation. Application of *DMRT1* morpholino antisense oligonucleotides (*DMRT1* knock down) blocked androgen-induced masculinization of XX gonads. These results suggest an important role of *DMRT1* in testicular differentiation in tilapia.

To identify the down-stream gene products of estrogen during ovarian differentiation, we have performed subtraction hybridization using mRNA derived from normal and estrogen treated XY gonads of tilapia. Interestingly, one of the up-regulated gene products was aromatase, indicating that endogenous aromatase activity is up-regulated in XY fry after the induction of XY sex reversal by estrogen. It is also of interest that one of the down-regulated gene products was *DMRT1*, providing evidence to support that *DMRT1* may function as a pivotal gene for male gonadal sex differentiation.

III. Sex-changing fish

The goby, *Trimma okinawae*, can serially change sex in either direction. Their gonads contain ovarian and testicular portions simultaneously; however, individuals typically produce one gamete type, change sex and then produce the other type. They do not produce mature sperm and oocytes at the same time. Thus, *T. okinawae* provides an ideal model for studies on the mechanisms of sex change. Aquarium experiments were carried out in the laboratory. Two males (M+M) or two females (F+F) were kept in separate tanks. In the M+M aquarium, the smaller male changed its sex to female. On the contrary, the larger female changed its sex to male in the F+F aquarium. Behavioral changes occurred within 30 minutes of social manipulation. After pairing, the larger male or female attacked the smaller fish, which fled and often hid in a nest. After 30 min, however, the larger fish began to court the smaller fish. These results suggest that the sex of brain/behavior changes quickly and easily, regardless of gonadal sex. Brain sex of sequential hermaphrodite might be determined independent of gonadal effect and were induced only by social cues.

IV. Embryonic development of gonadotropin-releasing hormone neurons

Appropriate development of forebrain gonadotropin-releasing hormone (GnRH) neurons is essential for establishing and maintaining reproductive competence, yet the mechanisms underlying this process are poorly defined. We established transgenic medaka lines that expressed GFP under the control of the *gnrh1* and *gnrh3* promoters, and they allowed prolonged *in vivo* imaging of GnRH neuronal behavior at any stage of development in a noninvasive manner (Fig. 2). Our images revealed that preoptic *gnrh1* neurons and terminal nerve ganglion *gnrh3* neurons originate in the olfactory region, while the medial basal telencephalic *gnrh1* neurons arise from the rostral telencephalon. We also found that the diencephalic neuronal population transiently expresses *gnrh1* during embryogenesis, that the trigeminal ganglion neurons express *gnrh3*, and that *gnrh3* is maternally expressed in oocytes and embryos. Taken together, the transgenic medaka provides a useful model system for studying GnRH neuronal development including disorders of GnRH deficiency.

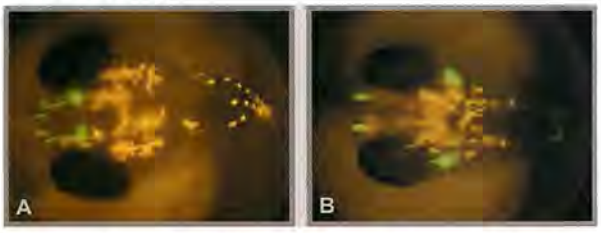

Fig. 2 Transgenic medaka embryos that express GFP under the control of *gnrh1* (A) and *gnrh3* (B) promoters.

V. Endocrine regulation of spermatogenesis

Using an organ culture system for eel, *Anguilla japonica*, 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. In turn, 11-KT activates Sertoli cells to stimulate the production of activin B. To clarify the mechanism of the initiation and progression of spermatogenesis, we examined the expression pattern of A-type cyclins and Dmc1 during gonadotropin-induced spermatogenesis in eel. Expression of cyclin A2 increased 1 day after the induction of spermatogenesis and reached a plateau after 6 days when many type B spermatogonia were found. In contrast, the expression of cyclin A1 mRNA was detected after 9 days coincident with the first appearance of spermatocytes. Cyclin A1 mRNA was localized in germ cells of all stages from primary spermatocytes to round spermatids, whereas cyclin A2 mRNA was specifically localized in spermatogonia, secondary spermatocytes, round spermatids, and testicular somatic cells, including Sertoli cells. Dmc1 protein was localized only in the earlier stages of primary spermatocytes; prior to this stage cyclin A1 mRNA was not detectable. These results suggest that cyclin A2, Dmc1, and cyclin A1 are expressed in spermatogenic cells sequentially before and during meiosis, indicating that the entry of spermatogonia into meiotic prophase can be distinguished by Dmc1 protein and cyclin A1 mRNA expression.

VI. Endocrine regulation of oocyte growth and maturation

Two major steroidal mediators, estradiol-17β and 17α, 20β-dihydroxy-4-pregnen-3-one (17α, 20β-DP) are synthesized by ovarian follicles during oocyte growth and final maturation in response to FSH and LH, respectively. 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) (Fig. 3), 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, 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.

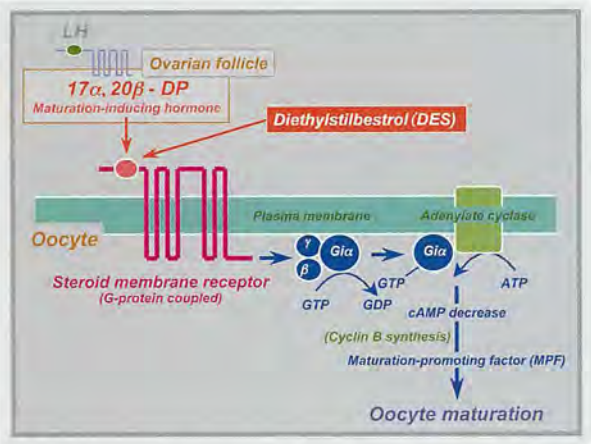

Fig. 3 Hormonal regulation of meiotic maturation in fish oocytes. Three major mediators, LH (pituitary), 17α,20β-DP (ovarian follicle) and MPF (oocyte), are involved. Both 17α,20β-DP and an endocrine-disrupting chemical, DES, may interact with a novel membrane progestin receptor.

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 (Fig. 3).

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DIVISION FOR SEX DIFFERENTIATION

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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, in the case of mammals. 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 several tissues including the gonads. The functions of the other transcription factors such as Ad4BP/SF-1, Emx2, M33, and Lhx9 were elucidated by the phenotypes of the gene disrupted mice. In addition, the expression profiles in terms of their gonadal 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.

Tentatively, we hypothesize that the sexually indifferent gonads determine their sexes under the control of two opposite signals, one of which is the signal for testicular differentiation and the other is the signal for ovarian differentiation (Fig. 1). Probably the nature of the signals might be a transcriptional activity of a certain transcription factor expressed in the fetal gonads, or otherwise might be activity of a certain type of growth factor. The study in this division has been performed mainly from the aspect of the transcriptional control as the signal for the sex differentiation. As indicated in Fig. 1, a number of transcription factors/DNA binding proteins are expressed in the developing gonads. Based on the symptoms of human patients and the phenotypes of gene knockout mice, *Ad4BP/SF-1*, *Emx2*, and *Wt1* are indispensable for the gonadal development. With respect to the signals for gonadal sex differentiation, *Sry*, *M33*, *Arx*, *Dhh*, *Sox9*, *Pdgf*, *Wt1*, and *Fgf9* are assumed to act as the testicular signals while Dax-1 and Wnt4 are the ovarian signals.

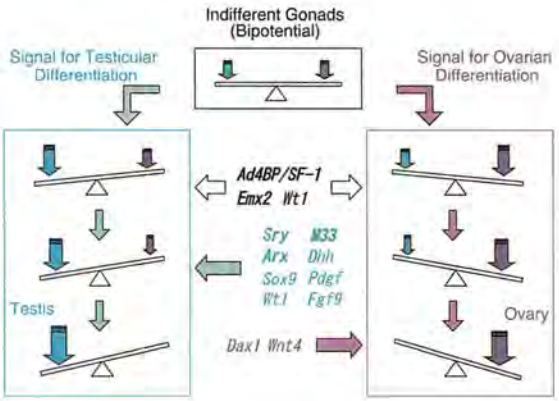

Fig. 1. Gondal sexes are determined by balance of signals for testicular and ovarian differentiation.

I. Functional analyses of Vinexin

In order to define the function of Ad4BP/SF-1, we have screened a two-hybrid library prepared form mouse embryonic gonads. Vinexin was one of the clones isolated by the screening. Two isoforms of Vinexin, Vinexin α and β, had previously been identified, however, the clone isolated in this study was distinct from those two isoforms in the 5' amino acid coding region. Thus, this novel isoform was tentatively designated Vinexin γ. Structurally, Vinexin γ is composed of 680 amino acids corresponding to the 57th to 736th amino acids of Vinexin α (76 kD). Analyses of the gene structure indicated that this difference is due to alternative usage of the first exon. In silico screening revealed the presence of Vinexin γ in other animal species; human SCAM-1 (SH3-Containing Adaptor Molecule-1) and rat SCAM-1. In situ hybridization and immunohistochemical studies showed that Vinexin γ started to be expressed in the sexaually indifferent gonads (E11.5), and thereafter expressed specifically in Sertoli cells in the testis (Fig. 2).

For the function of Vinexin, it has been discussed that

the molecule is involved in MAPK signaling through interacting with ERK. Thus, we investigated whether Vinexin γ interacts with the components of MAPK cascade. Interestingly Vinexin γ was found to interact with c-Raf as well as ERK, suggesting that Vinexin γ acts as a scaffold protein. Moreover, our data indicated that Vinexin γ activates MEK and ERK through interaction with c-Raf and ERK. Ultimately in culture cell study, it was revealed that *Sox9* transcription was induced by Vinexin γ. This up-regulation of *Sox9* expression disappeared when the cells were treated with a specific MEK inhibitor.

Fig. 2, Expression of Vinexin γ in the developing mouse gonads. Vinexin γ is expressed in the sexually indifferent gonads (E11.5) of both sexes (Male and Female). The expression continues at E12.5 gonads. In the male gonad, the expression of Vinexin γ is clearly restricted in Sertoli cell.

In order to reveal the function of Vinexin γ during gonadal development, we generated a Vinexin γ-specific gene-disrupted mouse. Unexpectedly, the homozygous mutant mice of both sexes were fertile with morphologically normal gonads. The mice showed decreased *Sox9* expression, and the decreased levels varied among individuals. Indeed, even in the most affected individuals, we failed to detect either complete disappearance of *Sox9* expression or developmental defects in the XY gonad. Since other MAPKs, JNK and p38, were not activated ectopically in the Vinexin $\gamma^{-/-}$ fetal XY gonad, it is unlikely that the Vinexin γ-dependent function of ERK was displaced by the other MAPKs. Together, the moderate phenotype displayed by the Vinexin $\gamma^{-/-}$ gonad suggested that MAPK activation is not critical for testis formation. Consistent with our observations, it was recently reported that a MAP kinase inhibitor did not inhibit testis cord formation in gonadal organ culture.

Alternatively, the relatively moderate phenotype displayed by the Vinexin γ-disrupted mice might be due to the following two possibilities. Firstly, Vinexin α and Vinexin β might function redundantly. Indeed, these two isoforms share three tandem repeats of SH3 domains, and moreover, Vinexin β was still evident in the gonad of Vinexin γ-disrupted mice. However, given that Vinexin γ contains its specific N-terminal half and the expression in the fetal testis does not appear to overlap with that of Vinexin β, it is unlikely that the function of Vinexin β is completely redundant with that of Vinexin γ. Secondly, the functions of two closely related proteins, Arg-binding protein 2 (ArgBP2) and c-Cbl-associated protein (CAP/ponsin/SH3P12), should be noted. Structurally, besides the presence of three SH3 domains in common at their C-termini, these proteins share a sorbin domain with Vinexin γ in their N-terminal halves. Moreover, expression of *ArgBP2* and *CAP/ponsin/SH3P12* was detected in the fetal gonads (E12.5) of both sexes by RT-PCR and whole-mount *in situ* hybridization, although these signals were weak. Thus, it might be possible that their features functionally and spatially overlapped with Vinexin γ failed to display drastic gonadal defects in the Vinexin γ-disrupted mice. Taken together, Vinexin γ seems to be implicated in regulation of *Sox9* gene expression by modulating MAPK cascade in mouse fetal gonads.

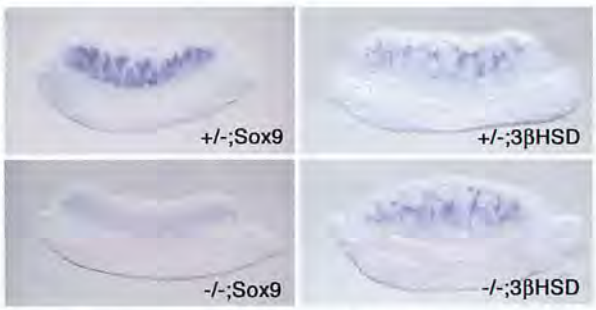

Fig. 3, Expression of Sox9 and 3βHSD in Vinexin γ knockout mice. The developing gonads (E12.5) of the knockout (-/-) and heterozygous (+/-) mice were subjected to in situ hybridization probed with Sox9 (Sertoli cell marker gene) and 3β-hydroxysteroid dehydrogenase (3βHSD) (Leydig cell marker gene). Representative decreased expression patters are shown for Sox9 in the knockout. The expression of 3βHSD in Leydig cells seemed not to be affected by Vinexin γ knockout.

II. Characterization of VMH-specific enhancer of Ad4BP/SF-1 gene

It has been generally accepted that genes are divided into two types according to their expression profiles. Firstly, there are the so-called house-keeping genes with ubiquitous expression among tissues and constitutive expression during developmental processes. In contrast, other genes display tissue-specific and/or developmental stage-specific expression. Many genes have been revealed to contain enhancer sequences, which may explain the molecular basis of regulation of these spatially and temporally restricted-expression.

Ad4BP/SF-1 is a transcription factor essential for animal reproduction. Based on the phenotypes observed in gene-disrupted mice, *Ad4BP/SF-1* is thought to be involved in establishment of the hypothalamic-pituitary-gonadal axis. However, the mechanisms underlying tissue-specific expression of *Ad4BP/SF-1* are largely unknown. We have investigated the *cis*-regulatory regions of the mouse *Ad4BP/SF-1* gene by transgenic mouse assays, and recently we identified a fetal adrenocortical-specific, pituitary-specific, and

ventromedial hypothalamic nucleus (VMH)-specific enhancers. In the present study, we identified a VMH-specific enhancer in intron 6 of the *Ad4BP/SF-1* gene. Functionally, the enhancer was characterized by driving VMH-specific expression of the *lacZ* reporter gene when the enhancer was localized with the basal promoter of *Ad4BP/SF-1*. Moreover, the VMH-specific expression was also reproduced with the *hsp68* basal promoter, indicating that the VMH-specific expression can be driven primarily by this enhancer. Structurally, the sequence is conserved among animal species (mouse, human, and chicken), thus strongly indicating that the conserved sequences probably function as a VMH-specific enhancer beyond animal species.

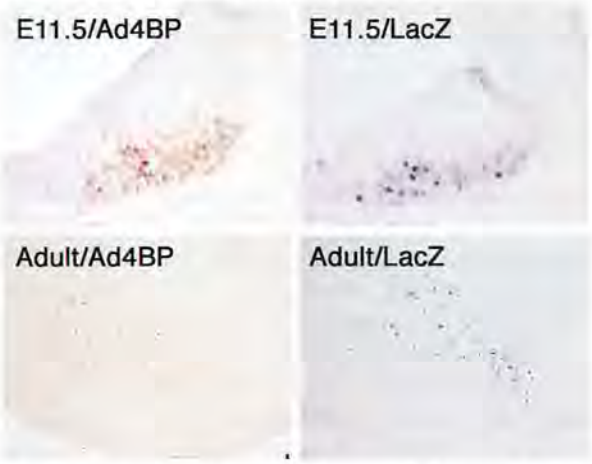

Fig. 4, Comaprison between endogenous Ad4BP/SF-1 and transgene-derived expression of lacZ. Transgene was constructed with a VMH-specific enhancer DNA fragment and lacZ gene as a reporter. Transgenic mice at fetal age (E11.5) and adult were used for the study. The endogenous expression of Ad4BP/SF-1 was examined immunohistochemically.

Further detailed transgenic analyses of the VMH enhancer revealed that the enhancer contains suppressive and activating elements. Mutation of the former element resulted in ectopic *lacZ* reporter gene expression in an area dorsal to the intrinsic expression domain and in the ventricular zone, while mutations in the latter containing ATTA motifs led to the disappearance of the reporter gene expression, suggesting the involvement of homeobox proteins. Brain regionalization is regulated primarily by the functions of growth factors together with regionally expressed homeobox proteins. Moreover, it has been shown that combined expression of these homeobox proteins is essential to establish progenitor cell identity and neuronal cell subtypes during forebrain patterning. Indeed, several homeobox proteins are known to be expressed in various regions of the developing brain. Among these, it is noteworthy that the expression domain of Nkx2.1 overlaps with that of *Ad4BP/SF-1* until E18.5. Nkx2.1 is a member of the Nkx homeoprotein family, and plays important roles in ventral forebrain development. In particular, in *Nkx2.1*-null mice, the ventromedial and dorsomedial nuclei were underdeveloped and fused in the midline. The expression profile of *Nkx2.1* and the phenotypes of the gene-disrupted mice prompted us to analyze the effect of gene disruption on the VMH enhancer function. Disappointingly, however, expression of the *lacZ* reporter gene driven by the VMH enhancer persisted even in the *Nkx2.1* knockout background, suggesting that *Nkx2.1* is not essential for the enhancer function. In this context, it is quite interesting to consider the potential redundant function of another family member, Nkx2.4, of which expression is overlapped with Nkx2.1 in the developing forebrain. Meanwhile, considering that the *lacZ*-positive cells in the ventral diencephalon of the *Nkx2.1* knockout mice were fewer in number than in the wild-type from the early developmental stage of VMH, Nkx2.1 could be partially involved in the VMH-specific enhancer function. Through the function of the VMH enhancer, Nkx2.1 might regulate the amount of Ad4BP/SF-1 expressed, and thereby stimulate VMH cell proliferation. Indeed, it was recently reported that cell numbers in adrenocortical primordium are tightly dependent on the amount of Ad4BP/SF-1.

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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 which trigger intracellular signaling, transcription factors which act in the nucleus to regulate gene expression are thought to be essential for the determination of cell fates. Our main interest is to know how pattern formation and morphogenesis during development is regulated by these growth factors and transcription factors. We address this problem using several model animals, including frog, fly and acidian, employing embryology, genetics, molecular and cellular biology, and biochemistry. In addition, we have recently introduced genomics technologies 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 the cell movement called "convergent extension". As convergent extension begins, cells are polarized and aligned mediolaterally, followed by the mutual intercalation of the polarized cells. Recent studies suggest that several extracellualr signals are essential for the polarization, one of which is fibroblast growth factor (FGF). We therefore attempted to identify FGF target genes to understand the molecular mechanism underlying the FGF function using our *Xenopus* cDNA microarray. As a result, we identified a gene encoding a neurotrophin receptor homolog (NRH) and demonstrated it to be an essential component of FGF to control protrusive activity of cells undergoing gastrulation.

Another pathway regulating gastrulation is 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. Subsequently, we identified a protein that binds to XPK by yeast two-hybrid screening. The XPK binding protein was found to be a member of the Ste20 kinase family and named as *Xenopus* prickle-interacting kinase, XPIK. Loss-of-function of XPIK resulted in severe defects in gasrtulation causing *spina bifida*. 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 two thousand 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. Wnt signaling pathway regulates actin cytoskeletal dynamics during gastrulation

The noncanonical Wnt pathway has been implicated in the regulation of *Xenopus* gastrulation movements. Loss of function of its signaling components, such as Dishevelled, Rho GTPases and c-Jun N-terminal kinase, leads to a severe gastrulation defect. We investigate the molecular mechanism of how the Wnt pathway regulates this morphogenetic process. Convergent extension of the dorsal mesoderm is one of the important mechanisms during gastrulation. In this movement, cells are polarized, elongated and then, intercalated. We have established a technique to observe this process at the cellular level. This method enabled us to show that loss of function of the Wnt signaling components caused a defect in the

polarization of the mesodermal cells. During convergent extension, the formation of lamelipodia- and filopodia-like protrusions from the mesodermal cells are observed. We found that the blockade of the Wnt signaling pathway significantly reduced the protrusive activity. On the contrary, overexpression of Xwnt11 and its receptor Frizzled promoted the protrusive activity. These results indicate that the noncanonical Wnt pathway plays an essential role in the convergent extension movements through the regulation of actin cytoskeletal dynamics.

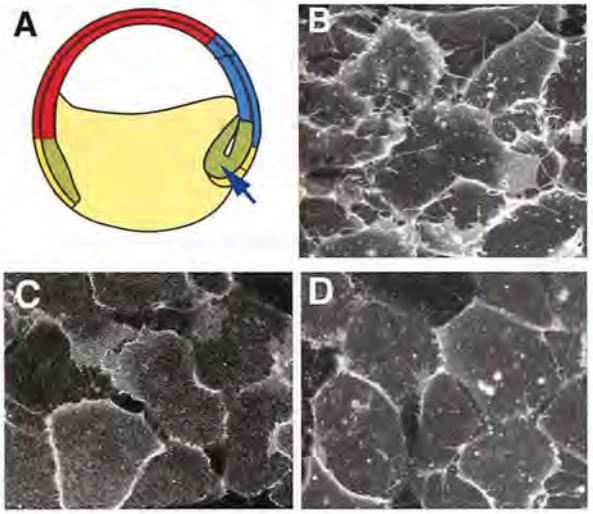

Figure 1 The Wnt signaling pathway regulates protrusive activity in the mesodermal cells. (A)The dorsal mesodermal cells indicated by the arrow were observed. Control cells (B) form lamelipodia- and filopodia-like protrusions, whereas this activity was significantly reduced by the expression of the dominant negative mutant of Xwnt11 (C) or Dishevelled (D).

III. One of the DPP signal downstream components TNA/TONAS participates roles in protein SUMO modification and epigenetic regulation

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. Two mutant alleles of the *tonalli* (*tna*) gene were isolated in this screening. *tna* is a previously reported mutant and a member of Trithorax group genes. Trithorax group components have been shown to be involved in the epigenetic regulation including Histone modification and chromatin remodeling. We isolated vertebrate homologues of *tna* and named as tonalli related SP-RING finger protein, 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. We have been investigating function of TNA/TONAS family SP-RING finger proteins by genetical and biochemical approarches. The SP-RING motif was originally found in the PIAS family SUMO-E3 ligase proteins. We found that TONAS can bind E2 enzyme ubc9 and has activity stimulating SUMO-2/3 specific conjugation to TONAS itself. SUMO-2/3 conjugated TONAS proteins were predominantly recovered in cytoplasmic fraction. The result indicates thata SUMO-2/3 modification somehow regulates nuclear export or cytoplasmic retention for SUMO-2/3 modified substrates. Our ongoing study also suggests that TNA/TONAS proteins participates a role in complex Histone modifications.

IV. Function of *Brachyury*-downstream notochord-specific genes in morphogenetic movements of the *Ciona intestinalis* 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.

Formation of the chordate body is accomplished by a complex set of morphogenetic movements of constituent cells. Convergent extension is a major component of axis elongation. In the urochordate ascidian *Ciona intestinalis*, *Brachyury* (*Ci-Bra*) plays a key role in the formation of the notochord, which undergoes typical convergent extension. Nearly forty *Ci-Bra* downstream notochord genes have been identified. The present functional analyses of 32 genes revealed that 24 (75%) of them are involved in convergent extension; nine genes including *Ci-prickle*, *Ci-PTP* and *Ci-talin* caused failure of convergent extension when their function was suppressed with specific morpholinos, while the function of 15 other genes including *Ci-netrin*, *Ci-ERM* and *Ci-pellino* was required for intercalation/extension. In addition, knockdown of *Ci-Noto7* resulted in the failure of convergence but intercalation/extension took place normally suggesting a separable phase of convergent extension.

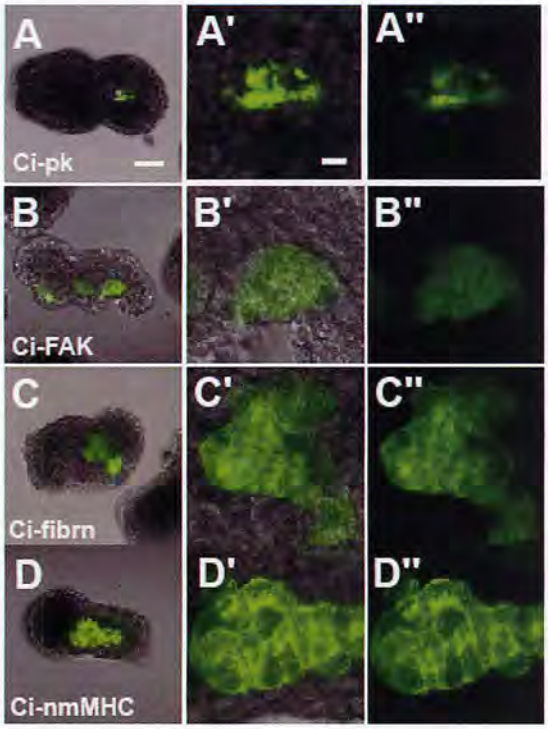

Figure 2 Failure in convergent movements caused by functional suppression of four genes. (A) *Ci-pk*, (B) *Ci-FAK*, (C) *Ci-fibrn*, (D) *Ci-nmMHC*. (A-D) Whole embryos with EGFP expression, (A'-D') enlargement of notochord region, and (A"-D") their dark-field images. Scale bars, 50 μm in A and 10 μm in A'.

V. *Xenopus* functional genomics (Xenomics)

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

In order to achieve the goal, we first collected more than 170,000 ESTs from the early embryo cDNA libraries. These ESTs were assembled into more than 30,000 contigs and singlets. Among them, we have selected full-length cDNAs excluding redundancy, and have read the full insert sequence for about 3,000 novel genes. To explore the spatiotemporal expression profile of each gene, we have carried out whole mount *in situ* hybridization (WISH) of embryos at different stages of early embryogenesis. So far, we have investigated the expression patterns of more than 2,000 genes. We have also started to investigate the subcellular localization of each gene product using GFP-labeled expression vector. We constructed the comprehensive database XDB3 that stores EST sequences, assembled sequences, full insert sequences and WISH data with the descriptions of presumed gene function. We also created microarray with PCR-amplified cDNAs, and carried out a series of microarray experiments on the collaboration basis. In order to investigate how the gene expression is regulated by the cell-cell interaction, we combine the microarray technology with classical embryo manipulations including surgical operations.

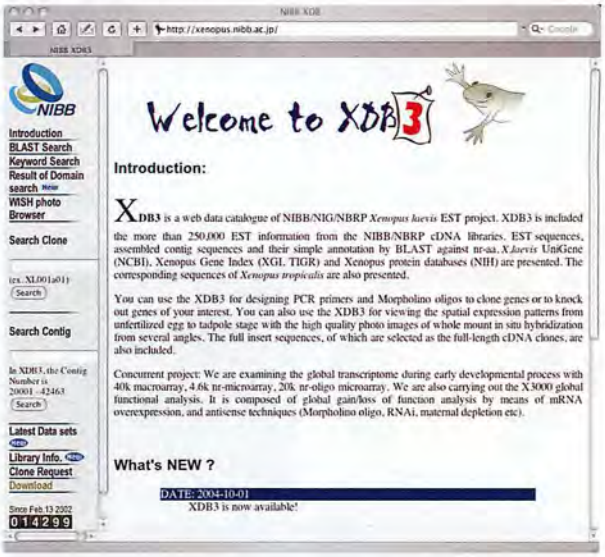

Figure 3 Xenopus Data Base 3 (http://xenopus.nibb.ac.jp).

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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 *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.

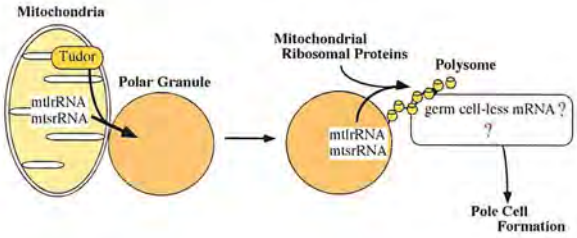

Fig. 1 Role of mitochondrial rRNAs in pole cell formation

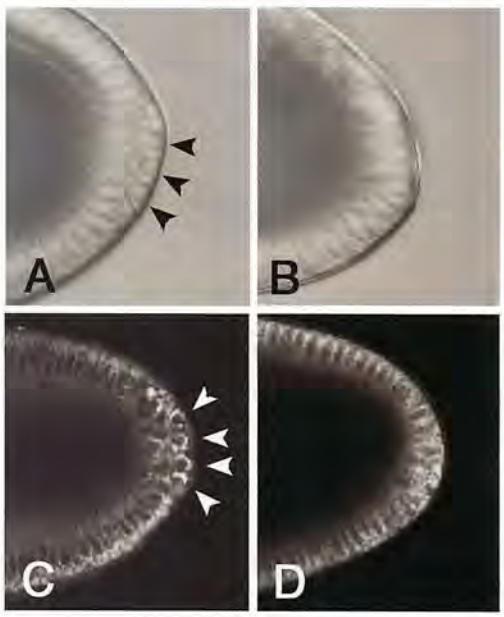

Fig. 2 Injection of kasugamycin causes a defect in pole cell formation but not in mitochondrial activity. Embryos at stage 4-5 were injected with DW (*A, C*) and KA (*B, D*) and photographed using a compound microscope equipped with Nomarski optics. (*A, B*) Pole cells are not discernible in the KA-injected embryo, although somatic cells are intact. (*C, D*) Injected embryos were stained with Rhodamine 123, which was not affected by KA treatment. We found that CH treatment also inhibited pole cell formation, but did not affect Rhodamine 123 staining (data not shown). Arrowheads in (*A, C*) indicate the pole cells.

We have 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 (Fig. 1). To address this issue, we have been examining the effect of *Chloramphenicol* and *Kasugamycin* (KA) 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 (Fig. 2). 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.

Recently, we found that one of the target RNAs translated on the mitochondrial-type of ribosomes was *germ cell-less* (*gcl*) mRNA, which is stored in polar granules and encodes a protein required for pole cell formation.

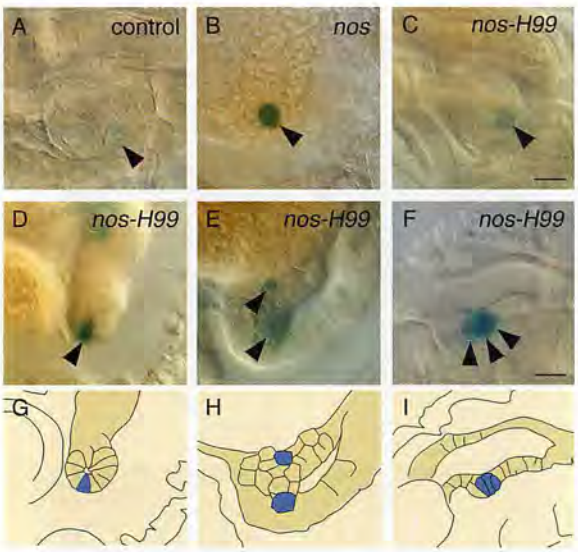

Fig. 3 *nos*-H99 pole cells have the ability to adopt somatic cell fate and to enter into the embryonic gonads. Pole cells from *nos* (*B*), *nos*-H99 (*C-F*) and control embryos (*A*) were transplanted into hosts, and the hosts were developed until stage 15-17. The transplanted pole cells were identified as blue cells after heat treatment and staining with X-gal (arrowheads). The transplanted control (*A*) and *nos*-H99 pole cells (*C*) were observed within the gonads, while *nos* pole cells remained outside the gonads (*B*). Some *nos*-H99 pole cells were integrated within somatic tissues (*D-F*). (*D*) *nos*-H99 pole cell within midgut epithelium is shown. Note that *nos*-H99 pole cell and the neighboring midgut epithelial cells were columnar in shape at stage 17. *nos*-H99 pole cells within gastric caeca (*E*) and tracheal epithelium (*F*) are also shown. The lower cell pointed by arrowhead in (*E*) was out of focus. *G-I*, Drawings tracing the integrated *nos*-H99 pole cells (blue) and the surrounding somatic cells in (*D-F*), respectively. Scale bar; 10 μm.

II. Role of Nanos protein in pole cell differentiation

Pole cells differ from the soma in regulation of transcriptional activity. 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) remains inactive in early pole cells. Thus, the ability to express zygotic mRNA-encoding genes is suppressed only in pole cells in early embryos.

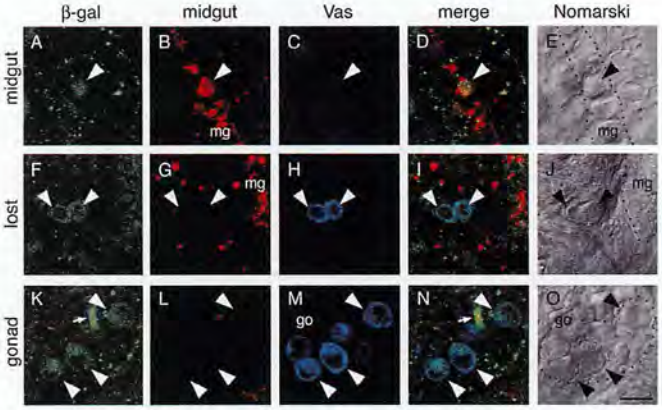

Fig. 4 *nos*-H99 pole cells within midgut epithelium expressed midgut marker genes but lost germline marker, Vas. We examined expression of midgut marker genes and Vas protein in *nos*-H99 pole cells within midgut epithelium (*A-E*), haemocoel ("lost" pole cells) (*F-J*) and the gonad (*K-O*) of host embryos. The transplanted pole cells were identified as the cells expressing β-gal (marked by arrowheads). The embryos at stage 13-15 were triple-stained with probes for the midgut marker genes (*blnt-n/ mex1*; red) (*B, G, L*) and antibodies against β-gal (green) (*A, F, K*) and Vas protein (blue) (*C, H, M*). *nos*-H99 pole cell integrated within midgut epithelium (mg), like the neighboring host cells, was cuboidal in shape at stage 14 (*E*). The "integrated" pole cell expressed midgut marker genes (*B*), but not Vas protein (*C*). In contrast, *nos*-H99 pole cells, which were found within haemocoel in the vicinity of midgut (mg) (*J*), maintained Vas expression (*H*), but never expressed midgut marker genes (*G*). Within the gonad, *nos*-H99 pole cells and the host pole cells were both negative for midgut markers (*L*). Arrows in (*K*) and (*N*) indicate non-specific staining of tracheal lumen. Merged images (*D, I, N*) and photographs taken with a compound microscope equipped with Nomarski optics (*E, J, O*) are shown. Dotted lines indicate midgut epithelia (mg) in (*E*) and (*J*), and the gonad (go) in (*O*). Scale bar; 10 μm.

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. *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.

We have 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 protein required for 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.

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 (Fig. 3, 4). 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.

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

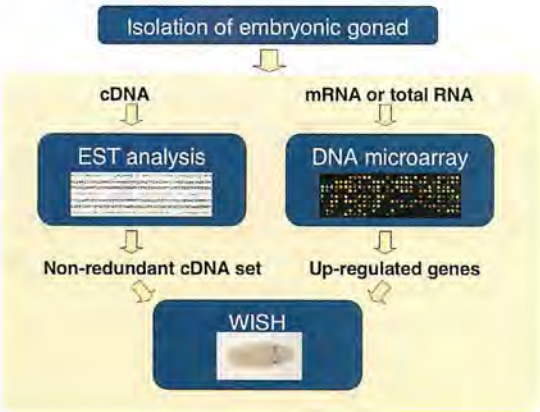

Fig. 5 Transcriptome analysis of the embryonic gonads

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) (Fig. 5). Approximately 20,227 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.

We also started to identify genes expressed predominantly in pole cells, using DNA microarray technique (Fig. 6).

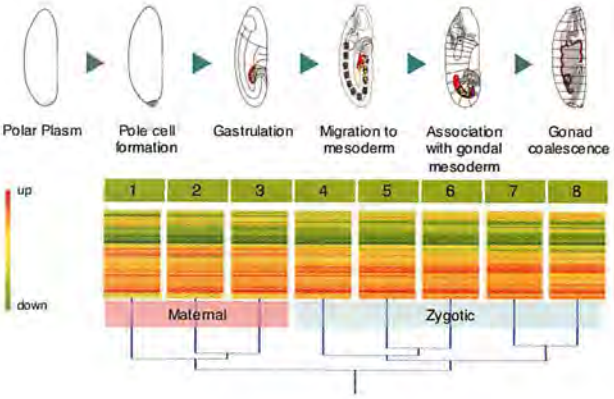

Fig. 6 Clustering analysis of expression profile of all of the predicted genes in the *Drosophila* genome. We divided embryonic stages into 8 groups, and isolated pole cells from the embryos at each stage. These pole cells were used for microarray analysis. As a reference, we used RNA from whole embryos of these 8 groups. Single line represents single transcript. Red line shows "upregulated" in pole cells. Hierarchical clustering analysis shows that they can be divided into two large groups. This is due to the onset of zygotic transcription in group-4 pole cells.

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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 Functional Analysis of molecular targets of Wnt signaling during development

Wnt signaling plays important roles in many aspects of vertebrate development. For revealing the molecular mechanisms underlying each developmental event triggered by Wnt signaling, identification and functional analysis of genes activated or repressed by this signal are important. However, only a few systematic approaches have thus far been reported for identification and functional analysis of Wnt-responsive genes in vertebrate development.

The gene-trap methodology is a powerful strategy for the systematic identification and functional analysis of genes in the post genomic era, because this methodology offers the identification of a novel gene, the analysis of its expression pattern, and the generation of its functional mutation in a single experimental approach as described below. In this methodology, random insertion of a gene-trap vector leads to the tagging, and frequently to the disruption, of genes across the genome. Therefore, if ES cells are used for the generation of insertion events, embryonic and adult whole bodies containing tagged and disrupted alleles can be produced. Such an insertion event affords the following advantages for identification and functional analysis of a trapped gene. 1) Nucleotide sequences of a trapped transcript and an insertion site can be determined by 5' rapid amplification of cDNA ends (RACE) and plasmid rescue method, respectively. The process of identification of a trapped gene has been substantially eased by the recent completion of the genome database. 2) The expression pattern of a trapped gene during development can be easily monitored by the expression of protein tag derived from a gene-trap vector in embryos generated from trapped ES cells. 3) An insertion event has the potential to be mutagenic. Because of these strong advantages, the gene trap methodology has been applied for several studies, including large-scale insertional mutagenesis programs.

Although the gene trap methodology basically involves the random integration of a gene-trap vector, this strategy has also been expected to be available for identification and characterization of genes regulated by particular signals. In a modified gene-trap strategy, called induction gene trap, genes are selected by their response to specific secreted signal proteins, including BMP2, activin, nodal, and FGF, added to the culture of gene-trapped cells. Although the *in vivo* correlation between selected genes and signals has remained unclear in these cases, *in vitro* prescreening of trapped ES cell lines with secreted signal proteins would be effective for enrichment of genes regulated by specific signals even in normal development.

Here, we examined whether gene trap methodology, which would be available for systematic identification and functional analysis of genes, is effective for screening of genes responsive to Wnt signaling during mouse development. We screened out 2 individual clones (Clone 43 and Clone 5) among 794 gene-trapped ES cell lines by their *in vitro* response to WNT-3A proteins.

To examine whether the *in vivo* expression of these trapped genes was actually regulated by Wnt signal, we examined their expression precisely in mouse embryos. Expression of *Clone 43* was temporarily observed in the inner cell mass of blastocysts. Later, *Clone 43* was specifically expressed in ductal structures in the mid-gestation stages. This gene was expressed during a number of aspects of the ductal morphogenesis in kidney development. The urogenital expression of *Clone 43* was observed first in the nephric duct and mesonephric tubules at E10.5 (Figure 1A) and later in the Wolffian duct, the ureter, the collecting duct, and the distal portion of tubules connected to the collecting duct in the metanephros (Figure 1B, C, E). The expression was restricted to the epithelium, i.e., was not found in the mesenchyme, in

these organs (Fig. 1E, F). *Clone 43* was also expressed in the ductal epithelium of the salivary glands (submandibular, sublingual, and parotid glands) at E 15.5 (Fig. 1F, G).

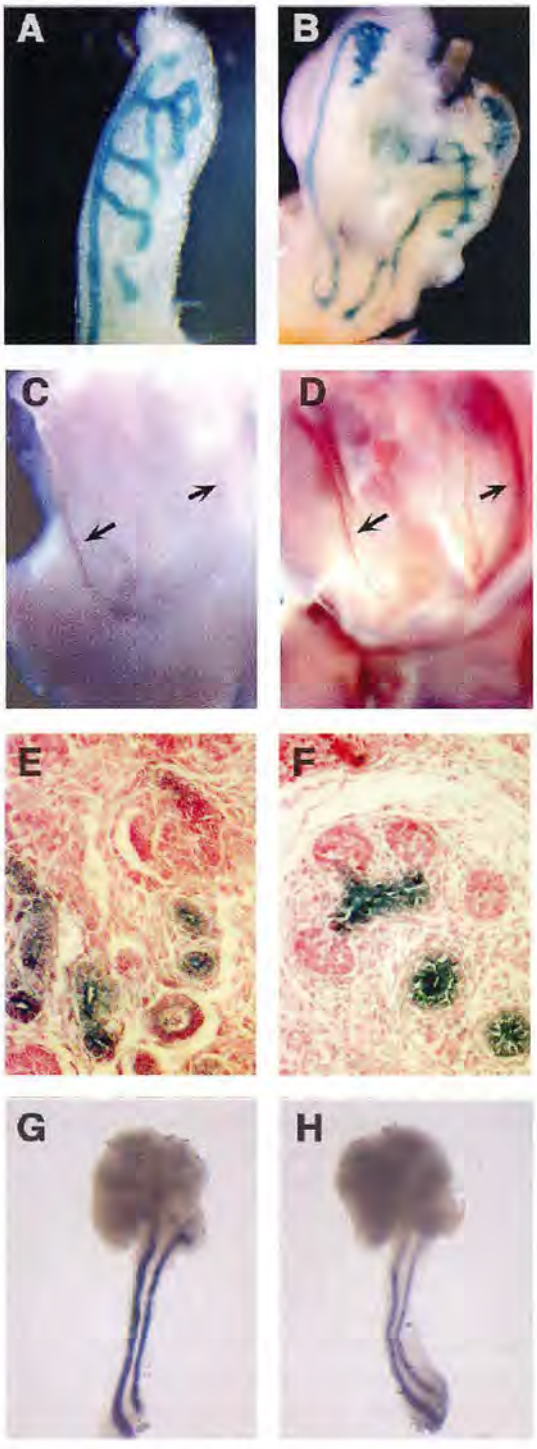

Figure 1 Expression of Clone 43 in the ductal development. (A, B) During urogenital development, *lacZ* reporter was expressed first in the nephric duct and mesonephric tubules at E10.5 (A). At E13, *lacZ* reporter of clone 43 was also expressed in the Wolffian duct, mesonephric tubules, the ureter, and the collecting duct (B). (C, D) *Clone 43* (C) was co-expressed with*Wnt-7b* (D)in the Wolffian duct. (E, F) *Clone 43* is expressed in the epithelial cells in the developing kidney (E) and SMG (F). (G, H) Expression of *CRTR-1* mRNA (G) and *Wnt-5b* (H) in the SMG and SLG at E13.5.

Interestingly, the expression of several *Wnt* genes was also observed in the ductal structures in which *Clone 43* was expressed. In the urogenital development, *Wnt-7b*, which has been reported to be expressed in the Wolffian duct, the ureter, and the collecting duct at E13.5, was co-expressed with this trapped gene (Figure 1C, D). On the other hand, in the submandibular gland (SMG), the expression of a number of *Wnt* genes, including *Wnt-2, 2b, 3, 4, 5b, 6, 10b, 14, 16,* was detected by RT-PCR analysis. Among these *Wnt* genes, *Wnt-5b* exhibited an expression pattern spatially and temporally correlated with that of *CRTR-1*. *Wnt-5b* was expressed at mid-gestation stages, for instance E13, in the stalks of the SMG and the sublingual gland (SLG), where *Clone 43* was expressed (Fig. 1G, H). Thus, the ductal expression of *Clone 43* was well correlated with the expression of *Wnt-7b* and *Wnt-5b* in the kidney and the salivary gland, respectively

The expression pattern of *Clone 5*, also suggested its close correlation with that of *Wnt* genes. Expression of *Clone 5* was first observed at E 8.5 in rhombomere 5. At E9.5, the expression of this gene was still detected in rhombomere 5 although it was relatively dispersed. Rostral to rhombomere 5, the lacZ-positive cells straggled in the superior membrane of the rhombencephalon. At E10.5-11.5 the expression was observed with strong intensity in scattered neural crest cells over the dorsal diencephalon and mensencephalon, as well as in relatively weak intensity over the dorsal spinal cord. Interestingly, these scattered signals were strong along the dorsal midline adjacent to the roof plate where *Wnt-1* and *Wnt-3a* were expressed. *Clone 5* gene was also expressed in mesenchyme in the telencephalic flexure. This mesenchymal expression represents another example of correlation between this gene and *Wnt*, since *Wnt-3a* is expressed in the neuroepithelium, adjacent to the mesenchymal cells, in the telencephalic flexure. The expression of *Clone 5* gene was also observed in the meninx at E13.5. Taken together, the close correlation between gene expression of the 2 trapped genes and that of several *Wnt* genes strongly suggests that the trapped genes screened by their *in vitro* response to WNT proteins were also responsive to Wnt signals *in vivo*.

Furthermore, the expression of these 2 genes was significantly decreased in embryos deficient for several *Wnts* or in cultures of embryonic tissues treated with a Wnt signal inhibitor. For instance, the positive LacZ signal of *Clone 5* gene was absent in neural crest cells migrating from the hindbrain and the spinal cord in the *Wnt-1/Wnt-3a* double mutant (Figure 2H). In contrast, the lacZ signal was present in the wild-type and in *Wnt-1* and *Wnt-3a* single mutant embryos (Figure 2E-G). These results strongly suggest that *Wnt-1* and *Wnt-3a* were redundantly required for the expression of the clone 5 trapped gene in this region. In addition, the X-gal staining in the mesenchymal cells in the telencephalic flexure was significantly reduced in the *Wnt-3a* null mutant, as well as in *Wnt-1/Wnt-3a* double mutant, compared with that in the wild-type and *Wnt-1* single mutant embryos (Figure 2A-D).

To examine whether the trapped genes were required for proper development of cells or organs where they were expressed, we generated homozygous mutants for the trapped genes by crossing heterozygous males and females. In homozygotes for the *Clone 43* trapped allele, the expression of this gene was almost completely diminished, whereas the expression of a truncated transcript fused to the β-geo gene in the trap vector was strongly detected. Interestingly, 70% of homozygotes for the *Clone 43* trapped allele died before 5 weeks after birth. Most of in the *Clone 43* homozygous mice exhibited hypoplasia of the kidney at postnatal day30, whereas the heterozygous and wild-type mice in the same litters showed no obvious defect in their kidney. Thus, *Clone 43* is likely to be required for proper development of the kidney, in which this gene is expressed. On the other hand, no obvious defect has yet been observed in homozygotes for the clone 5 trapped allele.

Thus, the *in vivo* expression of the 2 trapped genes, which were screened by their *in vitro* response to Wnt, was also dependent on the Wnt activity. Furthermore, homozygotes for a trapped allele showed a morphological phenotype in the kidney, where the trapped gene was expressed overlappingly with Wnt-7b. These results indicate that an inductive gene trap in ES cells is likely to be effective for screening and functional analysis of genes induced by Wnt signaling during embryogenesis.

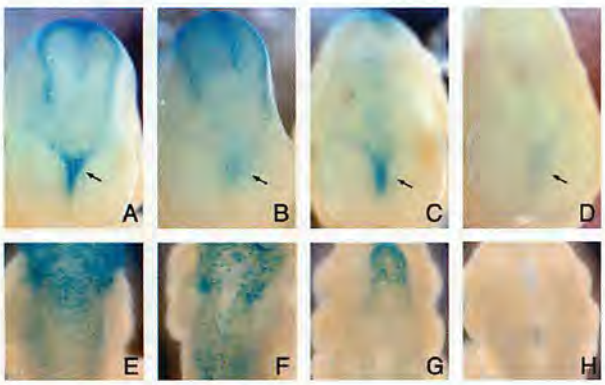

Figure 2 Down-regulation of the expression of *Clone 5* gene in *Wnt-3a* and *Wnt-1/Wnt-3a* mutants. (A-D) Frontal views of the expression of *Clone 5* in the telencephalic flexure (arrow) at E11.5. (E-H) Dorsal views of the expression of *Clone 5* gene in the hindbrain at E11.5. Wild type (A, E), *Wnt-3a* null mutant (B, F), *Wnt-1* mutant (C, G), *Wnt-1* and *Wnt-3a* double-null mutant (D, H).

II 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 (Figure 3). 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.

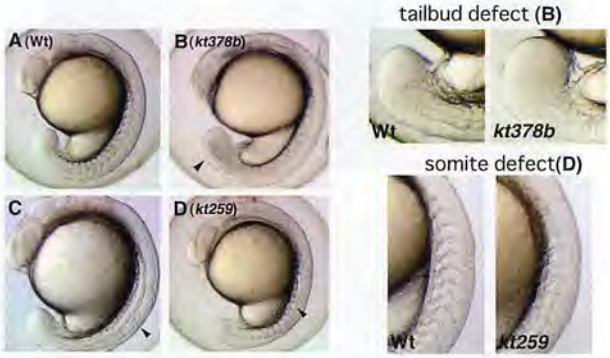

Figure 3 Zebrafish mutant embryos which display abnormal shape of the tailbud (kt378b) and a segmentation defect of somites (kt259).

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LABORATORY OF DEVELOPMENTAL REGULATION

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Protein palmitoylation during embryogenesis

Covalent attachment of fatty acids is a feature of many proteins in eukaryotic cell. The two modifications involve acylation with myristate and palmitate. Myristate is usually linked through an amino-terminal glycine residue via an amide linkage which is relatively stable. Palmitate is usually linked through cysteine residue via a thioester linkage, and the post-translational process is referred to as protein palmitoylation. Protein palmitoylation is a dynamic modification. The turnover rate of bound palmitate through thioester linkages is faster than that of the modification with myristate through amide linkages. Most small GTP-binding, G proteins and G protein-coupled receptor proteins are known to be modified with palmitate via thioester linkages. Protein palmitoylation is thought to be important in the regulation of signal transduction. However the molecular mechanisms how protein palmitoylation regulate development is unknown.

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 growth cones. Addition of an inhibitor of protein palmitoylase to the medium of cultured primary neural cells reduces the axonal growth of neurons. From these findings, we speculated that palmitoylation of the signaling proteins is critical for development of axons.

GAP-43 is a neuron-specific protein. Cys 3 and Cys 4 of GAP-43 are modified with palmitate in developing neurons. Transfection of GAP-43 cDNA into non-neural cells such as COS-7 cells is reported to cause the induction of filopodia-like formation. Since Cys residues are necessary for the induction of filopodia-like formation, protein palmitoylation is discussed to regulate the induction filopodia-like formation. Filopodial processes is thought be important for the development of neural growth cones. Therefore we focused to analyze the regulation mechanisms of the induction of filopodia-like formation in non-neural cells.

Transfection of protein palmitoylase cDNA into non-neural cells

We prepared the full-length protein palmitoylase cDNA for the expression in cultured cells. The cDNA contained a long open reading frame 2,504 amino acids. Protein palmitoylase and GAP-43 cDNAs were transfected into COS-7 and HeLa cells and the transient expression of these proteins were induced.

We confirmed that expression of protein palmitoylase reproducibly alters COS-7 morphology. Filopodia extention is observed in about 5% of control COS cells, but in 15% of protein palmitoylase-expressing COS cells under our conditions. To characterize the effect of protein palmitoylase on non-neural cells, we examined cellular morphology at various times after trypsinization and replating. Before contact with a substratum allowing adhesion, about 15% of control COS-7 cells exhibit filopodia. Transfection with protein palmitoylase increases this to 25%. The presence of filopodia is not significantly altered by varying the length of time to 120 minutes during which the cells are maintained in suspension after trypsinization. After contact with an adherent substratum the percentage of cells with filopodia rapidly declines in both the control and protein palmitoylase transfected cells. The rate of decline is approximately the same in both groups. The effect of protein palmitoylase is to alter the initial morphology after trypsinization, and not to alter the influence of the substratum on the cell shape.

Filopodia are exhibited by few protein palmitoylase expressing COS cells after 30 minutes of incubation with an appropriate substratum. However the shape of protein palmitoylase expressing cells can still be distinguished from control cells by another attribute, the degree of cell spreading. Protein palmitoylase expression appears to delay cell spreading. When control COS cells are plated on poly-L-lysine-coated glass, cell spreading occurs over 60 minutes. The time required to achieve the same degree of spreading is prolonged in the protein palmitoylase expressing cells, although the degree of spreading eventually reaches the same level. One day after plating, cell shape is indistinguishable between control and protein palmitoylase expressing cells.

Induction of filopodia-like formation in cultured cells by peptide transfection

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. By using the method, we have prepared the peptides which are modified with myristate (C14:0), palmitate (C16:0), stearate (C18:0) and arachidate (C20:0) via thioester linkages. The method also modified the peptide with unsaturated fatty acid such as palmitoleate (C16:1). The modified peptides were separated by reverse phase HPLC and these peptides were eluted from the column depend on their chain length.

We are attempting to see the effect of filopodia-like formation in COS-7 cells by peptide transfection, which were chemically modified with fatty acids. Since the efficiency of the induction of filopodia-like formation by transfection with palmitoylated peptide is low, we are trying to elevate the efficiency.

LABORATORY OF MOLECULAR GENETICS FOR REPRODUCTION

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

Since sex determination gene of mice was identified in 1990, research has intensively been focused on the testis development. However, for instance, the differentiation of the precursor cells determining sex, the interaction of somatic cells and germline, the differentiation of germline to germ cells having the capability to undergo meiosis, and so on, there remain many important and facinating problems to be solved.

Our laboratory aims to reveal the molecular mechanims regarding to the formation of gonads, especially emphasizing on visualizing specific cell lineages in medaka living embryos and by applying moelcular genetics to medaka embryos.

I. Monitering the formation of gonads through fluorescent germline.

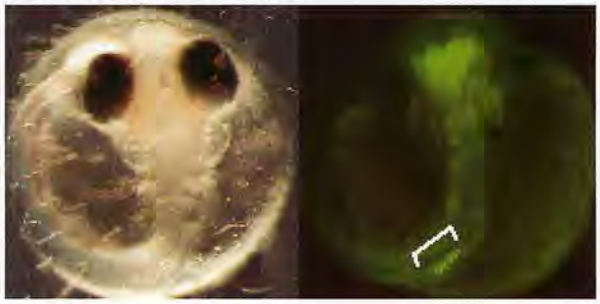
One of the advantages to use medaka is that embryos are transparent and can be seen from outside. This encouraged us to develop transgenic medaka exhibiting GFP exclusively in germline and succesfully established the trangenic lines (*olvas*-transgenic medaka). During the establishment, we found that two genomic regions, 5' promoter region and 3'UTR region, are necessary for proper expression of germline-specific gene, *olvas* (medaka *vasa* homologue). Furthermore we identified an element, GSE (germline-specific element), in the 3'UTR, which mediates both maintenance of RNA exclusively in germline and activation of translation through enhancement of poly(A) elongation. Both mechanisms act independently on GSE and consequently bring about efficient translation in germline,

Injection of *in vitro* synthesized GFP RNA with GSE at its 3' end allows to monitor more clearly and brightly the development of germline from early embryogenesis. Bright fluorescence enables us to identify primordial germ cells easily and isolate ESTs that express in germline. We continue to analyze the ESTs currently.

II. Germline Development

Primordial germ cells have been characterized as the large cells having electron-dense amorphas structure called germ granules (also called germplasm or nuage). We isolated several ESTs whose products are components of germ granules and revealed that 3'UTRs of some ESTs possess *cis*-element functionally similar to *olvas* GSE. We synthesized RNA of *EST-GFP-3'UTR* fusion genes and injected it into fertilized eggs. The GFP-fused products were localized in germ granules as has been detected by antibodies.

Fig.1 GSE in 3'UTR functions to translate RNA exclusively in germline. The picture below right shows fluorescent germline in a gonad.



By keeping track of the structure of the granules with a laser confocal microscopy, we could outline germline development and revealed several developmental stages of germline. Firstly, germline separates from somatic cell lineages at morula stage and is suggested to be fated to primordial germ cells as epiboly movement proceeds at early gastrulation stage. The primordial germ cells near the germ ring move to anterior lateral plate mesoderm and then migrate posteriorly along the embryonic body. Localization of the components on the germ granules differs from that found in the germ cells of the indifferent gonads, suggesting that an additional step is required to differentiate into germ cells which has the capability to undergo meiosis.

III. Origin of gonadal mesoderm

Many focus have been concetrated on the mechanisms of differentiation into male gonadal mesoderm after sex detemination gene is activated. However, the origin and the lineages of the gonadal precursor cells have remained to be elucidated.

As described in section II, germ cells migrate into a gonadal region. This suggests that cells producing guidance substance for primordial germ cells become the precursor of gonadal mesoderm. We constructed a fate map showing where the precursor of gonadal mesoderm is derived using uncaging method. The result indicates that most posterior portions of lateral plate mesoderm converge medially toward an embryonic body to locate bilateral regions where migrating primordial germ cells target. These regions correspond to posterior ends in expression domain of guidance substance. After primordial germ cells meet the precursor cells of gonads in the lateral plate mesoderm, the precursor cells and

primordial germ cells move dorsally around a developing hindgut, and finally reach the region where the indifferent gonad will form.

It has been suggested by the analysis of mice mutant possessing few germ cells that somatic precursors autonomouly develop to gonadal mesoderm. It is, however, likely that in the process of organogenesis gonads should interact between somatic mesoderm and germ cells. To challenge this problem with our skills and biological backgrounds, we are generating medaka without germ cells by ablation of fluorescent germ cells with a laser or disruption of function of guidance substance.

IV. Analyses of mutant medaka affecting formation of gonad

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

In collaboration with ERATO Kondoh differentiation signaling project, we have been screening mutants affecting primordial germ cells and the formation of gonads. The defects were screended by expression of *olvas* gene in germ cells. The screening told us the presence, number and distribution pattern of germline at a somitogenesis stage and at ten days post hatching (10 dph). Nine mutants (19 alleles) and twelve mutants (14 alleles) were identified for primordial germ cells and gonads, respectively.

The mutants affecting formation of gonads fall into four groups according to their phenotypes. 1) mutants having overproliferating germ cells, 2) mutants with reduced number of germ cells, 3) mutants with fragmented gonads and 4) mutants with scattered germ cells in the gonadal region. We are now characterizing mutant phenotypes and the modes of inheritance.

One mutant, *totoro*, is very interesting in that the similar phenotype has not been described before in other animals. *totoro* has a swelled large abdomen filled with a gonad. Our analyses show that the phenotype is semidominantly inherited. Gonads are sex-reversed in genetic males and fullfilled with numerous oocytes regardless of its genetic sex. The positional cloning is ongoing with ERATO project and the characterization of the phenotype is also in progress.

Fig.2 sex reversed *totoro* mutant showing a large abdomen, which is occupied by a gonad with numerous number of oocytes.

Another mutant, *zenzai*, is in good contrast with *totoro* mutant and is unique in that germ cells are not maintained in the gonad. The mode of inheritance of the phenotype indicates that the allele is recessive. The characterization and the positional cloning are also in progress.

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We have been studying the molecular and cellular mechanisms underlying the development of the vertebrate central nervous system, mainly using the visual system. 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. The scope of our interests also encompasses various functions of the matured brain, including sensation, behavior, learning and memory.

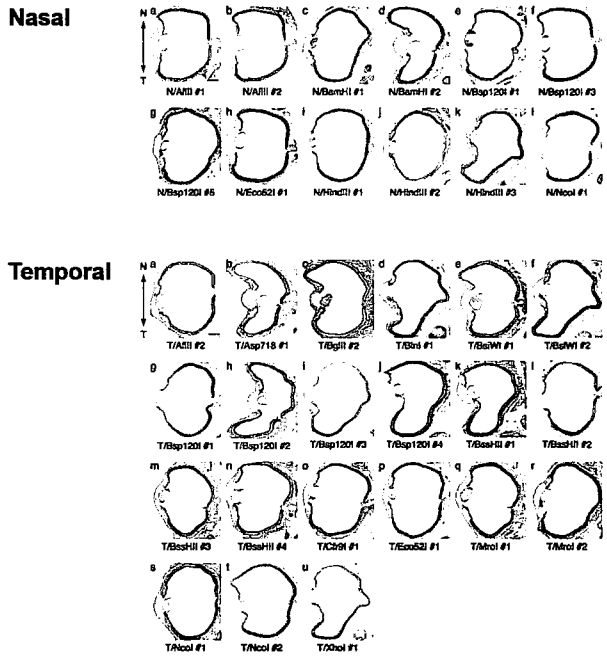
I. Regional specification in the retina

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

Regional specification along the nasotemporal and dorsoventral axes precedes the topographic retinotectal projection in the developing retina. To understand the molecular basis of topographic retinotectal projection, an overall view of the asymmetrically expressed molecules in the developing retinas is needed. 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.

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

This year, we published the whole of results as one paper. These included transcription factors, secretory factors, transmembrane proteins, and intracellular proteins with various putative functions. Their expression profiles revealed by *in situ* hybridization are highly diverse and individual (Fig. 1). Moreover, many of them begin to be expressed in the retina from the early developmental stages, suggesting that they are implicated in the establishment and maintenance of regional specificity in the developing retina. We have already described on several molecules in published papers, but the study to know their hierarchical order to establish the regional specification in the retina is still in progress. The molecular repertoire revealed by this work will provide candidates for future studies to elucidate the molecular mechanisms of topographic retinotectal map formation.



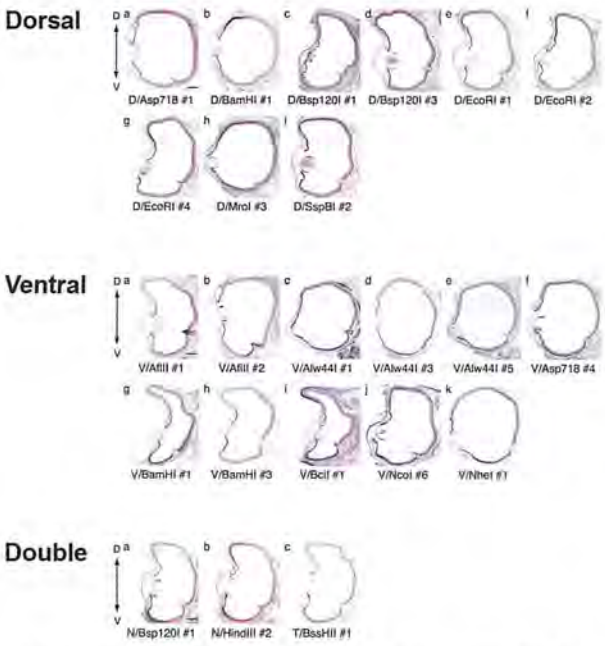


Fig.1. *In situ* hybridization analysis of the expression of identified genes in E8 retina. Dig-labeled cRNA probes were hybridized to horizontal sections (Nasal and Temporal) or coronal sections (Dorsal,Ventral and Double). Horizontal sections are oriented with nasal up and temporal down. Coronal sections are oriented with dorsal up and ventral down. The double gradient expression of some genes at E8 was verified in (Double). Scale bars: 500 μm.

II. Topographic retinotectal projection

In the chick embryos, the first retinal ganglion cells become postmitotic at day E2 and their axons leave the retina at E3. The earliest axons arrive at the most anterior part of the tectum at day E6 and advance over its surface in the posterior direction. These retinal axons form the stratum opticum (SO), which covers the entire tectum at E12. At the onset the chick retinotectal projection has a general topographic order, and axonal sprouting begin predominantly at the vicinity and towards the site of normal terminal zone. In all vertebrates studied, however, the developing axons make trial branches, many of which are not entirely within the retinotopic area, and elimination of ectopic branches and elaboration of appropriate branches is shaped in an activity dependent manner. We have already identified some molecules among the region-specific molecules in the retina that show abnormal targeting, branching or synaptic arborization when their expression was experimentally elevated or suppressed *in vivo*.

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

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

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

It is well known that 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. In 2002, we found that Ptprz expressed in the gastric epithelial cells functions as a receptor for VacA: Mice deficient in Ptprz do not show mucosal damage by VacA, indicating that erroneous Ptprz signaling induces gastric ulcers.

Although members of the protein tyrosine phosphatase (PTP) family are known to play critical roles in various cellular processes through the regulation of protein tyrosine phosphorylation in cooperation with protein tyrosine kinases (PTKs), the physiological functions of individual PTPs are poorly understood. This is due to a lack of information concerning the physiological substrates of the respective PTPs. Therefore, the development of a standard method applicable to all PTPs has long been awaited. We published a genetic method to screen for PTP substrates which we named the "yeast substrate-trapping system". This method is based on the yeast two-hybrid system with two essential modifications: the conditional expression of a PTK to tyrosine-phosphorylate the prey protein, and screening using a substrate-trap PTP mutant as bait (Fig. 2). This method is probably applicable to all the PTPs, because it is based on PTP-substrate interaction *in vivo*, namely the substrate recognition of individual PTPs. Moreover, this method has the advantage that continuously interacting molecules for a PTP are also identified, at the same time, under PTK-noninductive conditions. By using this method, we successfully identified several substrate molecules, together with many interacting molecules for Ptprz. The identification of physiological substrates will shed light on the physiological functions of individual PTPs.

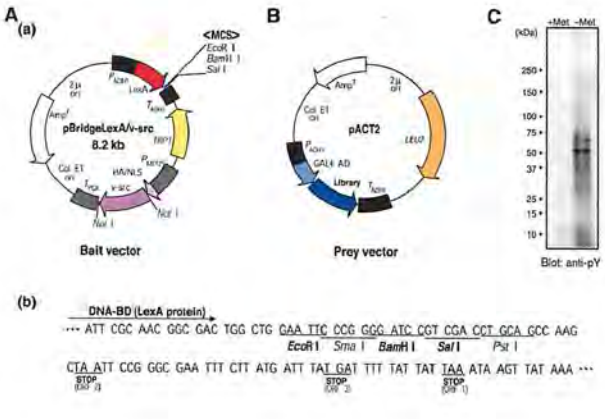


Fig. 2. Bait and prey vectors and tyrosine phosphorylation by the PTK induction in the yeast. (A,a) Structure of the pBridgeLexA/v-src vector. The PTP substrate-trap mutant is inserted into the multiple cloning site (MCS) in-frame with LexA. (A,b) DNA sequence of the MCS region in the vector. Unique restriction sites are shown in bold. The *Sma*I and *Pst*I sites are not shown in bold, because both sites exist in the v-src sequence. (B) Structure of the pACT2 vector (CLONTECH). (C) Cellular proteins in the yeast are highly tyrosine phosphorylated when v-src is induced in the absence of methionine (-Met), while almost no tyrosine phosphorylation was observed in the presence of methionine (+Met). L40 cells containing pBridgeLexA/v-src were cultured in the medium both with and without 1 mM methionine for 24 h, and the tyrosine phosphorylation of cellular proteins was analyzed by Western blotting with anti-phosphotyrosine 4G10 antibody.

IV. Na^+-level sensing in the brain

Dehydration causes an increase in the sodium (Na) concentration and osmolarity of body fluid in mammals. For Na homeostasis of the body, controls of Na and water intake and excretion are of prime importance. However, the system for sensing the Na level within the brain that is responsible for the control of Na- and water-intake behavior remains to be elucidated. We reported previously that the Na_x channel is preferentially expressed in the circumventricular organs (CVOs) in the brain and that *Na*$_x$ knock-out mice ingest saline in excess under dehydrated conditions. Subsequently, we demonstrated that Na_x is a Na-level-sensitive Na channel.

We revealed this year that the subfornical organ (SFO) is the principal site for the control of salt-intake behavior, where the Na_x channel is the Na-level sensor. Infusion of a hypertonic Na solution into the cerebral ventricle induced extensive water intake and aversion to saline in wild-type animals but not in the knock-out mice. Importantly, the aversion to salt was not induced by the infusion of a hyperosmotic mannitol solution with physiological Na concentration in either genotype of mice. When Na_x cDNA was introduced into the brain of the knock-out mice with an adenoviral expression vector, only animals that received a transduction of the *Na*$_x$ gene into the SFO among the CVOs recovered salt-avoiding behavior under dehydrated conditions. These results clearly show that the SFO is the center of the control of salt-intake behavior in the brain, where the Na-level-sensitive Na_x channel is involved in sensing the physiological increase in the Na level of body fluids.

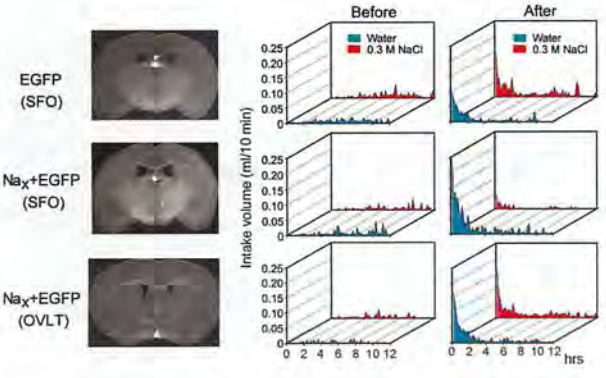


Fig. 3. Abnormal salt-intake behavior of *Na*$_x$ knock-out mice was rescued by introduction of the *Na*$_x$ gene to SFO. The coronal sections of the brain showing the loci infected by the expression of EGFP (left column). Time course of water and saline (0.3 M NaCl) intake by the infected mice before and after 48 hr dehydration (middle and right columns, respectively). Behavioral data are the average of six mice that were successfully infected in a specific site in the brain by an adenoviral vector encoding *egfp* (EGFP) or by vectors encoding *Na*$_x$ and *egfp* (Na_x and EGFP).

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DIVISION OF BRAIN BIOLOGY

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Our aim of research is to understand molecular 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. We have obtained genes that showed marked differences within primate neocortical areas. Our second approach is to understand informational processing in the brain underlying learning behaviors by examining gene expression.

I. Genes expressed in specific areas of the neocortex

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

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

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

i) One gene, designated *occ1*, is specifically expressed in the occipital cortex, particularly in V1 area, in the primate brain. 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. Interestingly, the specific expression of excitatory cells in the primary visual cortex was only observed in primates (Takahata et al., in the meeting of Soceity for Neuroscience in North America , SFN meeting, 2004).

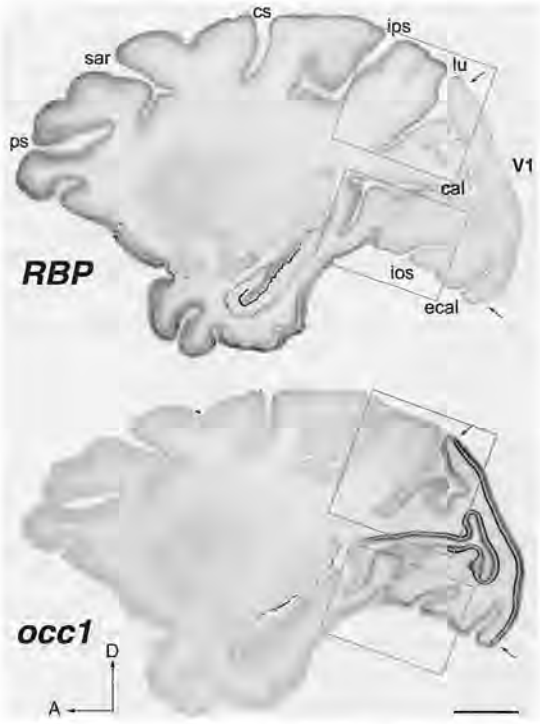

Fig. 1 Expression pattern of *occ1* and *Rbp* in the neocortex.

In situ hybridization pattern of *occ1* and *Rbp* in the primate neocortex. The two genes are expressed complimentary *occ1* is markedly expressed in the visual cortex. On the other hand *Rbp* is specifically expressed in association areas. ps: principal sulcus, sar: superior arcute sulcus, cs: central sulcus, ips: inferior parietal sulcus, lu: lunate sulcus, cal: calcarin sulcus, ios: inferior occipital sulcus, ecal: external cal (Komatsu et al., Cerbral Cortex, 15, 96-108, 2005).

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

iii) This year, we further report the third gene, *Rbp* (retinol-binding protein), which was preferentially expressed in association and higher areas in the neocortex (Komatsu et al., 2005). *Rbp* also shows characteristic features. i) Its expression is high in sensory association and higher association areas and limbic areas, but low in the primary sensory areas.

Expression is complementary to that of occ1 and to parvalbumin immunoreactivity (PV-IR) in primary sensory areas. ii) In early sensory pathways, the expression is limited to superficial layers only (in particular, layer II). With progression into higher sensory areas, the expression is expanded into layers 3 and then 5. iii) In higher-order association areas, *Rbp* is expressed throughout all layers except layer 4. iv) This characteristic distribution of *Rbp* is mainly formed during postnatal development. *Rbp* probably regulates the concentration of retinoic acid (RA) by the delivery of retinol, which is converted into RA in cells. Although the role of RA in the mature brain is not yet known, the characteristic expression of *Rbp* within association areas may provide a clue to the molecular basis of the formation and function of the association areas.

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 similar 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) *Rbp* is expressed in association areas 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. However, since many sensory stimuli per se cause c-Fos induction, 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 difference of 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 the difference. Therefore, we prepared ourselves for using two behavioral systems, which represent declarative and non-declarative memory.

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

Although this AVD task system is quite powerful to analyze the problem described above and useful for studying underlying molecular and cellular mechanisms because of advantages of using rodents, one concern was 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 (visual Vs auditory) difference.

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

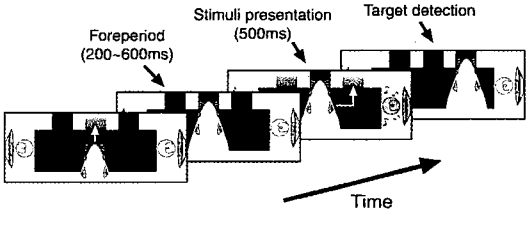

Fig.2 Newly developed AVD task.

A rat faces to a panel with a visual cue and/or an auditory cue located in the same position, being asked to poke its nose into one of the holes to obtain reward (a pellet) (The figure is cited from Sakata et al., Exp Brain Res, 159, 409-417, 2004).

Various oscillations are observed depending on brain-states. Spike wave complexes (Sws), 7-11Hz cortical oscillations with harmonics in awake but immobile rats, have been widely regarded as a model of paroxysmal activities in absence epilepsy. However, several studies have suggested that SWs in the primary somatosensory cortex are analogous to human mu rhythms. Because SWs have been frequently observed depending on vigilance levels, SWs in rats might represent normal brain-states related to the sleep-waking cycle. To elucidate behavioral contexts to induce SWs and temporal relations between SWs and neuronal ensemble activities, we recorded local field potentials (LFP) and multi-unit activities (MUAs) in the medial prefrontal cortex and electroencephalogram (EEG) in the bilateral regions of rats. Long-term recordings of EEG revealed that SWs were prominently generated in frontal and parietal regions and that SWs frequently followed non-REM sleeps. Occurrence probabilities of SWs significantly increased after the rats performed cognitive tasks. Our results suggest that SWs are one of the brain-state-specific oscillations rather than pathological activities. We also observed that MUAs were organized into phase-locked patterns in cycles of these oscillations. MUAs recorded from electrodes apart to each other were synchronized during SWs. (Sakata et al., SFN Meeting. 2004).

(2) 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., SFN 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 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, cerebral cortex. 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. Combining with c-Fos mapping technique and neuronal specific markers or DiI labeling, we revealed specific activation of bilaterally projecting cortico-striatal neurons and particular types of striatal neurons upon change of steps. These results suggesting cortico-striatal circuits actively participate in complex motor learning (Kitsukawa et al., SFN Meeting, 2004).

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In mammals, several social behaviors are dependent on sex. Cooperative regulation of many autosomal genes as well as those on the sex chromosomes constitutes their differences. We are currently investigating the epigenetic status of the critical brain subareas responsible for the sex difference of the behaviors by analyzing genomic DNA methylation patterns.

DNA methylation of nuclear receptors

In mammals, DNA methylation, mainly occurring on CG dinucleotides, is a fundamental mechanism that differentiates the gene expression pattern in respective cell. DNA methylation is a restraint of the pluripotency because once the patterns are established during development it is maintained through cell division. On the other hand, for example, some fishes that contain much less methylation activity are found to easily and reversibly change the sex status according to the environmental context. In rodents, endocrine disturbance at the fetal and/or postnatal stages irreversibly changes the behaviors such as the lordosis (in females) and the mounting (in males) after the pubertal stages that are normally dependent on genetic sex. In some cases, lordosis can be observed even in males and *vice versa*. These clearly indicate that sex-dependent patterns of behaviors are not directly dependent on "sex-specific" genes but rather established through epigenetic processes. The sex-dependent patterns of behaviors must be acquired through highly irreversible processes during development. We hypothesize that the long-term effects of the sex steroid at the perinatal stages on behaviors after puberty are somehow marked at the genome level. In this year of 2004, we report herein the DNA methylation pattern of steroid receptor genes (estrogen receptor α, ERα; androgen receptor, AR; progesterone receptor, PR) in the male and female brain subareas.

In general, the binding affinity of the transcription factors is reduced by hypermethylation that are frequently observed on promoter region of either ERα, AR or PR in tumor cells (Fig. 1). We analyzed the DNA methylation status of their 5'-flanking regions in the rat using the bisulfite sequencing method. In either of the genes, hypomethylated status was observed between –200 and +1 (in relation to the transcription start site) in preoptic area, bed nucleus of the stria terminalis (BNST), ventromedial hypothalamic nucleus (VMH), hippocampus, arcuate nucleus, and amygdala, suggesting that the core region of the promoters are maintained hypomethylated in normal tissues in contrast to the tumor cells. We also found that there exist the regions between –1,000 and –200, in which DNA methylation patterns differed depending on brain subarea. For ERα, no differences are found within male brain subareas examined. In contrast, hypermethylation was specifically observed in the BNST of female rats (Fig. 2). Similarly, we discovered the sex-dependent pattern of DNA methylation in the AR and PR, where hyper- and hypomethylation was found in VMH of female and male, respectively.

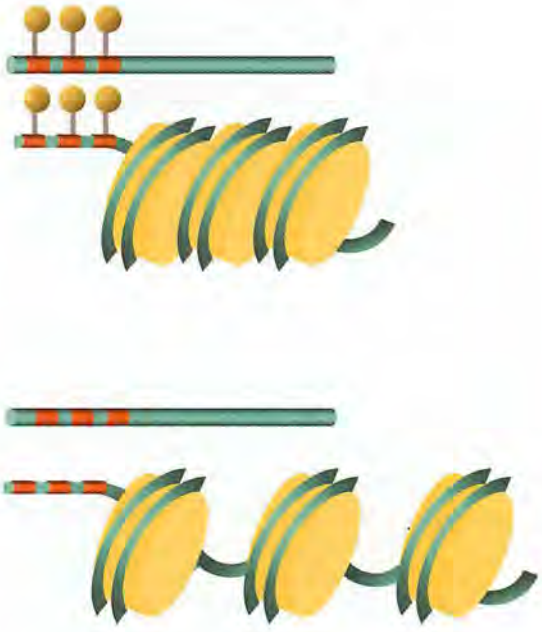

Fig. 1. Schematic representation of DNA methylation and chromatin structure. Upper panel shows the inactive state that reduces the transcriptional activity of the associated gene. Lower panel shows the DNA methylation-free state that allows DNA binding proteins, including transcription factors, accessible to the *cis*-regulatory element. Lollipops denote the methylated cytosines.

Several reports described the significance of gonad-derived factors, such as testosterone, at the perinatal stage on brain masculinization. To determine if the sex-dependent DNA methylation status of nuclear receptors is established by gonadal factors, we made use of adult male rats castrated at the onset of birth. DNA methylation pattern of AR in VMH was reversed to that observed in the females, indicating that the epigenetic status follows the genetic sex through endocrine pathway, at least partially. However, no significant differences were found in the DNA methylation status of ERα and PR in the examined brain subregions between normal and castrated males. Therefore, these suggest that prenatal factors also function to set up the masculized/defeminized pattern of DNA methylation in the brain.

In conclusion, we found the area- and sex-specific DNA methylation patterns of nuclear receptors. We are currently exploring the molecules that determine the DNA methylation pattern using the target *cis*-elements for differential methylation found in this study.

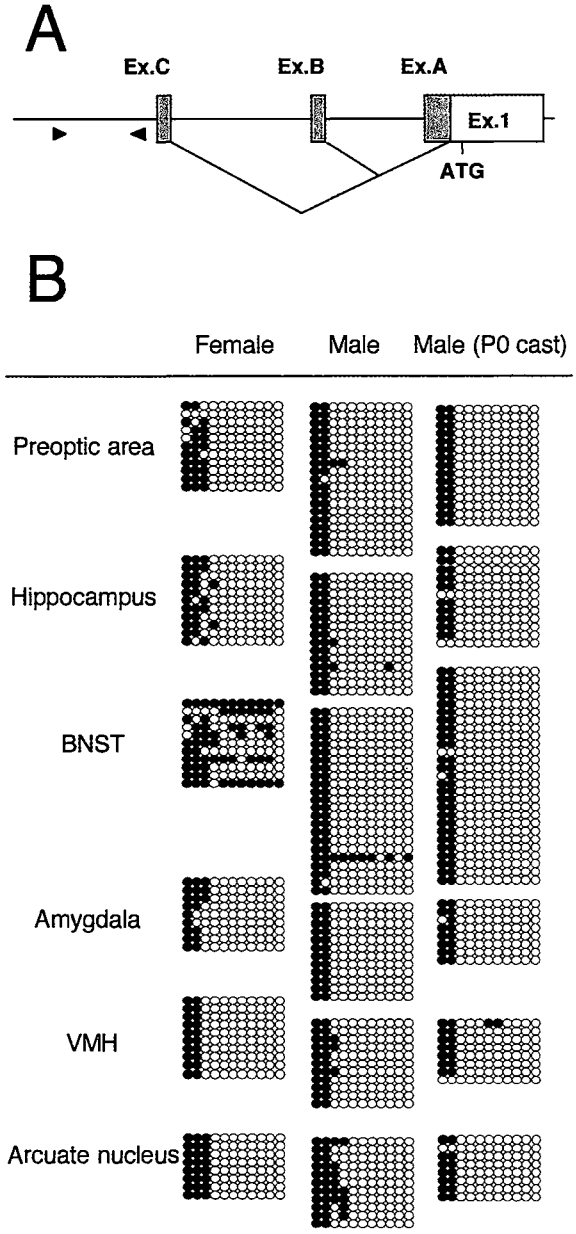

Fig. 2. DNA methylation analysis of rat estrogen receptor α
A. Genomic structure of rat estrogen receptor α. Ex. denotes exon. Arrowheads indicate the relative positions of the primers for the bisulfite sequence analysis. B. Bisulfite sequencing of estrogen receptor α in adult rats. Methylation patterns of CG dinucleotides are shown. Each row of circles represents a single cloned allele with open circles for non-methylated cytosines and filled circles for methylated cytosines. P0 cast, adult male castrated at postnatal day 0.

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Kanari, K., Kikusui, T., Takeuchi, Y., Mori, Y. (2005) Multidimensional structure of anxiety-related behavior in early-weaned rats. *Behavioral Brain Res.* **156,** 45-52.

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LABORATORY OF NEUROPHYSIOLOGY

Associate Professor: *WATANABE, Eiji*
JST Technical Staff: *TAKEUCHI, Kazumi*

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

To explain the properly regulated animal behaviors, neurobiologists have postulated the existence of both osmoreceptors and specific sodium receptors in the brain. However, the molecular entities of osmoreceptors and specific sodium receptors have long been enigma. In 2000, we first clarified by using gene-targeting technology that Na_x sodium channel is a probable candidate for the specific sodium receptor in the brain.

Na_x sodium channel had long been classified as a subfamily of voltage-gated sodium channels (NaChs) that serve to generate action potentials in electrically excitable cells such as neuronal and muscle cells. Comparing with the other NaChs, however, Na_x channel 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 Na_x channel, *Na$_x$*-gene deficient mice were generated by gene-targeting technique and the physiological phenotypes have been examined. Behavioral studies suggested that the Na_x channel plays an important role in the central sensing of body-fluid sodium level and regulation of salt intake behavior. Na_x-deficient mice ingest hypertonic sodium chloride solution in excess in comparison with wild type-mice. LacZ reporter gene knocked into *Na$_x$*-gene locus revealed that *Na$_x$* gene is expressed in the circumventricular organs, which is the specialized central organs involved in sensing of sodium concentration and osmosity in the body fluids.

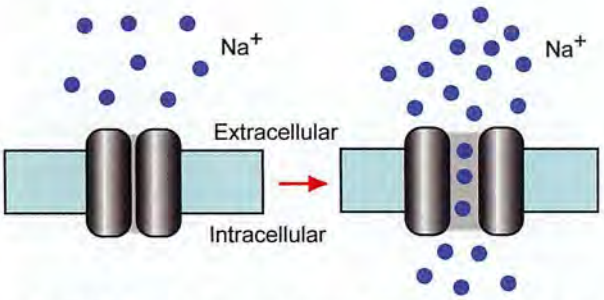

Figure 1. Na_x is a sodium channel sensitive to extracellular sodium level. When the extracellular sodium concentration is increased, Na_x channel opens the gate pore and generates the sodium ion influx into the cells. This view was hypothesized by ion-imaging studies.

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

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

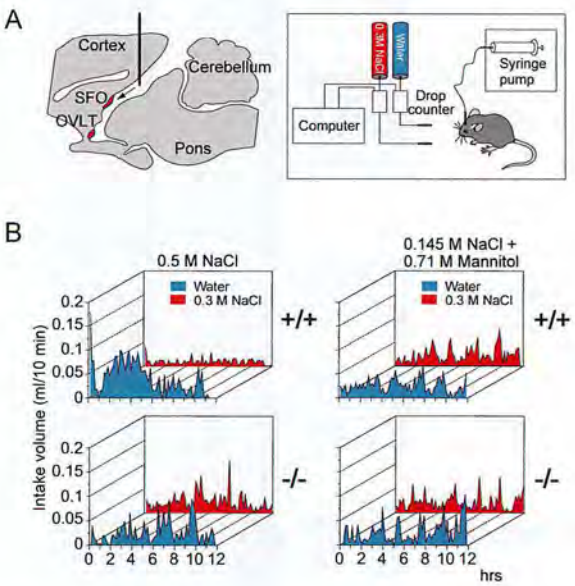

Figure 2. *Na$_x$*-knockout mice are insensitive to increases of the sodium level in the cerebrospinal fluid. (A) Left, Location of the cannula for intracerebroventricular infusions. The tip of the cannula was positioned at the lateral ventricle. Right, a schematic representation of the experimental set up for the two-bottle test. Two drinking tubes were presented to free-moving mice infused with sodium solutions into the ventricle for 12 hrs. (B) Averaged time course of water and saline (0.3 M NaCl) intake in wild-type (+/+) and knockout (-/-) mice during intracerebroventricular infusions of a hypertonic (0.5 M) NaCl solution, or hypertonic mannitol (0.145 M NaCl + 0.71 M mannitol) solution. Each point shows the average quantity per 10-min period.

In this year of 2004, we developed an automatic measurement equipment for intake volume of drinking solutions (Fig. 2A, right). Using this equipment, we showed that the subfornical organ is the principal site for the control of salt-intake behavior, where the Na_x channel is the sodium-level sensor. Infusion of a hypertonic sodium solution into the cerebral ventricle induced extensive water intake and aversion to saline in wild-type

animals but not in the knockout mice (Fig 2B, left). Importantly, the aversion to salt was not induced by the infusion of a hyperosmotic mannitol solution with physiological sodium concentration in either genotype of mice (Fig. 2B, right). When Na_x cDNA was introduced into the brain of the knockout mice with an adenoviral expression vector, only animals which received a transduction of the *Na_x* gene into the subfornical organ among the circumventricular organs recovered salt-avoiding behavior under dehydrated conditions. These results clearly show that the subfornical organ is the center of the control of salt-intake behavior in the brain, where the sodium-level-sensitive Na_x channel is involved in sensing the physiological increase in the sodium level of body fluids.

Currently, in order to understand how the subfornical organ translates extracellular sodium-level sensed by Na_x channel to the neural activities, we identified subcellular localization of Na_x channel in the subfornical organ. Immuno-electronmicroscopic and double immunostaining studies clearly showed that Na_x channel was exclusively localized to perineuronal lamellar processes extended from astrocytes and tanycytes in the organs (Fig. 3). Importantly, glial cells derived from the subfornical organ were capable of sensing extracellular sodium-level, as analysed by ion-imaging method. In addition, we found that the Na_x-expressing glial cells enveloped GABAergic interneurons in the subfornical organ. Finally, in the subfornical organ, neuronal population activated by water deprivation was different from GABAergic interneurons, as monitored by Fos immunoreactivity. Together with previous observation that the subfornical organ of *Na_x* knockout mice is hyperactive under water deprivation, these results indicate that the glial Na_x channel senses increased sodium-level in the body fluid and inhibit the subfornical organ neuronal activity through control of GABAergic inhibitory interneurons. A novel communication style utilized the sodium-level sensitive sodium channel appears to occur between inexcitable glial cells and excitable neural cell.

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

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

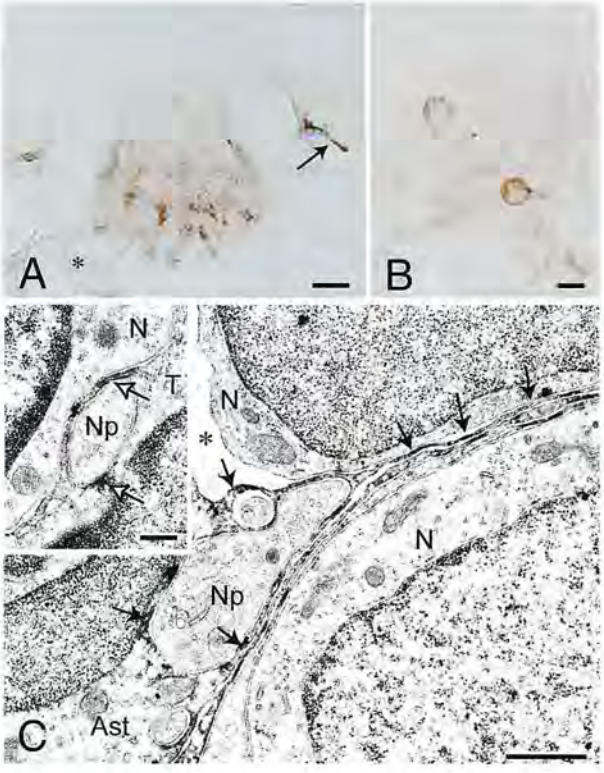

Figure 3. Na_x channel is localized to the perineuronal processes of astrocytes and tanycytes in the subfornical organ. (A and B) Coronal tissue sections of the subfornical organ are stained with anti-Na_x antibody. Immunopositive signals are widely observed within the subfornical organ (A) and most of intensive signals are accumulated in the shape of ring (B). An arrow indicates immunopositive tanycyte-layer peeled off from the subfornical organ and an asterisk indicates the choroid plexus. (C) Immuno-electron microscopic studies using anti-Na_x antibody. A core region of the subfornical organ is shown. Neurons and their processes are surrounded by immunopositive thin processes of astrocytes. In inset of C, ventricular surface of the subfornical organ is shown. A neuronal process is enveloped with immunopositive thin processes of a tanycyte. Arrows point at immunopositive signals. V, ventricle; N, neuron; S, synapse; T, tanycyte; Ast, astrocyte; Np, neural process; An asterisk in C is an artificial void region occurred during fixation or staining procedures. Scale bars, 50 μm for A, 10 μm for B, 1 μm for C and 0.4 μm for inset.

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LABORATORY OF NEUROCHEMISTRY

Associate Professor: *SASAOKA, Toshikuni*

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

I. Role of dopaminergic transmission in eating behavior

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

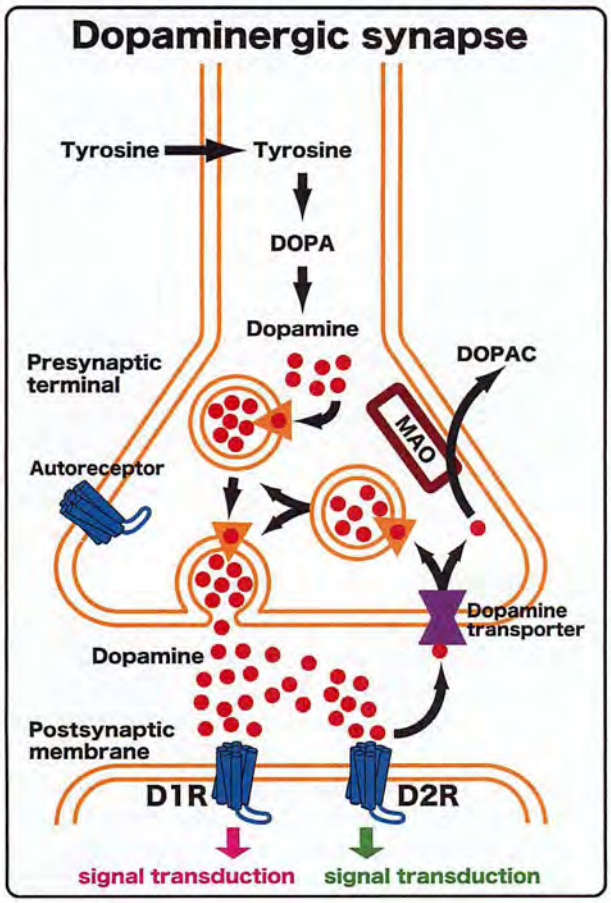

Figure 1. Schematic drawing of dopaminergic synapse.

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

In order to understand a role of dopaminergic transmission in behavior of interest, it is necessary to delete both D1-like and D2-like receptors. We have generated D1R/D2R double knockout (DKO) mice by crossing between D1R knockout (KO) and D2R KO mice, and observed that D1R/D2R DKO mice exhibited impairment in eating behavior and premature death. To investigate molecular mechanism of eating we have generated mutant mice in which the expression of dopamine receptor could be controlled temporally by tetracycline system on the D1R/D2R DKO background. We plan to establish and analyze the mutant mice.

II. Developing a novel conditional mutagenesis method in mice.

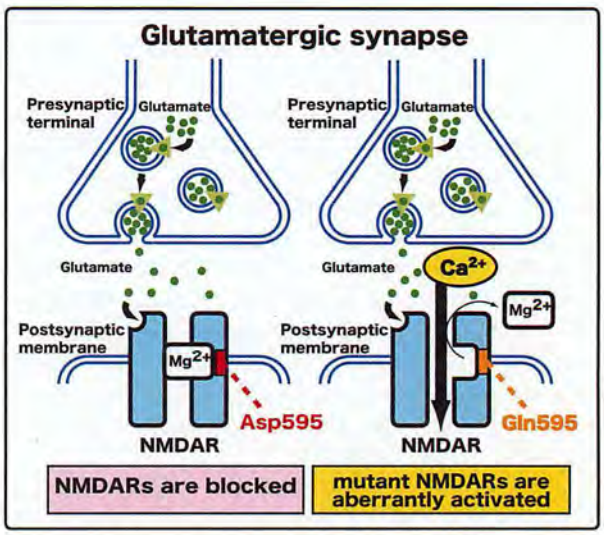

Figure 2. A single amino acid substitution (595th asparagine to 595th glutamine) of NR2A subunit leads to an aberrant activation of NMDAR.

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. The NMDARs are widely expressed in the nervous system, fundamental to excitatory neurotransmission, and play a number of important roles at different brain loci and time points. The NMDARs act as a coincidence detector and are not only important for neuronal differentiation, migration, and survival but are also critical for activity dependent synapse formation. It is suggested that the aberrant activation of NMDAR causes excitotoxicity, leading to neuronal death in various neurological diseases.

That the Ca^{2+} permeability through NMDAR is blocked by magnesium (Mg^{2+}) in a voltage-dependent manner indicates an essential role of NMDAR as a coincidence detector. Functional NMDARs consist of NMDAR1 (NR1) subunit and at least one subunit of NMDAR2A-2D (NR2A-NR2D). The NR1 subunit is ubiquitously expressed in the brain, while NR2 subunits have a more specific spatial distribution. It has been shown that the NR1/NR2A complex expressed in cultured cell is highly sensitive to the voltage-dependent Mg^{2+} block and that the substitution of asparagine (Asp595) by glutamine (Gln595) in the second transmembrane domain of the NR2A subunit results in a reduction of the Mg^{2+} block of the NR1/NR2A complex (Figure 2).

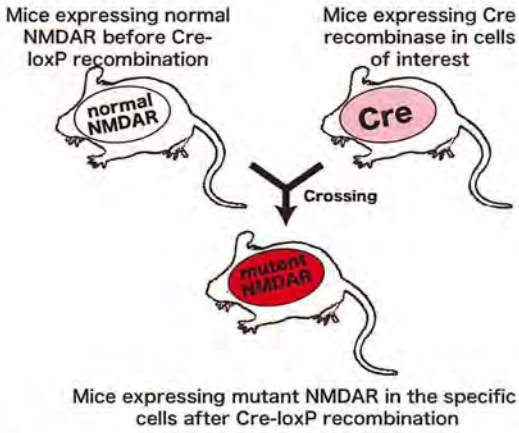

Figure 3. Conditional mutagenesis in mice. First, mutant mice expressing normal NMDAR molecule before Cre-loxP recombination were generated. Second, transgenic mice expressing Cre recombinase in cells of interest were generated. Third, these two mouse lines were crossed to generated mice expressing mutant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

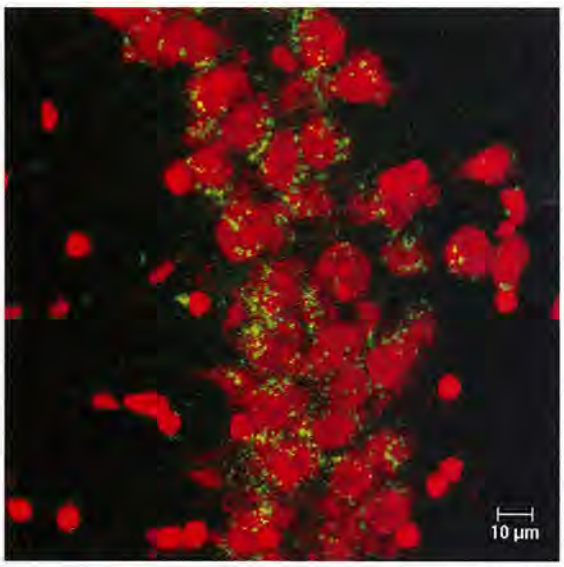

Figure 4. Detection of cells in which recombination was executed by Cre-loxP system by expression of green fluorescent protein (green) in the hippocampus. Neurons were stained by NeuroTrace (red).

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

We develop conditional mutagenesis method in mice using Cre-loxP recombination (Figure 3 and 4). By our method, we accomplished conditional substitution of the amino acid in mice and our mutant mice exhibited aberrant NMDAR activation and a neurological phenotype, similar to that of mouse models of neurological disorders. The development of our mutant mice should contribute to understanding the function of the critical amino acid residue and the molecular mechanism of neurological disorders.

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

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

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

To understand a physiological role of the SGC, we have generated the β-SG KO and γ-SG KO mice. The β-SG KO and γ-SG KO mice developed progressive muscular dystrophy and all SGs and sarcospan were absent in the sarcolemma of the β-SG KO and γ-SG KO mice. The dystrophin complex isolated from the skeletal muscles of β-SG KO mice was biochemically 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.

We plan to analyze a molecular architecture and a physiological role of dopamine receptor complex as a whole by applying analytical method of the SGC.

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Mizuno, Y., Guyon, J. R., Watkins, S. C., Mizushima, K., Sasaoka, T., Imamura, M., Kunkel, L. M., Okamoto, K. (2004) Beta-Synemin localizes to regions of high stress in human skeletal myofibers. *Muscle and Nerve* **30**, 337-346

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

I. Spontaneous mutants in morning glories.

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

I. nil had been introduced into Japan from China approximately in the 8th century as a medicinal herb, the seeds of which were used as a laxative, and the plant became a traditional floricultural plant in Japan around the 17th century. The plant has an extensive history of genetic studies, and a number of its spontaneous mutants related to the color and shape of the flowers have been isolated. Genetic studies on the color of *I. nil* have shown that blue flower coloration was mainly controlled by two genetic loci, *Magenta* and *Purple*. Recessive *magenta* and *purple* mutants bloom magenta and purple flowers, respectively, and double mutants carrying both *magenta* and *purple* alleles display red flowers (Figure 1C). The *Magenta* gene encodes flavonoid 3'-hydorxylase, which hydroxylates the 3' position of the B-ring of anthocyanidin precursors. The *Purple* gene encodes a vacuolar Na^+/H^+ antiporter called InNHX1 that increases the vacuolar pH during flower opening, causing a shift towards the bluer coloration. Among the various colors of *I. nil* flowers, the most favorite hue for Japanese floriculturists has been reddish-brown or purplish-grey petals (Figure 1B and D) since the early 19th century, and the flower coloration is mainly caused by recessive *dusky* mutations. We noticed that the petals in all *dusky* mutants often contained intensely pigmented globules, which appeared to affect flower hue. We found that the *Dusky* gene encodes UDP-glucose:anthocyanidin 3-*O*-glucoside-2"-*O*-glucosyltransferase (3GGT), which catalyzes the the conversion of anthocyanidin 3-*O*-glucosides into anthocyanidin 3-*O*-sophorosides (Figure 1E) and that all of the *dusky* mutants tested carry the 4-bp insertion mutations GGAT or CGAT at an identical position near the 3' end of the gene, which resulted in frameshift mutations (Figure 1F). The expected 3GGT enzymatic activities were found in the crude extracts of *Escherichia coli,* in which the *3GGT* cDNA was expressed, and the introduced *3GGT* cDNA could efficiently produce 3GGT that could convert cyanidin 3-*O*-glucoside into cyanidin 3-*O*-sophoroside in transgenic petunia plants.

In the *purple* mutant deficient in the *InNHX1* gene for the vacuolar Na^+/H^+ antiporter, the vacuolar alkalization occurs only partially, and reddish-purple buds become purple open-flowers. While most of the plant *NHX* genes characterized are generally expressed in leaves, stems and roots and induced by NaCl treatment, the *InNHX1* gene is predominantly expressed in the flower limbs at around 12 hour before flower-opening. It is expressed very scarcely in leaves, stems and roots, and no induction occurs in response to NaCl treatment. We identified a novel vacuolar Na^+/H^+ antiporter gene *InNHX2*, which is expressed in leaves, stems and roots and is induced in response to NaCl treatment. In addition, relatively higher expression of *InNHX2* was observed in the flower limbs shortly before flower-opening. We also discovered that both the InNHX1 and InNHX2 proteins could catalyze both Na^+ and K^+ transport into vacuoles. These results suggest that InNHX2 performs dual functions: to confer salt tolerance on the plant and to promote partial vacuolar alkalization in the petals.

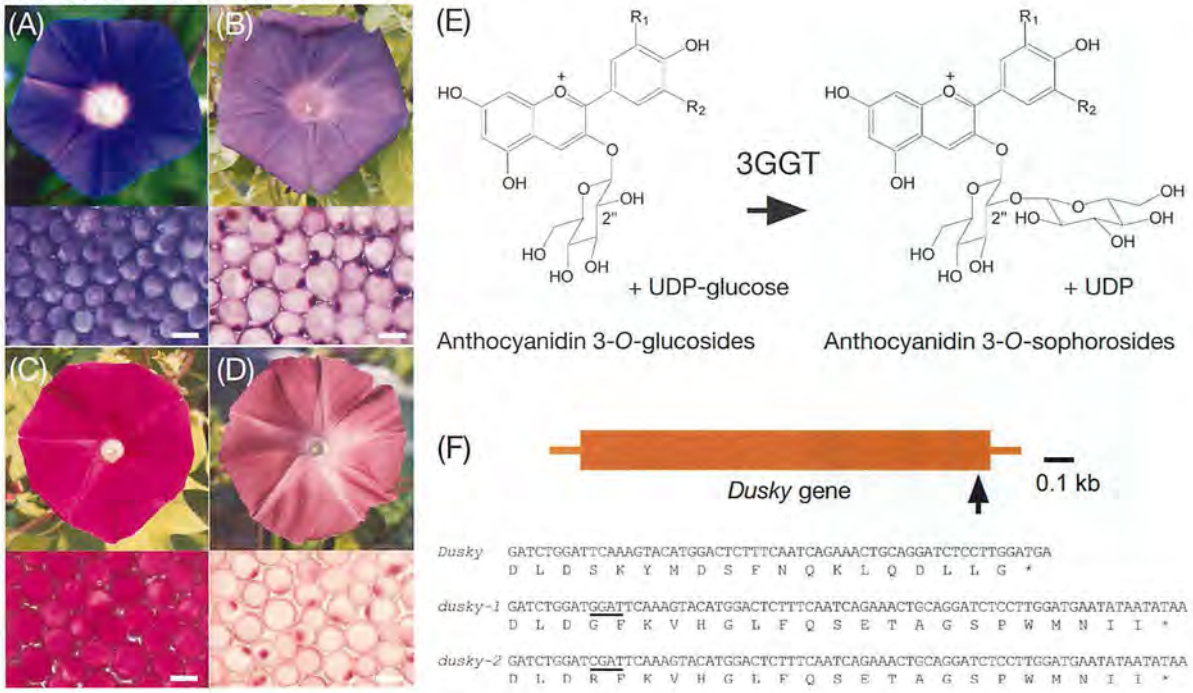

Fig.1. Flower phenotypes and the *Dusky* gene for the 3GGT enzyme in *I. nil*. **A-D.** Flower phenotypes (above) and microscopic photographs of adaxial epidermal cells of flower petals (below). (**A**) The wild-type line with the *Magenta*, *Purple*, and *Dusky* alleles. (**B**) The line with the *Magenta,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

II. Modification of endogenous natural genes 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 the *Adh1* and *Adh2* genes, which belong to a small multigene family and reside adjacent to repetitive *Copia*- and *Gypsy*-like retroelements (Figure 2). The *Adh1* and *Adh2* genes reside on chromosome 11 in the same orientation with the 30 kb of interval, and the coding sequences of *Adh1*and *Adh2* are similar to each other. The results obtained indicate that the gene targeting by homologous recombination occurred more efficiently in *Adh2* than that in *Adh1*.

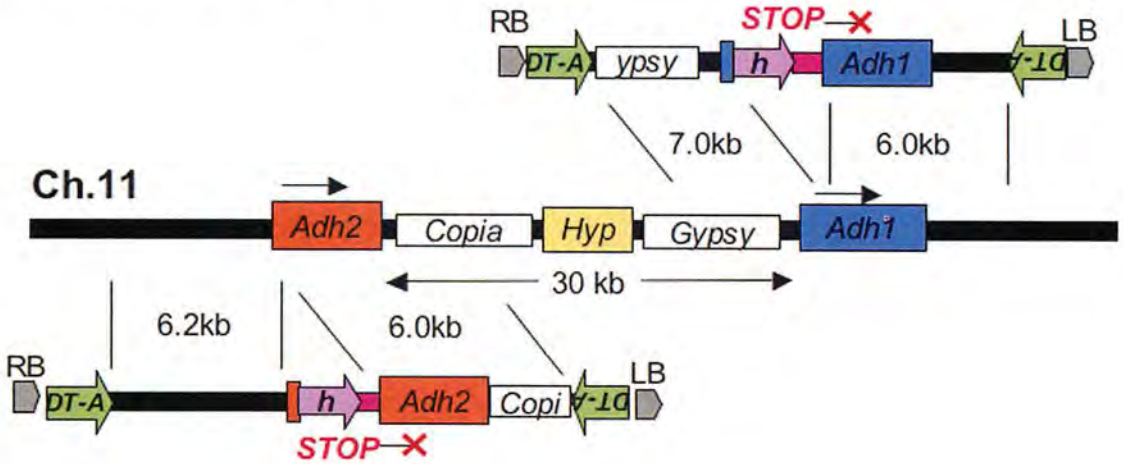

Fig.2. Strategy for gene targeting of the *Adh1* and *Adh2* genes in rice. The symbols *h* and *DT-A* on the T-DNA regions of the vectors used indicate the positive and negative selection markers, respectively. The hypothetical gene flanked by the retroelements on chromosome 11 is indicated by *Hyp*.

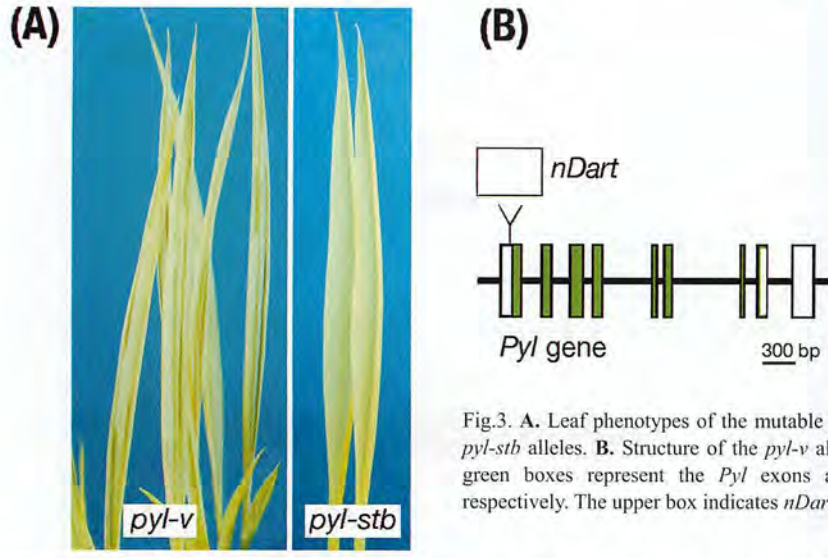

Fig.3. **A.** Leaf phenotypes of the mutable *pyl-v* and the stable *pyl-stb* alleles. **B.** Structure of the *pyl-v* allele. The white and green boxes represent the *Pyl* exons and coding region, respectively. The upper box indicates *nDart1*.

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

Leaves of seedlings in the *virescent* mutant of rice are initially pale-yellow green due to partial deficient in chlorophyll and gradually become green with the growth of the mutant. We have been characterizing a spontaneous mutable *virescent* allele, *pale-yellow leaf-variegated (pyl-v)*, conferring pale yellow leaves with dark green sectors in its seedlings (Figure 3A). The *pyl-v* mutant was isolated among progeny of a hybrid between *indica* and *japonica* rice plants. The leaf variegation is regarded as a recurrent somatic mutation from the recessive pale-yellow allele to the dark green revertant allele. From the *pyl-v* line, we also obtained a stable *pyl-stb* (*pyl-stable*) line that exhibits pale-yellow leaves without variegation (Figure 3A), which appeared to carry no active autonomous element acting on the nonautonomous DNA element inserted into the *Pyl* gene. The availability of the genomic sequences of both *japonica* and *indica* subspecies facilitates map-based cloning of the *pyl-v* allele. We identified an active nonautonomous DNA transposon of about 0.6 kb, named *nDart1* (*nonautonomous DNA-based active rice transposon one*), in the untranslated exon 1 of the *Pyl* gene on chromosome 3 (Figure 3B), and excision of the new DNA transposon from the *pyl* gene appears to be responsible for conferring the leaf variegation. We also showed that the transposition of *nDart1* could be controlled under natural growth conditions. 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. In this respect, it is important to emphasize here that tissue culture is necessary in all of the currently available rice reverse genetic approaches including transposon tagging systems employing exogenous or endogenous transposons. We are currently attempting to develop a novel transposon tagging system in rice.

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DIVISION OF GENOME DYNAMICS

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The genomes of higher organisms contain significant amounts of repetitive sequences which, in general, are unstable. At present, neither the physiological function(s) of repeated sequences nor the mechanism controlling instability are 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 the rRNA repeated genes, (2) the mechanism of replication fork block-dependent recombination, a key reaction that increases or decreases the copy number of rRNA genes, and (3) development of *in vivo* artificial gene amplification systems. In 2004, work on the following four subjects has advanced our knowledge of the dynamics of the genome.

I. Transcription-mediated hyper-recombination in *HOT1*, a recombinational hotspot in *S. cerevisiae*.

Recombination hotspots are DNA sequences which enhance recombination around that region. *HOT1* is one of the best-studied mitotic hotspots in yeast. *HOT1* consists of two elements; I and E. They are two dis-continuous regions in an rDNA unit. The I-element corresponds to a RNA polymerase I (PolI) transcription promoter which is responsible for 35S ribosomal rRNA gene (rDNA) transcription. The E-element overlaps the enhancer for PolI transcription, containing a replication fork barrier site (FRB) where Fob1 protein, required for fork blocking at RFB, binds specifically. *HOT1* stimulates recombination when inserted at novel locations in the genome. For example, when HOT1 is integrated into one of the repeated *his4* genes, the fragment enhances recombination between the repeats ~ 100 times. In a PolI defective mutant the *HOT1* hotspot activity is abolished, therefore transcription of *HOT1* is thought to be an important factor for the recombination stimulation. However, it is not clear whether the transcription itself or other pleiotropic phenotypes stimulate recombination. To investigate the role of transcription, we made a highly activated Pol I transcription system in *HOT1* by using a strain whose rDNA repeats are totally deleted (rdnΔΔ). In the rdnΔΔ strain, *HOT1* transcription was increased about 14 times compared to wild-type. Recombination activity stimulated by *HOT1* in this strain was also elevated, about 15 times, compared to wild-type. These results indicate that the level of Pol I transcription in *HOT1* determines efficiency of the recombination. Moreover, Fob1p, which is essential for both the recombination stimulation activity and transcription of *HOT1*, was dispensable in the rdnΔΔ strains. This suggests that Fob1p is functioning as a PolI transcriptional activator in the wild-type strain.

II. *SIR2* regulates recombination between different rDNA repeats, but not recombination within individual rRNA genes in yeast.

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

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

We examined the mechanism involved in the increased frequency of recombination in rDNA repeats that is observed in mutants defective in *SIR2* functions. We measured the frequency of *FOB1*-dependent arrest of replication forks, consequent DNA double-strand breaks, and formation of DNA molecules with Holliday junction structures, and found no significant difference between *sir2Δ* and *SIR2* strains. Formal genetic experiments measuring mitotic recombination rates within individual rRNA genes also showed no significant difference between these two strains. Instead, we found a significant decrease in the association of the cohesin subunit Mcd1p (Scc1p) to the rDNA in *sir2Δ* relative to *SIR2* strains. From these and other experiments, we conclude that *SIR2* prevents unequal sister-chromatid recombination,

probably by forming special cohesin structures, without significant effects on recombinational events within individual rRNA genes.

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

Gene amplification is involved in various biological phenomena such as cancer development and drug resistance. However, the mechanism is largely unknown because of the complexity of the amplification process. We developed a gene amplification system in *Saccharomyces cerevisiae* that is based on double rolling-circle replication (DRCR), utilizing break-induced replication (BIR), and this is depicted in the Figure below. This system produced three types of amplification products. Type-1 products contain 5−7 inverted copies of the amplification marker, *leu2d*. Type-2 products contain

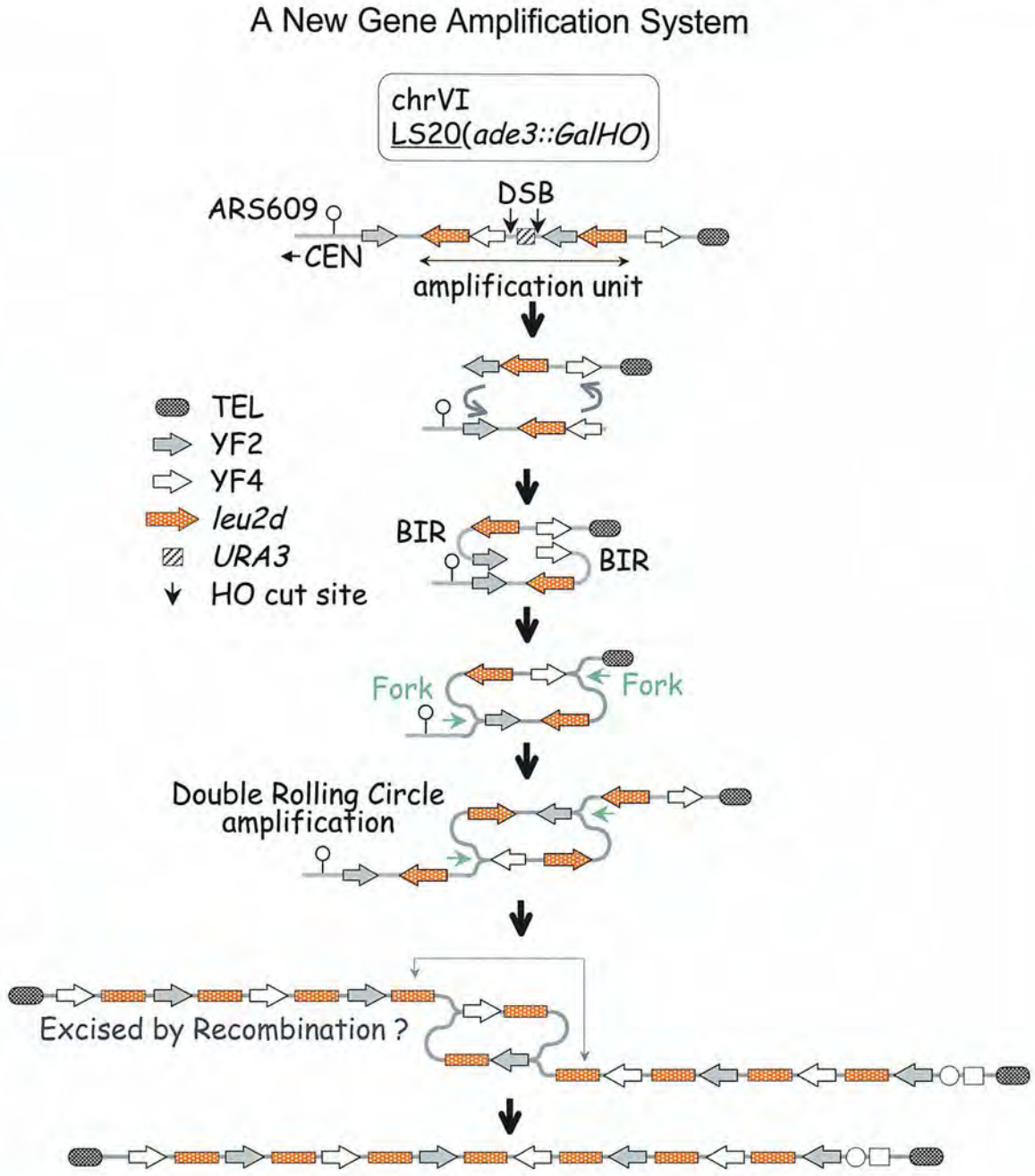

Figure: A new gene amplification system in which amplification through double rolling circle replication (DRCR) is triggered by break-induced replication (BIR). Top shows the structure of the right terminus region of chromosome VI where an amplification unit (-*leu2d*-YF4-HO-*URA3*-HO-YF2- *leu2d*-) was inserted. Induction of double strand breaks (DSBs: shown by the two arrows) by HO endonuclease triggers BIR, which initiates DRCR. An amplification selective marker, *leu2d*, is used to select amplified clones. The DRCR process is expected to terminate by recombination between *lue2d* genes on each bidirectionally elongated arm. ARS: autonomously replicating sequence; CEN: centromere.

13 to ~100 copies of *leu2d* (up to ~730 kb increase) with novel arrangement present as randomly oriented sequences flanked by inverted *leu2d* copies. Type-3 products are acentric multi-copy mini-chromosomes carrying *leu2d*. Structures of type-2 and -3 products resemble those of homogeneously staining region (HSR) and double minutes (DMs) of higher eukaryotes, respectively. Interestingly, products analogous to these were generated at low frequency without deliberate DNA cleavage. These features strongly suggest that the processes described here may contribute to natural gene amplification in higher eukaryotes.

VI. Recombination enhanced by replication fork blockage at the *Ter* site on *E. coli* plasmids.

In order to elucidate the effects of replication arrest, which is of frequent and spontaneous occurrence, replication blocking sites have been frequently used. In *E. coli*, when both bi-directional replication forks are blocked at the two flanking replication blocking (*Ter*) sites, the next round of replication forks arrive at the *Ter* sites, producing a giant linear DNA molecule, whose terminus ends lead to recombination and overcome the blockage at the *Ter* sites through an unknown mechanism. On the other hand, eukaryotic chromosomes, and probably bacterial plasmids also, do not initiate such a second round of replication, at least under normal growth conditions. When plasmid replication is arrested at the fork blocking *Ter* site, it remains unknown whether recombination is enhanced or not. Therefore, we investigated whether recombination is stimulated when plasmid replication is inhibited by a 22 bp *TerA* site and, if the recombination is activated, what kinds of recombinational genes are required for the activation by using various rec-defective mutants as hosts. Furthermore, the recombinational genes involved in the SOS response, which is induced by plasmid replication blockage at the 22 bp *Ter* site, were also examined. The results were (1) the recombination is enhanced 5 – 7 fold by the fork block, (2) this enhancement is dependent on *recA, recF recO, recR* and *recJ*, but not *recBC* and *recQ*, (3) SOS induction is dependent on *recF* absolutely, and *recJ* and *recQ* partially, (4) the recombinational enhancement is disappeared when the 22 bp *TerA* sequence was replaced by the 0.6 kb original *TerA*-containing *E. coli* chromosomal fragment, which is consistent with the results of SOS induction. The last result indicates that in plasmids multiple rounds of replication do not occur, and furthermore fork blocking at the *Ter* site induces not only recombination but also the SOS response. This correlation suggests that there may be a common process between the initiation steps of the recombination and the SOS induction. A mechanism by which recombination and the SOS response were induced by replication blockage at the 22 bp *TerA* site, but not the 0.6 kb *TerA* containing *E. coli* chromosomal fragment, was proposed.

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DIVISION OF EVOLUTIONARY BIOLOGY

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All living organisms evolved from a common ancestor that lived more than 3.5 billion years ago, and the accumulation of mutations in their genomes has resulted in the present biodiversity. Traces of the evolutionary process are found in the genomes of extant organisms. By comparing the gene sequences and gene networks of different organisms, we can infer (1) the phylogenetic relationships of extant organisms and (2) the genetic changes that caused the evolution of morphology and development. The inferred phylogenetic relationships provide important insights into problems in various fields of evolutionary biology. Our group focuses on biogeography, the evolution of morphological traits, and systematics in a wide range of taxa. Concerning the evolution of morphology and development, we hope to explore the genetic changes that led to the evolution of the plant body plan. We have selected 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 (1) cytosolic calcium icon concentration and cytoskeleton organization during chloroplast movement in the moss *Physcomitrella patens* and (2) the functional divergence of photoreceptors and motor proteins involved in chloroplast movement in the moss from angiosperms are in progress by a team directed by Y. Sato.

II. Evolution from unicellular to multicellular organisms

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

III. Evolution from cells to tissues

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

Organization of microtubules

Cortical arrays of microtubules are essential for morphogenesis in plants. We found that, by live imaging, microtubules in the arrays are formed as branches on pre-existing microtubules. γ-tubulin, a protein that is essential for the formation of microtubules in animal cells, is located at the branching point and in the cytoplasm, 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 in cytoplasm 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 in progress by a team directed by T. Murata.

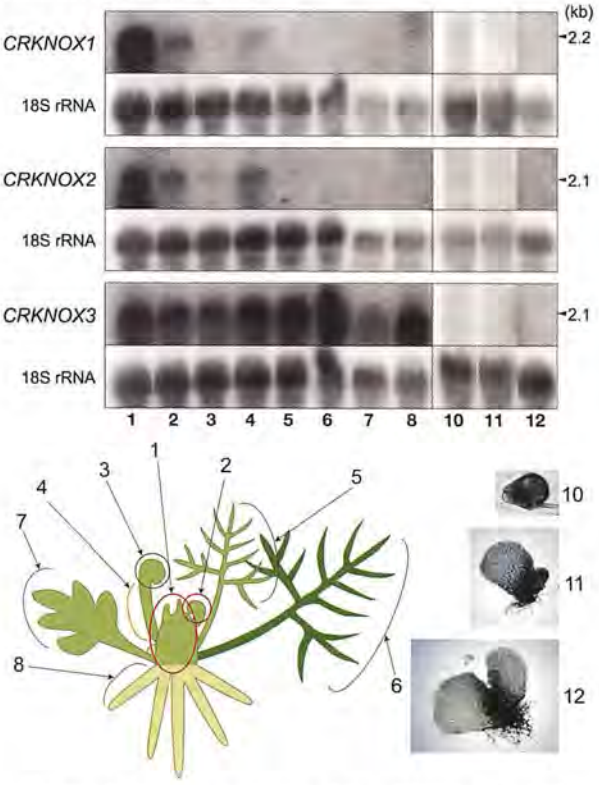
IV. Evolution of molecular mechanisms in the development of vegetative organs

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. Three lines, exhibiting reporter gene (*uid*A) expression preferentially in the apical cells, were isolated from previously established gene- and enhancer-trap lines, and identified as encoding kinesin- and ubiquitin-like proteins, and an unknown protein. Functional analyses of these genes are currently under investigation by a team directed by Y. Hiwatashi. The disruption of the kinesin-like gene did not show any phenotypic differences from the wild type. This is likely caused by the functional redundancy of closely related genes, and the analyses of double disruptions are in progress. Disruption of the gene encoding ubiquitin-like protein suggests that the gene is involved in cell division and elongation through microtubule organization with the proteasome complex.

Evolution of shoot meristem

The angiosperm shoot apical meristem is a dome of small, proliferating cells whose organization is highly structured into layers and/or zones. The apical meristem of many seed-less vascular plants contains a single apical cell or initial that is apparent in both the gametophyte (haploid) and sporophyte (diploid) generations. Given the importance of the apical meristem in elaborating the three dimensional body plan of plants, the apical meristem is thought to be among the most important innovations for the evolution of land plants. In order to gain insights into the molecular mechanisms underlying the development and evolution of the plant meristem, we have identified and analyzed the expression of two class 1 and one class 2 KNOX (knotted-like homeobox) genes from the fern *Ceratopteris richardii*. Expression of both class 1 genes was detected in the shoot apical cell, leaf primordia, marginal part of the leaves, and vascular bundles by *in situ* hybridization, a pattern that closely resembles that of class 1 KNOX genes in angiosperms with compound leaves. The fern class 2 gene was expressed in all sporophytic tissues examined, which is characteristic of class 2 gene expression in angiosperms. Unexpectedly, all three *CRKNOX* genes were not detected in gametophyte tissues by RNA gel blot analysis. Arabidopsis plants overexpressing the fern class 1 genes resembled plants that overexpress seed plant class 1 KNOX genes in leaf morphology. Ectopic expression of the class 2 gene in Arabidopsis did not result in any unusual phenotypes. Taken together with phylogenetic analysis, our results suggest that 1) the class 1 and 2 KNOX genes diverged prior to the divergence of fern and seed plant lineages; 2) the class 1 KNOX genes function similarly in seed plant and fern sporophyte meristem development despite their differences in structure, 3) KNOX gene expression is not required for the development of the fern gametophyte, and 4) the sporophyte and gametophytic meristems of ferns are not regulated by the same developmental mechanisms at the molecular level (Sano et al. in press). To further investigate the evolution of meristem and KNOX genes, characterization of the moss Physcomitrella KNOX genes are in progress by a team directed by T. Nishiyama.



(Figure 1) Expression patterns of three KNOX genes in the fern *Ceratopteris richardii*. The tissues from which RNAs were extracted are shown in the schematic figures.

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 these genes in several basal angiosperms, the moss Physcomitrella, and three green algae (*Chara*, *Coleochaete*, and *Closterium*) are being analyzed.

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

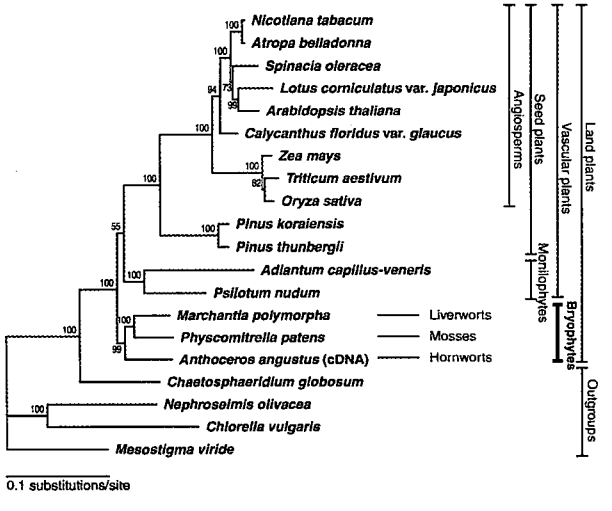
VI. Molecular mechanisms of speciation

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

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

VII. Phylogenetic analysis of land plants

Opinions on the basal relationship of land plants vary considerably and no phylogenetic tree with significant statistical support has been obtained. We performed phylogenetic analyses using 51 genes from the entire chloroplast genome sequences of 20 representative green plant species. The analyses, using translated amino acid sequences, indicated that extant bryophytes (mosses, liverworts, and hornworts) form a monophyletic group with high statistical confidence, and that extant bryophytes are likely sister to extant vascular plants, although the support for monophyletic vascular plants was not strong. Analyses at the nucleotide level could not resolve the basal relationship with statistical confidence. Bryophyte monophyly inferred using amino acid sequences has a good statistical foundation and is not rejected statistically by other datasets. We proposed bryophyte monophyly as the current best hypothesis (Nishiyama et al. 2004).



(Figure 2) Phylogenetic relationship of representative lineages in land plants inferred using 8,979 amino acid residues for 51 genes from 20 chloroplast genomes.

VIII. Evolution of RNA editing in land plants

We sequenced transcripts from all putative genes for proteins, rRNAs, and a selection of those encoding tRNAs in the chloroplast genome of the fern *Adiantum capillus-veneris*. We detected 349 RNA editing sites when the cDNA sequence was compared to that of the genomic DNA. The level of RNA editing in this fern is more than ten times that of any other vascular plant examined across an entire chloroplast genome. A previous study found even higher levels of editing in a hornwort (942 sites). This suggests that the relatively low levels of editing in seed plants (less than 0.05%) may not be typical for land plants, and that RNA editing may play a major role in chloroplast genome processing. Additionally, we found that 53 editing sites in *Adiantum* are homologous to editing sites in the hornwort, and some other land plants. This implies that a major component of RNA editing sites have been conserved for hundreds of millions of years.

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DIVISION OF SPECIATION MECHANISM (ADJUNCT)

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Research Associate: *TAKAHASHI, Kazuhiko*
Postdoctoral Fellow: *SASAKI, Takeshi*
Technical Assistant: *MIURA, Seiko*

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

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 could be sometimes very slow, whereas other examples suggest, by contrast, that their speciation and diversification could be often accomplished very rapidly by the process called adaptive radiation. What factors affected rates of speciation and phenotypic change during the evolution of vertebrates? Although many examples of adaptive radiation (and slow evolution as well) have been reported so far for various groups of vertebrate, its mechanism has been hardly clarified so far, especially from the molecular perspective. The final goal of the research of our division is to understand the processes and mechanisms of speciation of vertebrates using molecular approaches. In particular, we are dedicated to elucidate the mechanism of speciation of cichlids in Lake Victoria (Figure 1), based on our knowledge on the phylogenetic relationships among African cichlids as clarified by our retroposon analysis.

Figure 1. A species of cichlid fish (male *Pundamilia nyererei*) caught in Lake Victoria.

I. Inference of molecular phylogeny of vertebrate groups using the SINE method

Cichlid flocks of the East African Great Lakes, which consist of Lakes Victoria, Malawi, and Tanganyika, have attracted interest of evolutionary biologists for more than a century. These species exhibit extraordinary levels of diversity and high species endemicity to each lake as a result of explosive adaptive radiation that occurred independently and in parallel.

Our group has been conducting phylogenetic analysis of these fish using insertions of retroposons (SINEs; short interspersed elements) as markers for elucidation of their evolutionary history. Our studies confirmed that cichlids in Lakes Victoria and Malawi are closely related to each other, and that species in the both lakes are related to only a portion of the major lineages found in Lake Tanganyika, the oldest Great Lake estimated at 9-12MY. Interestingly, evidence from the above analyses suggested that explosive radiation is indeed in progress in the Victorian fauna at present (see Section II for the detail)

Another group of vertebrate that we analyzed using SINEs was the order Testudines (turtles), which constitutes one of the major reptile groups. Our analysis, focusing especially on the superfamily Testudinoidea, revealed several novel phylogenetic relationships among members of this group. Our result indicated that convergence of the major morphological traits that had not been successfully elucidated by the past morphological analyses occurred during their evolution. This study was the first application of the SINE method to a phylogenetic analysis of reptiles.

II. Analysis of Cichlids in Lake Victoria for elucidation of molecular mechanisms of their 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 an evolutionary very recent origin of this fauna is consistent with the observation by our group that most of selectively-neutral polymorphic alleles (presence/absence of retroposons at orthologous sites in the genome) are retained both within and among species of this lake. This fact 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 positive selection on this gene. In such a case, we may be able to regard it as a candidate gene that was responsible for the phenotypic differentiation of this species from other related species. Thus, investigation of cichlids in Lake Victoria is advantageous for the purpose of elucidation of molecular mechanisms of speciation and their following diversification during adaptive radiation.

The most impressive appearance of cichlids in Lake Victoria is vivid and beautiful coloration of their body in male adults (see Figure 1 for an example). Cichlids are known to depend largely on their vision when they choose individuals to mate with, and such color variations are considered to affect mate preference by females. This fact implies that body color of males plays an important role for recognition of individuals of their own species during mating with females. At the same time a similar logic can be applied to the visual system of females since the body color of males are recognized by females through their visual system. Thus, if one or both of these factors changed significantly, speciation might occur due to a change in their mate choice. Another interesting point on

the visual system of cichlids is that their evolution must have been affected by environmental difference in their habitat such as the turbidity and depth of lake water. An earlier study revealed that, in Lake Victoria, several cichlid species show differently fixed alleles, or significant differences in allele frequencies, of a visual pigment (LWS) gene among different populations within the same species. Although only a limited number of species were investigated in this research, it implies the existence of positive selection on this gene, based on our assumption as mentioned above.

In our division, we are interested in elucidating evolution of genes encoding six classes (LWS, SWS-1, SWS-2A, SWS-2B, RH2 and rhodopsin) of visual pigments (opsins), which are known to exist in the cichlid genome, as factors promoting their speciation in Lake Victoria. In order to start an extensive survey of these genes in Victorian cichlids, we conducted sampling of these fish around Mwanza Gulf in the Southern shore of this lake in Tanzania (Fig. 2). In our preliminary analysis of the coding region of the rhodopsin gene that was amplified from representative individuals by polymerase chain reactions (PCRs), four sites were found to show variations of nucleotide so far (Fig. 3). Interestingly, all of these variations were considered to have arisen by nonsynonymous mutations. This observation is not inconsistent with that the rhodopsin gene has been under strong positive selection during the course of evolution of the investigated species. It would be necessary to accumulate more data on the evolution of this gene in order to assume the environmental background that might be (or might have been) related to this phenomenon. A more extensive analysis on molecular evolution of opsin genes in Victorian cichlids is in progress in our division.

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Terai, Y., N. Takezaki, W. E. Mayer, H. Tichy, N. Takahata, J. Klein, and N. Okada (2004) Phylogenetic relationships among East African haplochromine fishes as revealed by short interspersed elements (SINEs). *J. Mol. Evol.* 58: 64-78.

Figure 2. Localities of collection of cichlids in Lake Victoria. The top panel shows a map of the entire lake. The area indicated by a red rectangle in this panel corresponds to the region shown as a magnified map in the bottom panel. Sampling was conducted by angling and trawling and by using gill nets. White circles denote localities where sampling both by using gill net and by angling was carried out, and orange circles show localities where trawling was done. Sampling was conducted in collaboration with Tanzania Fisheries Research Institute (TAFIRI).

Site	33	104	213	298
AA	S	V	L	A
Codon	AGC	GTC	TTA	GCT
	↓	↓	↓	↓
AA	N	I	S	S
Codon	AAC	ATC	TCA	TCT

Figure 3. Variations observed in the rhodopsin gene that was amplified from representative species of cichlids collected in Lake Victoria. All the variations were assumed to have been caused by nonsynonymous changes that alter amino acid sequences. Direction of mutation was postulated by comparison of the sequences with those from outgroup species in Lake Tanganyika. AA, amino acid.

LABORATORY OF MORPHODIVERSITY

Associate Professor: *KODAMA, Ryuji*

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

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

The outline shape of the adult wing is often different from that of the pupal wing. This difference is brought about by the programmed cell death of the marginal area of the pupal wing. The marginal dying area is called "the degeneration region" and the internal area, which 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 apoptotic cell death in mammalian cells, such as fragmented and condensed nuclei containing short DNA fragments detected by TUNEL staining. The cells in the degeneration region are actively engulfed by the macrophages in the cavity beneath the wing epithelium. The macrophages seem to be concentrated beneath the degeneration region by the strong adhesion between basal surfaces of the dorsal and ventral epithelium in the differentiation region, which occurs at the time of prominent cell death and excludes macrophages out of the differentiation region. Thus realised concentration of macrophages seems to accelerate the shrinkage of the degeneration 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.

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

DIVISION OF CELLULAR REGULATION

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Visiting Scientists: *PANICHKIN, Vladimir* [4)]
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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 environments. 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 molecular mechanisms of photodamage and repair of the photosynthetic machinery under severe stress conditions. In 2004, significant progress was made in the following areas using the cyanobacterium, *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*).

I. Crosstalk of signal transduction in the two-component systems.

Cells perceive the hyperosmotic signal and transduce it to regulate the expression of a large number of genes. Two-component systems that consist of a histidine kinase (Hik) and a response regulator (Rre) are widely distributed from bacteria to higher plants as the mechanism for intracellular signal transduction. The *Synechocystis* genome encodes 47 Hiks and 45 Rres. To identify Hiks and Rres that are involved in the perception and transduction of hyperosmotic signals, we screened knockout libraries of Hiks and Rres by RNA slot-blot and genome-wide DNA microarray analyses. We identified four two-component systems, Hik33-Rre31, Hik34-Rre1, Hik16-Hik41-Rre17 and Hik10-Rre3 and an additional potential two-component system, Hik2-Rre1. Interestingly, Rre1 can perceive the hyperosmotic signals from both Hik34 and Hik2. These results suggest that the crosstalk of hyperosmotic signals occurs in *Synechocystis* cells

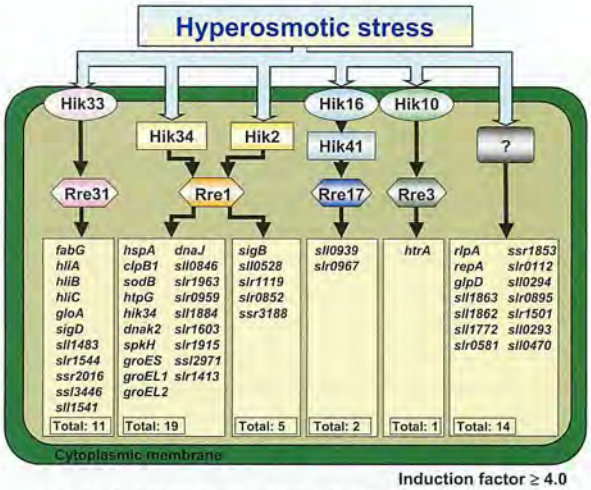

Fig. 1. A hypothetical scheme for the two-component signal transduction pathways that are activated in response to hyperosmotic stress and the genes whose expression is regulated in the respective pathways.

(Figure 1). Hyperosmotic stress probably induces several phenomena such as rigidification of membranes, decreases in cytoplasmic volume, and changes in hydration state of intracellular proteins. It seems likely that Hik34 and Hik2 might perceive such kinds of stimulus and transduce the signals to Rre1 to regulate the expression of a number of genes. Genes whose expression was regulated by Rre1 include a number of important genes, such as chaperons, proteases and a sigma factor. Therefore, it is understandable that cells have multiple pathways to activate Rre1-dependent transcriptional regulation to synthesize proteins, which are indispensable for the acclimation to hyperosmotic stress. These results uncover a part of complicated mechanisms of signal transduction via crosstalk of multiple pathways of two-component systems.

II. The membrane rigidification regulates the cold-inducible gene expression.

Changes in the ambient temperature affect the physical properties of biological membranes. To obtain insights into the role of membranes in the mechanism of cold signal perception, we used a mutant of *Synechocystis*, in which the *desA* gene for the Δ12 desaturase and the *desD* gene for the Δ6 desaturase were both inactive as a result of targeted mutagenesis. Cells of the *desA*$^-$/*desD*$^-$ mutant synthesize only a saturated C16 fatty acid and a monounsaturated C18 fatty acid regardless of the growth temperature, whereas wild-type cells synthesize di-, tri- and tetra-unsaturated C18 fatty acids in addition to the monounsaturated C18 and saturated C16 fatty acids [Tasaka et al. (1996) EMBO J. 15, 6416-6425]. Fourier transform infrared spectrometry revealed that the *desA*$^-$/*desD*$^-$ mutation rigidified the plasma membrane at physiological temperatures. We applied DNA microarray technique to examine effects of the membrane rigidification on the induction of gene expression upon cold shock. The results demonstrated that the cold

inducibility of a part of cold-inducible genes, such as the *crh* gene for an RNA helicase and the *rbp1* gene for an RNA-binding protein, was unaffected by the rigidification. However, the expression of the other cold-inducible genes, such as *hliA*, *hliB* and *slr1544*, was enhanced by membrane rigidification. Moreover, the expression of certain heat-shock genes, namely, the *hspA*, *htpG* and *dnaK2* genes, was markedly enhanced by the membrane rigidification. We further inactivated the genes for Hik33 and Hik34, which had been identified as sensors of various signals and found that the mutation of these Hiks eliminated the membrane rigidification-dependent enhancement of expression of *hli* genes and heat-shock genes, respectively. These findings suggest that Hik33 and Hik34 perceive the change in membrane rigidification as an initial signal of the downward shift in temperature to regulate the cold-inducible gene expression.

III. Genome-based systematic analysis of Ser/Thr protein kinases and prediction of their function in signal transduction.

In addition to Hiks, cyanobacteria normally possess Ser/Thr protein kinases which may function as sensors and/or transducers of environmental signals. The complete genome sequence of *Synechocystis* harbors 12 putative genes for Ser/Thr protein kinases; seven of the 12 genes encode proteins that belong to a PKN2 subfamily, while the other five to an ABC1 subfamily of Ser/Thr protein kinases. These genes are termed in series as *spkA*, *spkB*, *spkC*, *spkD*, *spkE*, *spkF* and *spkG* for kinases of PKN2 type and *spkH*, *spkI*, *spkJ*, *spkK* and *spkL* for kinases of ABC1 type. The role of these genes for Ser/Thr protein kinases in *Synechocystis* remained largely unexplored.

We systematically mutated all of these genes for Ser/Thr kinase by insertion of antibiotic-resistance gene cassettes and investigated the impact of these mutations on the genome-wide expression of genes by means of DNA microarray analysis. Mutation of seven of the genes, namely, *spkA*, *spkC*, *spkD*, *spkG*, *spkH*, *spkJ* and *spkL*, markedly changed the gene expression with increases in the expression of some genes or with decreases in the expression of some other genes, whereas mutation of the other five *spk* genes did not significantly affect the profile of gene expression. These observations predict that at least seven Ser/Thr protein kinases are involved specifically in the regulation of gene expression possibly via a signal-transduction pathway. The stress inducibility of genes whose expression was affected by the *spk* mutations predicts the signal transduction pathway to which the individual Ser/Thr kinases belong.

IV. RNA helicase is required for cold acclimation in *Synechocystis*.

DNA microarray analysis revealed that cold stress induced the expression of a large number of genes. The *crhR* gene for RNA helicase was one of such highly inducible genes. We have observed the following characteristics of the *crhR* gene and its product CrhR (RNA helicase) of *Synechocystis*. (1) The *crhR* gene was highly induced, not only by cold, but also by hyperosmotic and salt stress. (2) The *crhR* mutant cells exhibited a phenotype of slow growth at low temperatures indicating an important function of CrhR under cold stress conditions. (3) DNA microarray and Northern blotting analyses indicated that the *crhR* mutation reduced the expression of genes for molecular chaperonins, *groES*, *groEL1* and *groEL2*. (4) CrhR regulated the level of mRNA of *groESL1* and *groEL2* by stabilizing the mRNA. (5) Further, we demonstrated that the *crhR* mutation decreased the GroEL levels during cold stress. (6) The *crhR* mutation downregulated the expression of *groESL1* and *groEL2* genes under salt and hyperosmotic stress conditions.

In micro-organisms such as *E. coli* and *Synechocystis*, the activity of translation is reduced when they are subjected to cold stress. During cold acclimation, several cold shock proteins are synthesized and association of these cold shock proteins with ribosomes resumes the translation. The cold-induced synthesis of GroESL might be important for the translation machinery to maintain the activity at a proper level under cold stress conditions. Thus the cold-induced expression of the *crhR* gene is very important for *Synechocystis* cells to acclimate cold stress.

V. Photodamage to photosystem II occurs in two-step mechanisms.

Light is necessary for photosynthesis but it also damages the photosynthetic machinery, in particular, photosystem II. The current hypothesis for the mechanism of photodamage to photosystem II postulates that excess light energy, which is absorbed by photosynthetic pigments but not utilized in photosynthesis, produces reactive oxygen species that damage the photochemical reaction center of photosystem II. Using monochromatic light generated by the Okazaki Large Spectrograph, we demonstrated that UV or blue light first inactivates the oxygen-evolving complex of photosystem II and then blue or red light, which is absorbed by photosynthetic pigments, inactivates the photochemical reaction center as the second step. These results lead us to conclude that the current hypothesis is not the case and that the photodamage to photosystem II occurs in two steps

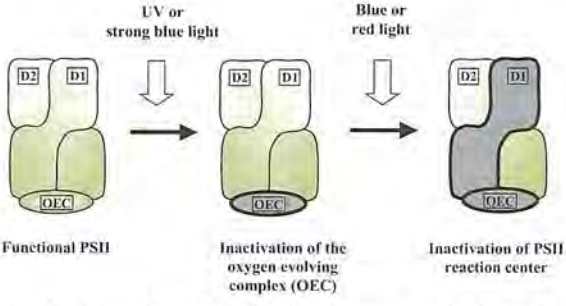

Fig. 2. Schematic model for the two-step mechanism of photodamage to photosystem II.

(Figure 2). The first step is the photodamage to the oxygen-evolving complex of photosystem II probably at the Mn-cluster and its protein environment. The second step is the photodamage to the photochemical reaction center II after the oxygen-evolving complex is inactivated.

VI. Environmental factors do not accelerate the photodamage to photosystem II, but inhibit the repair of photosystem II

Light inactivates photosystem II, and this phenomenon is referred to as the "photoinhibition". The extent of photoinhibition is a result of a balance between photodamage to and repair of photosystem II. We developed a system to monitor the photodamage and repair separately and examined the effect of various environmental stresses on photodamage and repair. In contrary to the current concept that environmental stress accelerates photodamage to photosystem II, we clearly demonstrated that environmental stresses do not damage photosystem II directly, but they inhibit the repair of photodamaged photosystem II at the level of translation of the *psbA* genes which encode the D1 protein, an important component of the photosystem II reaction center. Furthermore, we found in *Chlamydomonas* cells that the repair of photosystem II was inhibited when the Calvin cycle was inhibited.

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(1) Original articles

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A. Shapiguzov, A. A. Lyukevich, S. I. Allakhverdiev, T. V. Sergeyenko, I. Suzuki, N. Murata and D. A. Los (2005) Osmotic shrinkage of cells of *Synechocystis* sp. PCC 6803 by water efflux via aquaporins regulates the osmostress-inducible gene expression. *Microbiology* **151**: in press

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D. A. Los and N. Murata (2004) Membrane fluidity and its roles in the perception of environmental signals. *Biochim. Biophys. Acta (Biomembrane)* **1666**:142-157

Y. Nishiyama, S.I. Allakhverdiev and N. Murata (2005) Regulation by environmental conditions of the repair of photosystem II in cyanobacteria. Photoprotection, Photoinhibition, Gene Regulation, and Environment (eds., B. Demming-Adams, W.W. Adams and A.K. Mattoo), *Kluwar Academic Publishers*

Y. Nishiyama, S. I. Allakhverdiev and N. Murata (2005) Inhibition of the repair of Photosystem II by oxidative stress in cyanobacteria. *Photosynth. Res.*, in press

DIVISION OF MOLECULAR ENVIRONMENTAL ENDOCRINOLOGY

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KOBAYASHI, Kaoru
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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.

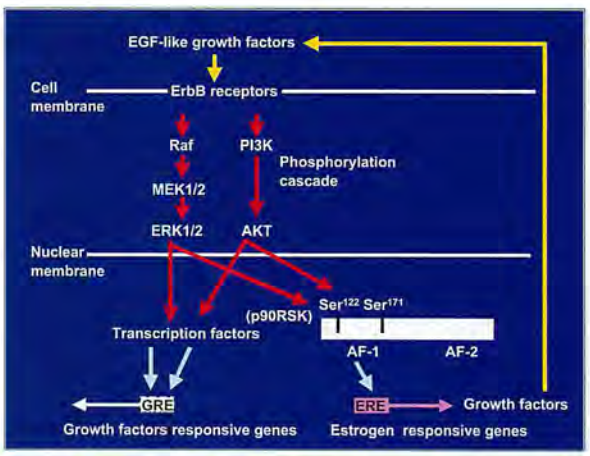

FIG.2 A hypothetical model for the estrogen-independent ER activation pathway in mouse vagina. EGF-like growth factors activate the protein-phosphorylation cascade via erbB receptors. In nuclear, estrogen receptor is phosphorylated on serine 122 and 171 in AF-1 domain. Furthermore, transcription factors 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 ERα 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.

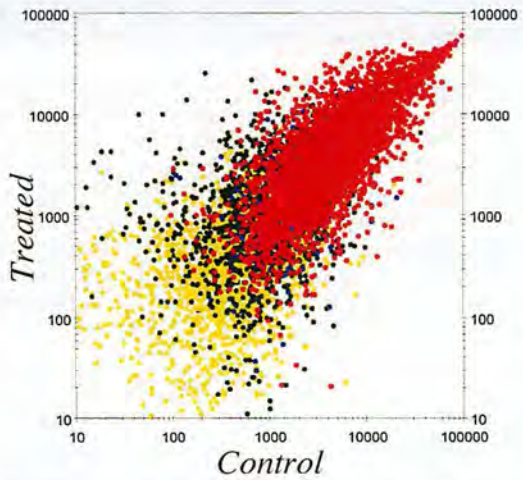

FIG.3 Scatter plot of average expression levels in control and chemical-treated uterus

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

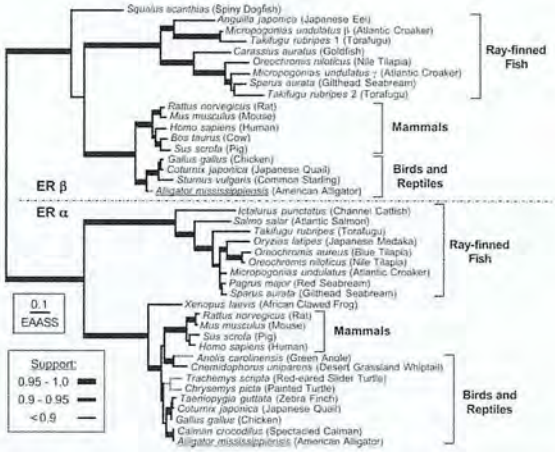

FIG. 4 Evolutionary relationships of estrogen receptor sequences.

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 sex differentiation 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*), the red belly turtle (*Pseudemys nelsoni*) and Japanese giant salamander (*Andrias japanicus*). 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.

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.

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DIVISION OF PLANT DEVELOPMENTAL GENETICS

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The leaf is the fundamental unit of the shoot system, which is composed with leaf and stem. Diversity of plant form is mostly attributable to variation of leaf and floral organs, which are modified leaves. Moreover, leaf shape is sensitive to environmental stimuli. 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 have 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).

Focusing on mechanisms that govern polarized growth of leaves in *Arabidopsis thaliana* we have identified genes for polar-dependent growth of leaf lamina: the *ANGUSTIFOLIA* (*AN*) gene regulates width of leaves and the *ROTUNDIFOLIA3* (*ROT3*) gene regulates length of leaves. Both *AN* and *ROT3* genes control leaf cell shape.

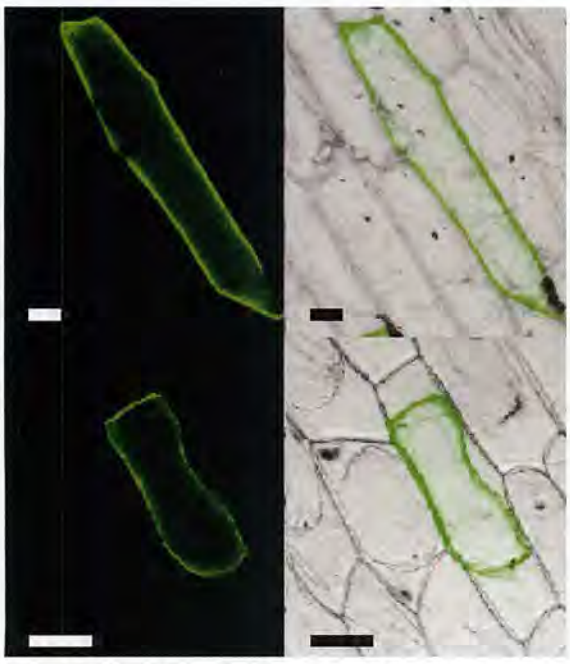

Fig. 1. Intracellular localization of ROT4::GFP in onion epidermal cells before (upper) and after (lower) osmosis.

How have these genes evolved the function of leaf-shape control? The *AN* is a homolog of animal *CtBP/BARS* gene family which has varied roles in morphogenesis and organelle control. To understand the common role(s) of *AN* subfamily in plant kingdom, we analyzed a homolog of *AN* from *Ipomoea nil, IAN,* and showed that the *IAN* exhibits the same function with the *AN* on the control of leaf shape when introduced to *Arabidopsis thaliana* (Cho et al., in press). On the other hand, the ability of the *IAN* to control branching pattern of trichomes was less efficient than the *AN*. Further comparative analyses of the *AN* homologs from various plants are now on going.

In addition to the above factors, in this year, we added *ROT4* gene to the list of the genes responsible for the polarity-dependent control of leaf shape (Narita et al., 2004). ROT4 is a member of novel peptide family (*RTFL: RoT-Four-Like*) which is specific to seed plants. Overexpression of the *ROT4* results in stunted leaves with normal width, due to decrease of number of leaf cells specifically to the longitudinal direction. All RTFL peptide conserve RTFL domain and even truncated forms of ROT4 that have RTFL domain show the same effect on leaf shape with full-length ROT4, the RTFL family is thought to have common role in control of leaf cell proliferation to the leaf-length direction. Interestingly, the ROT4 peptide appears to localize on plasma membrane when fused with GFP marker (Fig. 1). The role of the RTFL in the control of leaf-cell proliferation is under investigation.

How cell proliferation and cell enlargement are coordinated in leaf morphogenesis? 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 (Tsukaya, 2003). Thus, leaf size is, at least to some extent, uncoupled from the size and number of cells by the compensatory system(s). Recently, we have revealed that *ANGUSTIFOLIA3* (*AN3*) gene is involved in 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., submitted). Interestingly, the *an3* shows clear "compensation", namely, accelerated cell expansion in relation to decrease of number of leaf cells. By using various mutants with altered number and/or size of leaf cells, we are currently analyzing genetic system of the compensation.

On the other hand, we also focused on the effects of environmental factors on leaf morphogenesis. In darkness, expansion of leaf lamina is inhibited, while at the same time, petiole elongation is enhanced. This phenomenon is termed the shade-avoidance syndrome. We analyzed the nature of the shade-avoidance syndrome and found that phytochromes and cryptochromes specifically regulate the

contrasting growth patterns of the leaf blade and petiole in shade (Kozuka et al., in press; Fig. 2). Differed from photomorphogenesis of hypocotyl, cell elongation was stimulated in the petiole in dark conditions without an increase in the ploidy level. By examining the effects of sucrose on the growth of the leaf blade and petiole, we revealed that growth promotional effects of sucrose are highly dependent on the light conditions.

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

Fig. 2. Shade-avoidance syndrome in *Arabidopsis thaliana*. Left, under white light; right, under dark. Bar: 5 mm.

On the other hand, we are also interested in environmental adaptation of leaf size in wild plants. In the course of field research of natural evolution of leaf shape/size, we have revealed some aspects of biodiversity of plant forms (Tsukaya, 2004; in press; Tsukaya et al., 2004; Yokoyama et al., in press). Interestingly, many plants are known to have evolved small-sized leaves in some islands, such as Yakushima, Kinkazan and Miyajima islands. Similar phenomenon is also known in plants inhabited in precincts of shrines and temples in Japan. Typical example of the evolution of small-sized leaves in these environments is known in *Plantago asiatica.* To understand what factors have accelerated the evolution of the small-sized leaves, we collected *P. asiatica* and *P. major* from a number of localities in and around Japan and established more than thirty imbred lines to analyze the genetic background of the evolution. To nurse study of this field, co-organized with Dr. Araki of Kyoto University, we held a domestic meeting on *Plantago* Research in Okazaki Conference Center, in July 3, 2004, inviting twelve researchers from various universities. So called "Evo/Devo" study of leaf morphogenesis is also one of our research project in NIBB.

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

I. Chloroplast relocation movement

One of our major subjects is chloroplast photo-relocation movement, which is thought to be one of the simplest 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*

In *Arabidopsis* leaves, chloroplast movement is fluence rate dependent. Under lower light fluence rate, chloroplasts accumulate at the cell surface to maximize photosynthetic potential. Under high fluence rate, chloroplasts avoid incident light to escape photodamage. We examined the phenomenon of chloroplast avoidance movement and demonstrated a proportional relationship between fluence rate and the velocity of chloroplast avoidance movement. When a small area is irradiated with a microbeam, the majority of chloroplasts inside the beam begin moving towards the outside of the beam. Some chloroplasts, however, begin to move only after a relatively long lag time. The length of the lag period becomes longer under higher fluence rate light and occurred more frequently with chloroplasts located nearest the center of the microbeam. In addition, we showed that the amount of light-activated phot2, the photoreceptor for the avoidance response, likely plays a role in this phenomenon, as heterozygous mutant plants show a reduced avoidance velocity compared to that of homozygous wild type plants.

1-2 *Physcomitrella*

Phototropin is the blue light receptor that mediates blue and red light induce chloroplast movement in the moss *Physcomitrella patens*, which has four phototropin genes, PHOTA1, PHOTA2, PHOTB1 and PHOTB2. These genes were classified into two groups (PHOTA and PHOTB) on the basis of their deduced amino acid sequences. Then phototropin disruptants were generated by homologous recombination and used for analysis of chloroplast movement. It was found that blue light-induced chloroplast movement was mediated by phototropins. Both photA and photB groups were able to mediate chloroplast avoidance, although the photA group contributed more to the response. Red light-induced chloroplast movement was also significantly affected in photA2photB1photB2 triple disruptants. Because the primary photoreceptor for red light-induced chloroplast movement in *P. patens* is phytochromes, phototropins may be downstream components of phytochromes in the signaling pathway. The involvement of phototropins in the phytochrome signaling pathway was reported for the first time.

1-3 *Adiantum*

The avoidance movement response in *Adiantum* phot2 deficient mutants can be restored by transient expression of non-mutant AcPHOT2 cDNA, indicating that chloroplast avoidance movement in this fern is mediated by the Acphot2 protein. Further functional analyses of the Acphot2 protein were performed using this transient assay for chloroplast avoidance movement. The results suggest that the LOV2, but not the LOV1, domain of Acphot2 is essential for avoidance movement.

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. We identified active transposable elements, *miniaturePing* (*mPing*), *Ping* and *Pong* in rice. The *mPing* element was identified as the first active MITE from any organism. *mPing* is a short 430 base pair element with 15 base pair terminal inverted repeats that lacks a transposase. *mPing* elements are activated in calli derived from anther culture and excise efficiently from original sites to reinsert into new loci. *Ping* and *Pong* transposable elements were isolated as putative autonomous elements encoding an IS/PIF/Harbinger superfamily of transposases. Evolutionally, the number of copies of *mPing* elements has increased in *japonica* cultivars, but not in *indica* cultivars and their ancestral species, *Oryza rufipogon*. *Japonica* cultivars are the only rice varieties in which the transposable *Ping* element can be detected.

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

2- 2 *DNA interference in Adiantum*

Silencing of gene expression by RNA interference (RNAi) is a useful technique for determining the roles of genes of unknown function in a wide range of organisms. The dramatic increase in the number of genes of unknown function as a result of whole genome sequencing projects, as well as EST projects, means that faster approaches are needed for understanding gene function. We found a simple method for silencing genes using DNA fragments homologous to the target gene rather than RNAi. We have named this approach DNA interference or DNAi. It has the advantage of being faster and simpler than current RNAi approaches, and allows simultaneous silencing of multiple genes.

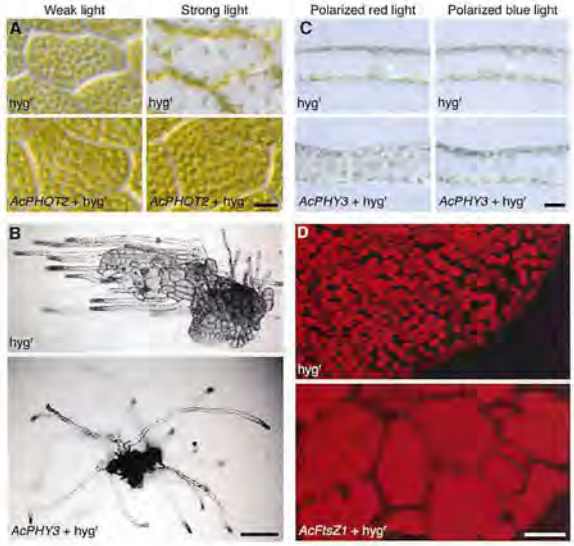

Fig. 1 Phenotypes by DNAi. (A) Chloroplast accumulation and avoidance movement in prothalli induced by weak white light (left) and strong blue light (right), respectively. Note that the avoidance response under strong light was inhibited by *AcPHOT2* introduction. (B) Red light-induced phototropism in protonemal cells generated from a prothallus. Red light was irradiated from the left. Note that phototropic response was inhibited by *AcPHY3* introduction. (C) Chloroplast relocation induced by polarized red (left) or blue light (right) in protonemal cells of (B). Note that chloroplast accumulation response induced by red light was inhibited by *AcPHY3* introduction. (D) Chloroplasts of a prothallus. Note that chloroplast division was inhibited by *AcFtsz* introduction. Both promoterless *AcPHOT2* (A), *AcPHY3* (B,C), and *AcFtsZ1*(D)and hyg^r gene or hyg^r gene only were introduced into these gametophytes. Bar in A, C and D: 20 μm, B: 200 μm. See Kawai-Tokuoka et al (2004) in detail.

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Kanegae, T. and M. Wada (2004) Photomorphogenesis of Ferns. In, Photomorphogenesis in Plants 3rd Edition Edited by Schafer and Nagy, Kluwer Academic Publishers, Dordrecht. In press.

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LABORATORY OF PHOTOENVIRONMENTAL BIOLOGY

Professor (Adjunct): *WATANABE, Masakatsu (Sep. 1 -)*
Associate Professor: *WATANABE, Masakatsu (- Aug. 31)*
Postdoctoral Fellows: *ISEKI, Mineo* [1)]
YOSHIKAWA, Shinya [2)]
SUZUKI, Takeshi [2)]
Visiting Scientist: *ITO, Shinji*
Technical Assistants: *KOUMURA, Yoshiko* [2)]
ITO, Makiko

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Photoreceptive and signal transduction mechanisms of phototaxis of unicellular algae are studied action spectroscopically (Watanabe 2004) by measuring computerized-videomicrographs 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 (Kräbs *et al.* 2004).

A novel blue-light receptor with an effector role was found from *Euglena gracilis* (Fig. 1; 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 α- and β-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.

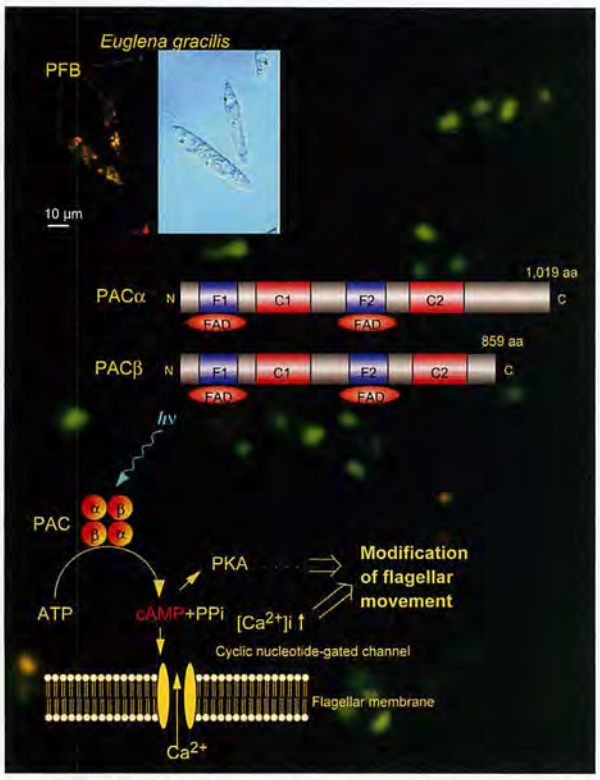

Fig.1 Schematic diagram showing domain structures of PAC subunits and working hypothesis of action mechanism of PAC to photocontrol *Euglena* flagellar movement.

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 through collaboration with Professor D.-P. Häder's laboratory of Phillips University, Erlangen. Germany (Fig. 2; Ntefidou *et al.* 2003 *Plant Physiol.* **133**, 1517-1521.).

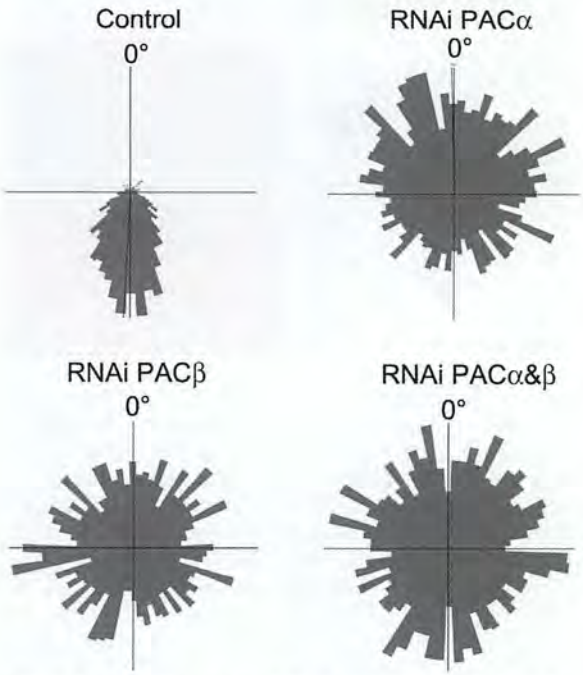

Fig.2 Swimming tracks of *Euglena* cells summarized in circular histograms under unilateral light irradiation. Control cells swimming with high precision away from the light source and RNAi-treated cells showing random swimming. (From Ntefidou *et al.* 2003, Plant Physiol. **133**, 1517-1521)

To gain an insight into the evolution of this unique protein, similar sequences were searched for in several euglenoids by RT-PCR using degenerate primers. Two similar transcripts were detected in each of the four phototrophic euglenoids, *Euglena stellata*, *Colacium sideropus*, *Eutreptia viridis*, *Eutreptiella gymnastica*, and in an osmotrophic (i. e., obtaining nutrients by absorption) one, *Khawkinea quartana,* but not in a phagotrophic euglenoid, *Petalomonas cantuscygni*. Each of them seemed to be orthologous to PACα and PACβ, respectively, and had the same domain structure as PAC

subunits each of which is composed of two flavin binding domains, F1 and F2, each followed by an adenylyl cyclase catalytic domain, C1 and C2, respectively. This fact implies that they constitute a functional photoactivated adenylyl cyclase like PAC. Phylogenetic analysis of the adenylyl cyclase catalytic domains revealed that they belong to a bacterial cluster, not to a trypanosomal one. In addition, two trypanosome-type adenylyl cyclases were discovered in *E. gracilis*. In contrast to PAC, deduced amino acid sequences of the trypanosome-type adenylyl cyclases indicated that they are integral membrane proteins with a membrane spanning region at the midpoint of them, followed by an adenylyl cyclase catalytic domain which seems cytoplasmic. Overall, we propose that PAC might have been transferred to euglenoids on the occasion of secondary endosymbiosis (Fig. 3; Koumura *et al.* 2004).

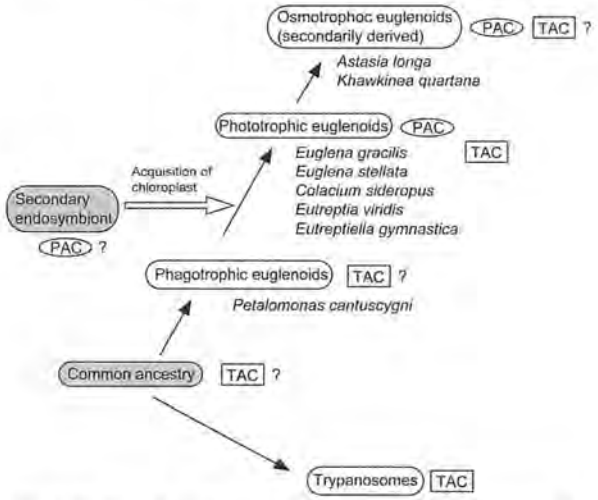

Fig.3 Working hypothesis on the origin of PAC. Presence of orthologs of PAC subunits ('PAC' in an oval) and trypanosome-type adanylyl cyclases ('TAC' in a rectangle) are indicated on the well accepted evolutionary history of euglenoids. (From Koumura *et al.* 2004).

Basic kinetic studies on photoactivation of PAC *in vitro* and *in vivo* were done (Yoshikawa *et al.* submitted): in vitro photoactivation by blue-light showed reciprocity between fluence rate and duration of irradiation in the fluence rate range of 2-50 μmol/m^2/s. Intermittent irradiation caused activation of PAC in a photon fluence dependent manner irrespective of cycle periods. The time course of the change in intracellular cAMP level agreed well with that of the step-up photophobic response. Overall, an increase in intracellular cAMP level evoked by photoactivation of PAC is a key event of the step-up photophobic response.

Publication List:

Koumura, Y., Suzuki, T., Yoshikawa, S., Watanabe, M. and Iseki, M. (2004) The origin of photoactivated adenylyl cyclase (PAC), the *Euglena* blue-light receptor: phylogenetic analysisi of orthologues of PAC subunits from several euglenoids and trypanosome-type adenylyl cyclases from *Euglena gracilis*. *Photochem. Photobiol. Sci.*, **3**, 580-586.

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LABORATORY OF STRESS RESPONSE

Associate Professor: *MIKAMI, Koji*

The aim of this laboratory is to understand molecular mechanisms of the flexibility in modification of the cell polarity during morphogenesis in plants under the changes in environmental conditions. As their sessile nature, growth and development of plants are largely influenced by environmental factors, thus plants must percept and response to these factors to regenerate the growth axis. In the responses to environmental stimuli, phosphoinositide-specific phospholipase C (PI-PLC) plays the essential role in the generation of two second messengers, inositol-1,4,5-trisphosphate and diacylglycerol through the hydrolysis of phosphatidylinositol-4,5-bisphospate (PIP_2). Plant PI-PLC is now thought to be involved in responses to gravity and light, both of which effect the modification of the cell polarity by generation of a physiological asymmetry. In moss plants, such as *Physcomitrella patens* and *Ceratodon purpureus*, changes in cytosolic Ca^{2+} concentration in caulonemal cells are associated with the generation of buds, the precursors of gametophores, which is regulated by blue light and cytokinin. In addition, the red-light-induced phototropic responses of protonemal tip cells are mediated by phytochrome. It has been shown that red-light-irradiation leads to an increase of the PI-PLC activity and the phototropic bending follows the production of a dynamic Ca^{2+} gradient in the protonemal tip cells. Moreover, *Physcomitrella* is now recognized as a model system for plants with easy application of molecular genetic approaches such as gene-targeted mutagenesis via the homologous recombination. Therefore, it is reasonable to employ the moss *Physcomitrella* for genetical and molecular investigations of the roles of plant PI-PLCs in the modification of cell polarity by environmental factors.

I. Structural characteristics of *Physcomitrella* PI-PLCs

Searching of the plant EST databases with BLAST algorithm against AtPLC1S from *Arabidiopsis thaliana* revealed four *Physcomitrella* cDNA clones, PPU141114, PPU140521, PPU070504 and PPU161218. For the first three ESTs derived from the same mRNA, the 5'-race and the subsequent 3'-race reactions resulted in the isolation of a corresponding full-length cDNA of 2,424 bp containing an ORF for a PI-PLC homologue of 633 amino acids and calculated molecular mass of 70.8 kDa, which was designated PpPLC1. In addition, a 2,423 bp full-length cDNA corresponding PPU161218 was isolated by the 5'-race reaction, which contains an ORF for a PI-PLC homologue of 639 amino acid and calculated molecular mass of 71.8 kDa, designated PpPLC2. The overall structure of PpPLC1 and PpPLC2 was similar to those of known plant PI-PLCs comprising the catalytic domain and the C2 domain. PI-PLC isoforms in mammals have been divided into four classes, β-, γ-, δ- and ε-types, all of which contain the catalytic domain that consists of X and Y regions and regulatory domains such as pleckstrin homology (PH), EF-hand and C2 domains. In contrast, plant PI-PLCs analyzed so far all show δ-type organization but lacking the PH and typical EF-hand domains. The N domain, which is conserved in *Arabidopsis* PLCs as a regulatory domain with structural similarity to the second loop of the EF-hand domain of rat PLCδ1, was found in the N-terminal extensions in PpPLC1 andPpPLC2, although there was an insertion in the N domain of PpPLC2. Since the PH and EF-hand domains are responsible for membrane-localization and Ca^{2+}-binding in mammals, the mode of activation of plant PI-PLCs is probably different from those in mammals.

II. *Physcomitrella* has typical and novel types of PI-PLCs

The *in vitro* activity of recombinant His-tagged PpPLC1 and His-tagged PpPLC2 was examined. His-tagged PpPLC1 hydrolyzed PIP_2 with maximum activity of 38 nmol/min/mg of protein at a physiological concentration of Ca^{2+} around 10 μM and lower activity at higher Ca^{2+} concentrations, consistent with other recombinant plant PLCs. In contrast, His-tagged PpPLC2 hydrolyzed PIP_2 with very low activity under the same conditions. When phosphatidylinositol (PI) was used as a substrate in the reactions with various concentrations of Ca^{2+}, both His-tagged PpPLC1 and His-tagged PpPLC2 showed a very low-hydrolyzing activity of 1.3 and 9.4 nmol/min/mg of protein, respectively, at 10 μM Ca^{2+}. However, at 1 mM Ca^{2+}, His-tagged PpPLC2 hydrolyzed PI very efficiently (22 nmol/min/mg of protein) than His-tagged PpPLC1 (3.5 nmol/min/mg of protein). Together with their structural characteristics, I conclude that PpPLC1 and PpPLC2 are typical and novel types of plant PLC, respectively.

In the X and Y regions, 11 amino acids have been identified as essential residues for mammalian PI-PLC activity via binding to Ca^{2+} and substrate. I found that all of them are well conserved in PpPLC1, although a conserved serine residue was replaced by asparagine residue at amino acid position 409 in PpPLC2. In fact, PLC-like proteins with amino acid substitutions of residues important for PLC activity have already been isolated from rat and human, which have no PIP_2-dependent PLC activity. Thus, it seems that the loss of PIP_2-dependent activity in PpPLC2 may be due to amino acid substitution in the catalytic domain and/or its abnormal N domain.

III. PpPLC1 is involved in cytokinin and gravity responses

To elucidate the physiological functions of PpPLC1, targeted knockout mutants of the *PpPLC1* gene were generated via homologous recombination. To construct a plasmid for gene targeting, a 35S-promotor-driven

neomycin phosphotransferase II (*npt*II) cassette providing G418 resistance was inserted into a 3509 bp fragment of the *PpPLC1* genomic clone. After transfomation of *Physcomitrella* protoplasts with the targeting construct, G418-resistance transgenic lines were obtained and screened by PCR to confirm the specific disruption of the *PpPLC1* gene.

Protonemal filaments grown under standard conditions showed a clear difference between wild type (WT) and *plc1*, the mutants failing to form gametophores (Fig. 1). The formation of gametophores in *Physcomitrella* follows two independent developmental steps. First, an immature side-branch is induced from an intercalary caulonemal cell, which was shown to be regulated by blue light (BL) through cryptochromes. Secondly, the side-branch initial differentiates into a gametophore bud. This is known to be a cytokinin-dependent step in *Physcomitrella*. By checking the response of protonemal cells to BL and cytokinin, it was concluded that the absence of gametophores in *plc1* is due to a defect in cytokinin signaling.

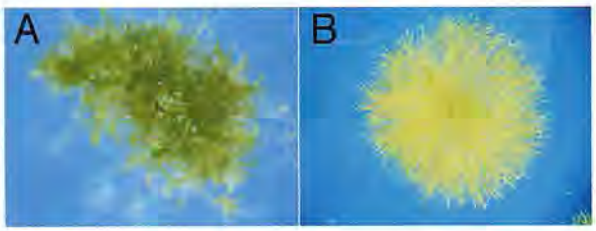

Fig. 1. Comparison of visible phenotypes between WT and *plc1* knockout. Leafy gametophores, which were well produced in WT (A), were not observed in *plc1* (B).

In addition, the protonemal filaments of *plc1* have a strongly reduced ability to response to the gravity (Fig. 2). WT plants produce uniformly negative-gravitropically growing protonemal filaments prior to unilateral red light irradiation, when protonemal filaments were cultured on vertical agar plates in the dark (Fig. 2A). However, the protonemal filaments of *plc1* have a strongly reduced ability to grow negatively gravitropically in the dark (Fig. 2B). Moreover, after rotating the cultures 90°, WT filaments responded with a subsequent change in the growth direction, according to the gravitropic stimuli, whereas the *plc1* continued to grow randomly (Figs. 2C and 2D). Similar results were obtained after growth in diffuse white light (100 μmol m^{-2} s^{-1}) for two weeks in a vertical orientation. Therefore, PpPLC1 plays an important role in the detection of and/or response to gravitropic stimuli in *Physcomitrella*.

In summary, PpPLC1 has diverse functions including the formation of gametophores from side branches in protonemal filaments and the response to gravity in apical tip cells. To understand molecular mechanisms of modification of the cell polarity, it is necessary to elucidate how plants percept cytokinin and gravity, how these signals are transduced in the cells and how PpPLC1 acts to modify the cell polarity. Whereas most of the knowledge about the physiological function of plant PI-PLC is based on inhibitor studies that might cause non-specific effects, *Physcomitrella* constitutes a most attractive experimental system for studying these points at the molecular level, which based on the efficient targeted gene disruption via homologous recombination to generate *plc1* null-mutants. Current works are focused on the mode of activation of PpPLC1 to elucidate how environmental stimuli lead the modification of the cell polarity in plants.

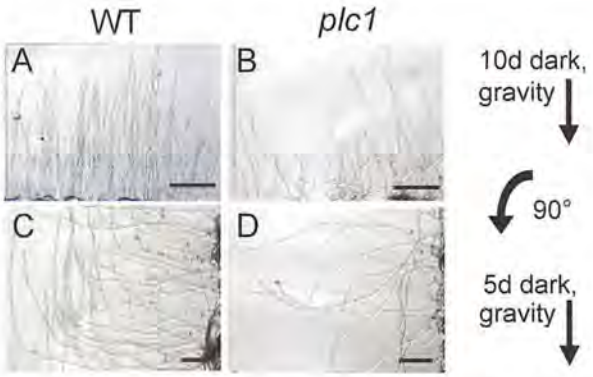

Fig. 2. Comparison of gravitropic response of protonemal filaments between WT and *plc1* knockout. Protonemal filaments of WT (A, C) and *plc1* (B, D) were cultured on vertical agar surfaces for ten days in the dark (A, B). Cultures were rotated after ten days for 90° and further kept in darkness for additional 5 days (C, D). Scale bars represent 500 μm.

Publication list:

Original articles

Mikami, K., Repp, A., Graebe-Abts, E. and Hartmann, E. (2004) Isolation of cDNAs encoding typical and novel types of phosphoinositide-specific phospholipase C from the moss *Physcomitrella patens*. *J. Exp. Bot.* **55**, 1437-1439.

Repp, A., Mikami, K., Mittmann, F. and Hartmann, E. (2004) Phosphoinositide-specific phospholipase C is involved in cytokinin and gravity responses in the moss *Physcomitrella patens*. *Plant J.* **40**, 250-259.

Review article

Mikami, K. and Hartmann, E. (2004) Lipid metabolism in mosses. In, *New Frontiers in Bryology: Physiology, Molecular Biology and Functional Genomics* (A.J. Wood, M.J. Oliver and D.J. Cove, eds.), Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 133-155.

DIVISION OF THEORETICAL BIOLOGY

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

I. Mathematical approach to biological phenomena

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

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

II. A theoretical study for gene regulatory networks and analysis of differentiated cell-states

The highly complex behavior of organisms is based on the interactive regulation of genes, functioning together to create a gene network. The regulation of gene expression levels is dependent on the concentrations of transcriptional regulators. Typically, multiple transcription factors can bind to the regulatory region of a gene; each factor may independently affect the gene expression levels. In this manner, the regulatory region of gene serves as an integration point, performing a logical computation of these transcription factors to determine the resulting gene expression levels. In addition, once translated, the encoded protein may control the transcription of other genes.

The complexity of gene networks has been considered to be a problem that may be solved from a theoretical point of view. Since the proposal of the Boolean model by Kauffman, multiple mathematical models have been invented and analyzed to study complex gene networks. There are two general classes of network models, discrete and continuous models. Continuous models are likely more realistic than discrete models as a representation of biological systems. However, the dynamical behavior of continuous models is analyzed much less than that of discrete models.

In this paper, I have developed a model for gene networks in a framework of continuous time and state variables. I analyze steady states appearing in this system in general way. The steady states specify distinct cell states defined by patterns of gene activity. I introduce three types of steady states observed in a continuous time and space model, whose values and stability can be evaluated from the structure of gene regulatory interactions. The expected numbers of steady states in a randomly generated gene network were derived in exact forms. I determined the distribution of the steady states in a randomly generated network.

The results were surprising; the number of steady states in a random gene network does not depend on gene number. In addition, the number of steady states is very small; the number of RSPs, in which the activity of each gene is either on or off, is only one. Most randomly generated gene networks will have only a few (or no) steady states, despite the presence of many genes in the system. Thus, increases in the number of genes do not augment the number of steady states for gene activity. The gene expression steady states may correspond to differentiated cell type identities. Thus, this result implicates that the number of genes is not the driving force increasing the diversity of differentiated cell types or the complexity of organisms.

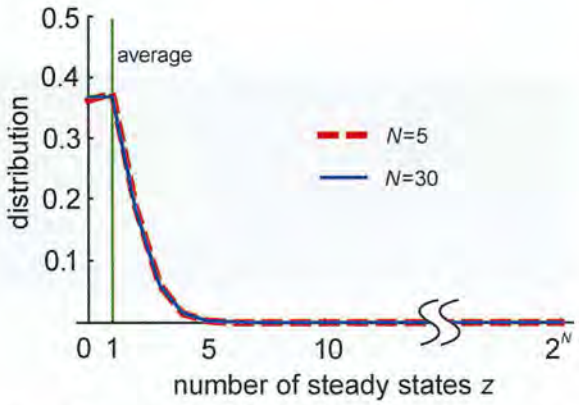

Figure 1. Distribution of the number of regular stationary points (RSPs). The distribution demonstrates a case that the regulation of each gene in each domain is independent. The two lines show the results of gene number N is 5 and 30. The distribution does not hardly depend on gene number

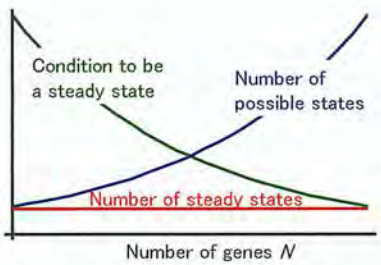

Figure 2. Intuitive explanation for the independence of steady state number on gene number. The number of domains 2^N, the maximum number of RSPs, increases exponentially with gene number N. The probability for each domain to contain an RSP, however, decreases with gene number. The two effects are opposing and equivalent. Thus, the effect of N on the number of steady states disappears.

In reality, multi-cellular organisms have a wide variety of differentiated cells. Why, then, do the networks in actual organisms have multiple steady states? It seems

likely that the networks within actual organisms are quite biased from the average randomly generated network. A gene network including a large number of self-regulatory genes has a large number of steady states. Thus, self-regulation within a gene network may be the main source of cell type diversity in actual organisms.

III. Mathematical models for pattern formation of leaf vascular networks.

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

Although a lot of experimental studies have attempted to identify the mechanism of vascular patterning in leaves, it is still a problem to solve. Jacobs revealed the importance of auxin for vascular differentiation. Auxin is one of the major plant hormones. In the higher plant, indole acetic acid (IAA) is one of the most important auxin chemicals. Auxin is diffusible molecule; however, it is considered that auxin is also transported along the particular direction, usually from apical to basal in a stem, from distal to proximal in a leaf. It is called auxin polar transport. In a leaf, auxin is considered to be produced in apical margins of leaves and transported toward the proximal regions. Many researchers considered that such auxin flow play important role in vascularization of plant.

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

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

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

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

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

Publication List

Mochizuki, A. (2005) An Analytical Study of The Number of Steady States in Gene Regulatory Networks. *J. Theor. Biol.* (In Press).

Tohya, S. & Mochizuki, A. (2005) A new model for leaf venation pattern. *J. Theor. Biol.* (In Press).

Fujita, H. & Mochizuki, A. (2005) Pattern formation by the positive feedback regulation between flow of diffusible signal molecule and localization of its carrier. *J. Theor. Biol.* (In Press).

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.

LABORATORY OF GENOME INFORMATICS

Research Associate: *UCHIYAMA, Ikuo*

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

I. Construction of microbial genome database for comparative analysis

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

We have been developing and maintaining a database system for comparative analysis of microbial genomes named MBGD (http://mbgd.genome.ad.jp/). The central function of MBGD is to create orthologous groups among multiple genomes (Figure 1), which is a crucial step for comparative genome analysis. The key components of MBGD include i) an algorithm that can classify genes into orthologous groups using precomputed all-against-all similarity search results, ii) a user interface that is designed for users to explore the resulting classification in detail, and iii) an incremental updating process for similarities between genes and other data, which enables the system to provide the latest data rapidly. By this approach, MBGD is now the world's largest database of its kind. Moreover, by specifying a set of organisms, users can obtain appropriate classification results that they want using the latest data available. This unique feature is especially useful for users whose interests are focused on some taxonomically related organisms. Indeed, we used this feature of MBGD in our comparative genomics studies among *Bacillus*-related species described below (see Figure 1).

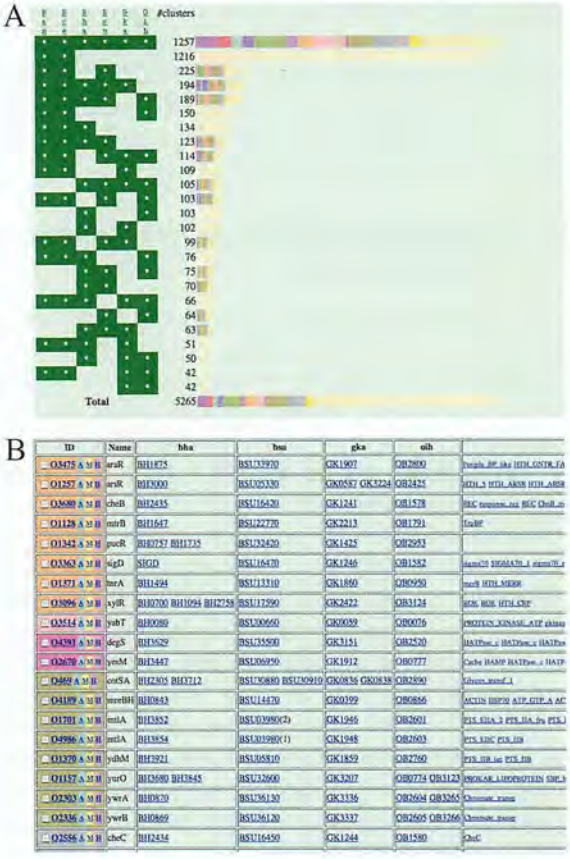

Figure 1 Ortholog cluster map (A) and ortholog cluster table (B) in MBGD. Here, the orthologous grouping was created using genomes of 6 *Bacillus*-related species including *G. kaustophilus*. In the ortholog cluster map (A), the grouping result is sorted according to the phylogenetic patterns, which represent presence or absence of the orthologs in each genome, and each cluster is assigned a color according to its function category. By clicking the bar graph of the map, the actual cluster table is shown (B), where each row represents an ortholog cluster and each column represents an organism.

We are also involved in a reannotation project for bacterial genomes that is currently in progress by the Japanese research community of pathogenic bacterial genomics (headed by Dr. Kuhara, Kyushu University). In this project, an orthologous grouping created by the MBGD system is used as a prototype grouping, which will be further modified and reannotated manually.

II. Revealing thermoadaptation trait by comparative genomics between thermophilic *Geobacillus kaustophilus* and mesophilic *Bacillus*-related species.

How thermophilic organisms adapt to high-temperature environments has long been an intriguing question in both academic and industrial fields. Recent studies revealed some remarkable genomic features strongly correlated with thermophily such as amino acid composition, codon usage and genomic dinucleotide composition. However thermophiles whose genomic sequences have already been determined are somewhat phylogenetically biased;

many of them belong to archaea or the deep-branching bacteria (*Aquifex* and *Thermotoga*). On the other hand, one of the effective approaches in revealing thermophilic trait is to compare genomes between closely related organisms including both thermophiles and mesophiles. This approach is also effective for understanding thermoadaptation from the viewpoint of evolution, although the genomic sequences from an appropriate set of organisms are needed, which have not yet been obtained.

In collaboration with Dr. Takami's group (JAMSTEC), we determined the complete genomic sequence of thermophilic *Geobacillus kausophilus* and compared it with those of phylogenetically related 5 mesophilic bacilli, *Bacillus subtilis*, *Bacillus halodurans*, *Oceanobacillus iheynsis*, *Bacillus cereus* and *Bacillus anthracis*. By the principal component analysis of the amino acid composition using 150 prokaryotic genomes including 20 thermophilic ones, we were able to find that the thermophilic *G. kaustophilus* were distinguishable from mesophilic bacilli by the borderline distinguishing thermophiles from mesophiles on the second principal axis, although on the whole the *Bacillus*-related species were located near this borderline (Figure 2). Further statistical analysis revealed some asymmetric amino acid substitutions between the thermophilic and the mesophilic bacilli, which are possibly associated with the thermoadaptation of the organism.

In addition, upon orthologous grouping of the 6 bacillar sequenced genomes, we found 839 genes (24%) in the *G. kaustophilus* genome being unique to that species, among which we were able to find some candidate genes that may contribute to thermophily presumably by enhancing the stability of nucleic acids.

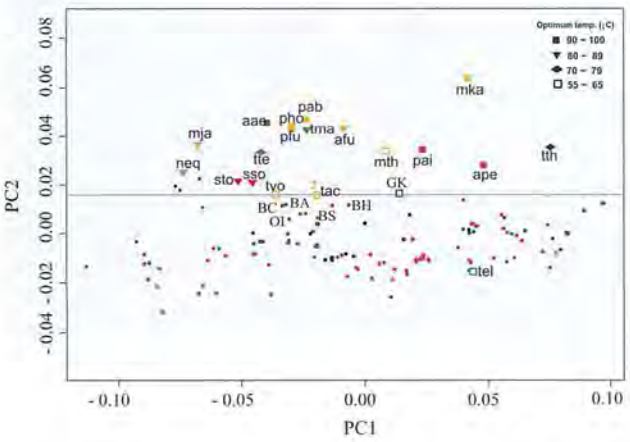

Figure 2 Principal component analysis of the amino acid composition using 150 prokaryotic genomes. Thermophiles are marked by symbols according to their optimal growth temperatures. An abbreviated name in upper case is assigned to each *Bacillus*-related species; GK: *G. kaustophilius*, BH: *B. halodurans*, BS: *B. subtilis*, BA: *B. anthracis*, BC: *B. cereus*, OI, *O. iheyensis*. An abbreviated name in lower case is assigned to each thermophile.

III. Identification of the common core structure of bacillar genomes

Recently several studies showed that horizontal transfer as well as vertical transfer has played important roles in the prokaryotic genome evolution. However, to obtain clearer picture of the bacterial genome evolution, we need further detailed investigations including extensive comparison of multiple genomes that are closely or moderately related to each other. In the collaborative work with Dr. Takami, we also performed such extensive comparison among *Bacillus*-related genomes for the purpose of drawing general picture of the bacterial genome evolution.

Aerobic endospore-forming gram-positive *Bacillus*-related species is known to be able to grow in a wide range of environments. The above-mentioned 6 bacilli whose genomic sequences have been determined include alkaliphilic *B. halodurans*, halotolerant *O. iheyensis* and thermophilic *G. kaustophilus*, in addition to well-known laboratory strain *B. subtilis* and pathogenic *B. anthracis* and *B. cereus*. These organisms except *B. anthracis* and *B. cereus* are moderately diverged each other and are belonging to distinct major clusters in the 16S rRNA phylogenetic tree. By simple pairwise dotplot analyses between them, one can easily see some large collinear regions along the diagonal lines of the plots so that the overall genomic structures are primarily well conserved between organisms. However, one can also find a substantial number of species-specific genes that are inserted in each of the genomes.

To investigate further, we are trying to identify common "core structure" of bacillar genomes, which is defined as a set of sufficiently long consecutive genomic segments in which gene orders are conserved among multiple genomes so that they are likely to have been inherited from the common ancestor mainly through vertical transfer. We have developed a novel algorithm for aligning conserved regions of multiple genomes by sorting orthologous groups so as to retain the conserved gene orders as many as possible. From this alignment, we were able to identify the common core structure of bacillar genomes comprising about 1500 genes.

It appears that most of the important genes are included in the resulting core gene set. For example, the set contains 246 out of 271 *B. subtilis* essential genes that were primarily determined by a systematic inactivation experiment. Further characterization of the core gene set is currently in progress.

Publication List:

Takami, H., Takaki, Y., Chee, G-J., Nishi, S., Shimamura, S., Suzuki, H., Matsui, S., Uchiyama, I. (2004) Thermoadaptation trait revealed by the genome sequence of thermophilic *Geobacillus kaustophilus*. *Nucleic Acids Res.*, **32**, 6292-6303.

LABORATORY OF DIRECTOR GENERAL

Director General: *KATSUKI, Motoya*
Postdoctoral Fellow: *ARAKAWA-KOBAYASHI, Satoko*
Graduate Student: *MORITA, Tomoko (Apr. '01 - Mar. '03)*
Supporting Staffs: *ETOH, Tomoo (Apr. '01 - Mar. '03)*
MIYAKAWA, Atsushi
KATSUKI, Kuniko
MIYAKAWA, Yuko

One of the largest themes of the 21st century is to promote the brain research, for example, researches on understanding a "mind." There are many approaches to elucidate the "mind," such as intelligence, memory, cognition, emotion and volition. Researches on developing an excellent artificial system for information-processing, researches on the ontogeny and the development of the brain, and researches on the protection of the brain from aging, neurological and psychiatric disorders are also important themes.

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

I. Dopaminergic system and feeding behavior

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

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

To examine involvement of dopaminergic system in neural development we generated mutant mice in which dopaminergic transmission can be shut off at a time point of interest. We utilized tetracycline controllable expression system and generated transgenic mice harboring conditional D1R expression on the *D1R/D2R* DKO background (Figures 1 and 2).

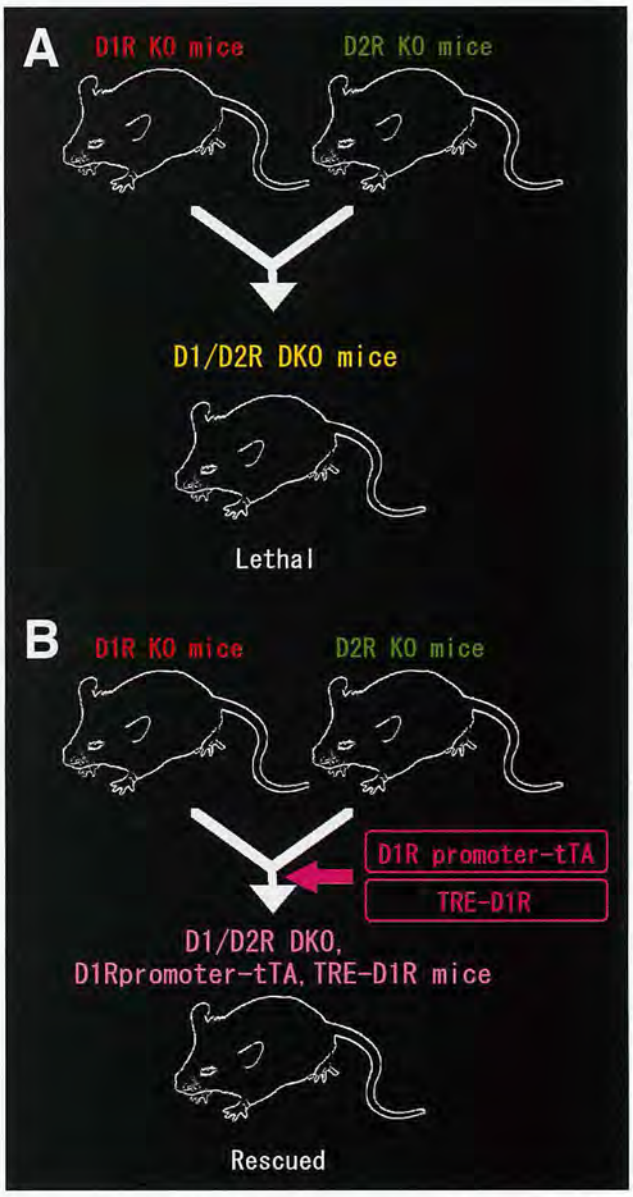

Figure 1. *D1R/D2R* DKO mice were rescued by conditional D1R expression. (A) *D1R/D2R* DKO mice showed impaired feeding and premature death. (B) To rescue *D1R/D2R* DKO mice transgenic mice harboring conditional D1R expression on the *D1R/D2R* DKO background were generated by tetracycline controllable expression system.

We screened potential transgenic lines with respect to tetracycline controllable expression and successfully established the transgenic mice harboring conditional D1R expression (Figure 3). We are producing the conditional D1R expressing mice on the *D1R/D2R* DKO background and plan to examine the mice by molecular biological, morphological and behavioral analyses.

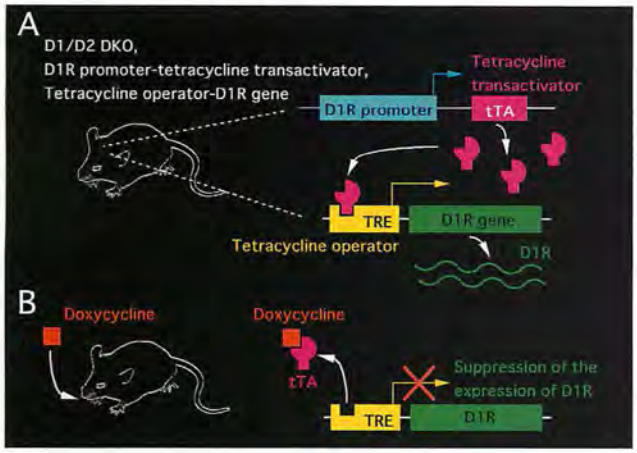

Figure 2. Tetracycline controllable gene expression system. (A) Transgenic mice were generated by introducing of the D1R promoter-driven tetracycline trasnactivator (tTA) and the tetracycline operator-driven D1R gene. In the absence of doxycycline the D1R gene was expressed by the action of tTA and the tetracycline operator. (B) By administration of doxycycline the binding of doxycycline to tTA led to the suppression of the expression of D1R.

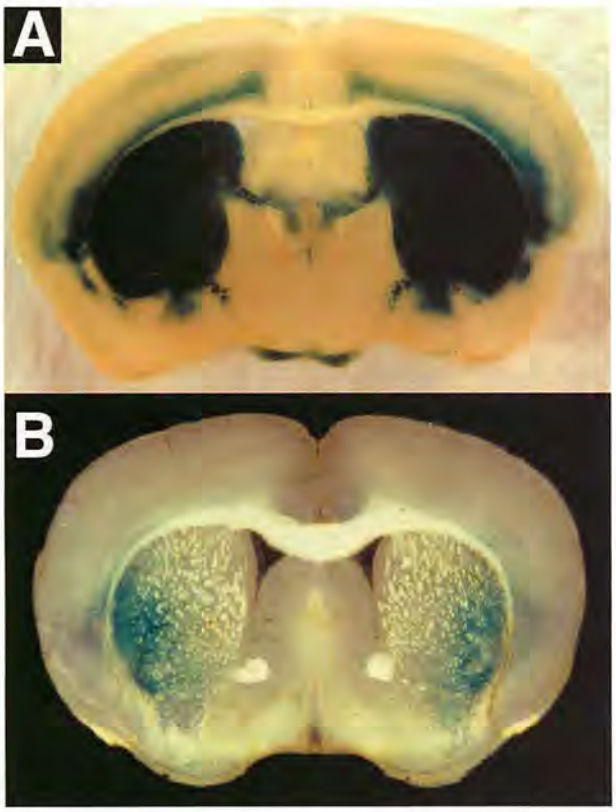

Figure 3. Mice carrying conditional D1R expression were generated using tetracycline system. Frontal sections of mouse brains with X-gal staining were shown. (A) The expression of transgene was seen in the striatum by the expression of a marker gene, *lacZ* (blue color). (B) By doxycycline administration the expression of transgene was suppressed in the striatum.

II. Analysis of the function of NMDARs

The NMDARs are widely expressed in the nervous system, fundamental to excitatory neurotransmission, and play a number of important roles. There are many reports on the involvement of the NMDARs in learning and memory. According to one hypothesis schizophrenia may involve a defect in NMDAR function. NMDARs consist of NR1 subunit and at least one subunit of NR2A-NR2D. The NR1 is ubiquitously expressed in the brain, while NR2 subunits have a more specific spatial distribution. We generated KO mice lacking each of NMDAR subunits and multiple KO mice lacking two subunits simultaneously, and found the *NR2A* homozygous, *NR2B* heterozygous mutant ($NR2A^{-/-}$,$NR2B^{+/-}$) mice exhibited behavioral alteration similar to that observed in patients with schizophrenia. We plan to develop a new experimental devise to assess behavioral alteration of the $NR2A^{-/-}$,$NR2B^{+/-}$ mice and study the molecular mechanism.

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

The *ras* proto-oncogene plays a critical role in cell growth control as a central component of mitogenic signal transduction pathways. In mammals there are H-, N-, K-*ras* identified as the *ras* family. H-, N-, K-*ras* have an overlapped spatial expression pattern as well as an overlapped function. We generated and analyzed KO mice lacking each of H-, N-, and K-*ras*. We found H-Ras was implicated in the regulation of long-term potentiation in the hippocampal CA1 region through NMDAR phosphorylation. To investigate the distinct function of the individual Ras protein in the brain we generated *ras* DKO mice expressing a single Ras and triple KO mice lacking all H-, N-, and K-Ras and analyzed developmental aspects of these mutant mice.

Publication list:

Konishi S, Naora H, Kimura M, Sato M, Nagasaki M, Yokoyama M, Otani H, Moritake K, Katsuki M. (2004) Expression of SV40 T antigen gene in the oligodendroglia induced primitive neuroectodermal tumor-like tumors in the mouse brain. *Congenit Anom (Kyoto).* **44**, 215-224.

Sato M, Tabata T, Hashimoto K, Nakamura K, Nakao K, Katsuki M, Kitano J, Moriyoshi K, Kano M, Nakanishi S. (2004) Altered agonist sensitivity and desensitization of neuronal mGluR1 responses in knock-in mice by a single amino acid substitution at the PKC phosphorylation site. *Eur J Neurosci.* **20**, 947-955.

Kuriwaki J, Nishijo H, Kondoh T, Uwano T, Torii K, Katsuki M, Ono T. (2004) Comparison of brain activity between dopamine D2 receptor-knockout and wild mice in response to dopamine agonist and antagonist assessed by fMRI. *Neurosignals.* **13**, 227-240.

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RESEARCH SUPPORT FACILITY

Head:	*NISHIMURA, Mikio*
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)
	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)

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 spectro-scopical 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 Origin 2000), database server (Sun Enterprise 450), file server (Sun Enterprise 220R), data visualization terminal and molecular simulation terminal (both are SGI Octanes). Some personal computers and color/monochrome printers are also equipped. On this system, we provide various biological databases and data retrieval/analysis programs, and support large-scale data analysis and database construction for the institute members. At the end of this year, a new computer system with enhanced performance was introduced as a replacement for the current system. The service of the new system will start at the beginning of the next year.

Computer laboratory also provides network communication 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 backbone network of Okazaki National Research Institute (ORION). Many local services including sequence analysis service, file sharing service and printer service are provided through this LAN. We also maintain a public World Wide Web server that contains the NIBB home pages (http://www.nibb.ac.jp/).

4. Plant Culture Laboratory

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.

II. Research Activities

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 scien-tists 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:

Cooperative Research Program for the Okazaki Large Spectrograph

Aoki, M., Furusawa, Y., Higashi, S-I. And Watanabe, M. (2003) Action spectra of apoptosis induction and reproductive cell death in L5178Y cells in the UV-B region. *Photochem. Photobiol. Sci.* 2004, **3**, 268-272.

Kami, C., Mukougawa, K., Muramoto, T., Yokota, A., Shinomura, T., Lagarias, J. C. And Kohchi, T. (2004) Complementation of phytochrome chromophore-deficient *Arabidopsis* by expression of phycocyanobilin: ferredoxin oxidoreductase. *Proc. Natl. Acad. Sci.* **101**, 1099-1104.

Kraebs, G., Watanabe, M. And Wiencke, C. (2004) A monochromatic action spectrum for the photoinduction of the uv-absorbing mycosporine-like amino acid shinorine in the red alga *Chondrus crispus*. *Photochem. Photobiol. Sci.* **79**, 515-519.

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Yoshihara, S. And Ikeuchi, M. (2004) Phototactic matility in the unicellular cyanobacterium Synechocystis sp. PCC 6803. *Photochem. Photobiol. Sci.* **3**, 512-518.

RESEARCH CENTER FOR INTEGRATIVE AND COMPUTATIONAL BIOLOGY

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

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

I. Research activity

The research center for integrative and computational biology was founded in 2001 under the situation of rapid progress in modern biology. The success in world wide genome projects has provided a huge amount of information of genes. Following this, it is expected to develop new medicine for intractable diseases or to exploit new cultivated plants resistant to noxious insects, and so on. In addition, it is the subject of present biology to solve higher-order phenomena that are constructed from complex interaction between many genes. To answer these requests, it is essential to decipher the enigma of huge amount of gene information, and to derive the essence for the biological behavior of cells or organisms.

Mathematical and computational sciences have strong property for this condition. Computational methods make it possible to process much larger data than human ability. Hypothetical experiments based on mathematical or computational models make it possible to consider conditions which are impossible in real experiments including the evolution of past organisms. We are continuing to research higher-order phenomena in biology by using mathematical or computational methods, and to develop new methodology for studying such complex phenomena.

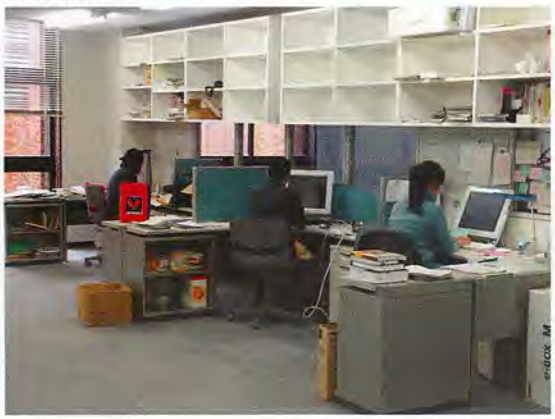

Figure 1. A laboratory room for computational studies.

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

II. Collaboration activity

Interaction between researchers is essential to make the best use of mathematical and computational methods for experimental biology. The theoretical methods should provide testable predictions, and the experimental biologists should test the predictions and return the results to theoretician for the next predictions. By repeating these predictions and tests, the integrative methods should be developed. The research center for integrative and computational biology has continued to enhance interaction between theoretical biologists and experimental biologists.

The center provides equipments for computation and experiments. We have some cluster machines and enough number of Unix based machines for our member. The member can also use computational equipments in Research Center for Computational Science in Okazaki. The center also provides experimental equips for the collaborations with experimental biologists.

Figure 2. one of equipment, a cluster machine.

We have held research conferences repeatedly. In 2004, we held two days symposium in March 8-9, entitled as "Mathematical and Computational Approaches to Biology", where dozens of researchers studying mathematical, computational, or experimental biology participated. The history of mathematical or computational methods for biology is still short. The center has continued to bring up and to encourage young researchers who can use these methods for biological phenomena.

CENTER FOR

TRANSGENIC ANIMALS AND PLANTS

Head: *TAKADA, Shinji*
Associate Professors: *WATANABE, Eiji*
SASAOKA, Toshikuni
TANAKA, Minoru
Technical Staffs: *HAYASHI, Kohji*
ICHIKAWA, Hiromi (Jun. '04 -)
Supporting Staffs: *YASUDA, Mie*
NOGUCHI, Yuji
SHIMIZU, Naoki (- Jul. '04)
YAMAGUCHI, Kyoko
YOSHIDA, Etsuko
KAWAMURA, Motofumi (Jun. '04 -)

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

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

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

Technical staffs and supporting staffs develop and promote research supporting activities. In 2003 two associate professors joined in the CTAP. A new CTAP building for transgenic animals in the Yamate area has opened in the end of 2003 (Figure 1).

The expected activities of the CTAP are as follows:

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

I. Research supporting activity (mouse)

In 2001 the NIBB mouse facility under the specific pathogen free (SPF) condition opened in the Myodaiji area and the production, breeding, analysis, cryopreservation and storage of genetically manipulated mouse strains has been conducted. The new CTAP building in the Yamate area strengthened research activities using genetically altered organisms (Figure 1). The building has five floors and a total floor space of 2,500m^2, in which we can generate, breed, store and analyze transgenic, gene targeting and mutant mice under SPF condition. This building is also equipped with breeding areas for transgenic small fish, birds and insects on the first floor. The mouse housing area of this building is constructed based on a barrier system in which the clean area and the semi-clean area are clearly separated and designed for an efficient and one-directional flow of mice, personnel, articles of animal housing and equipments from the clean area side to the semi-clean area side.

Figure 2. Autoclaves of a large scale for sterilization.

Since research activities in the CTAP building in the Yamate area started in 2004, 3,138 mice brought into the CTAP, and 7,894 mice (including pups bred in the facility) were taken out from the CTAP from April 01, 2004 to December 14, 2004.

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

A new mouse facility in the Myodaiji area is under the construction and will be open in the beginning of 2005. The facility provides research supporting activities to researchers in the Myodaiji area.

Figure 3. Breeding equipments for mutant mice under condition of specific pathogen-free.

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

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

Figure 4. Breeding equipments for transgenic small fish.

In 2004 (as of the end of November), 1,190 medaka (552 embryos and 638 adults) were brought to the facility and 19,776 medaka (15,757 fertilized eggs, 1,662 embryos, and 2,357 adults, including animals bred in the facility) were taken out from the CTAP. In a laboratory for chick embryos 20,349 fertilized chicken eggs were brought in and 213 animals (123 fertilized eggs, 85 embryos and 5 broods) were taken out from the CTAP. These animals were used for the research activities in neurobiology and developmental biology.

Academic activity

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

THE CENTER FOR ANALYTICAL INSTRUMENTS

(managed by NIBB)

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Technical Staffs: *MORI, Tomoko*
MAKINO, Yumiko
TAKAMI, Shigemi
Technical Assistants: *MORIBE, Hatsumi*
DODO, Yukiko
KAI, Tsutomu
Secretary: *HATTORI, Nobuko*

The Center serves for amino acid sequence analysis, amino acid analysis, and chemical syntheses of peptides and to support researchers in NIBB and NIPS. Newly installed instruments in 2004 is Genetic Anlyzer(ABI PRISM 3100). 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)

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

Automatic Nucleic Acid Isolation System (Kurabo NA-2000)

Genetic Anlyzer(ABI PRISM 3100,310)

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

Integrated Thermal Cyclers (ABI CATALYST Turbo 800)

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

Gas Chromatograph (Shimadzu GC-14APF-SC)

Glycoprotein Analysis System (Takara Glyco-Tag)

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

Integrated Micropurification System (Pharmacia SMART)

Flow Cytometer (Coulter EPICS XL)

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

Laboratory Automation System (Beckman Coulter Biomek 2000)

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)

TECHNICAL DIVISION

Head: FURUKAWA, Kazuhiko

Common Facility Group

Research 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)
TAKESHITA, Miyako (Technical Assistant)
NISHIMURA, Noriko (Technical Assistant)

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MAKINO, Yumiko (Subunit Chief)
TAKAMI, Shigemi
MORIBE, Hatsumi (Technical Assistant)
DODO, Yukiko (Technical Assistant)

Transgenic Animal Facility
HAYASHI, Kohji (Subunit Chief)
KAWAMURA, Mamiko (Technical Assistant)
ICHIKAWA, Hiromi (Technical Assistant)

Disposal of Waste Matter Facility
(MATSUDA, Yoshimi (Unit Chief))

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

Research Support Group

Chief: KOBAYASHI, Hiroko

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SUMIKAWA, Naomi

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KONDO, Makiko (Technical Assistant)

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MIWA, Tomoki (Unit Chief)

Reception

KATAOKA, Yukari
NAKANE, Kaori
TSUZUKI, Shihoko
HIRONAKA, Tomie
MUKOHDA, Yasuyo
UNO, Satoko

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.

OKAZAKI INSTITUTE FOR INTEGRATIVE BIOSCIENCE

(jointly managed by NIBB)

DEPARTMENT OF DEVELOPMENT, DIFFERENTIATION AND REGENERATION

DEPARTMENT OF BIO-ENVIRONMENTAL SCIENCE

CENTER FOR RADIOISOTOPE FACILITIES (CRF)

Head:	*TAKADA, Shinji*
	(Professor, concurrent post)
Associate Professor:	*OGAWA, Kazuo*
	(Radiation Protection Supervisor)
Technical staffs:	*MATSUDA, Yoshimi*
	(Radiation Protection Supervisor)
	SAWADA, Kaoru
	IINUMA, Hideko
Supporting Staffs:	*ITO, Takayo*
	KATAGIRI, Izumi
	KAMIYA, Kiyomi (Dec. 16 -)
	KANEUJI, Kimie (Apr. 16 -)

Technical and supporting staffs of the CRF maintain three 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.

Matsuda, Iinuma, Ito, and kaneuji are maintaining CFBI(Common Facilities Building I)-branch and LGER (Laboratory of Gene Expression and Regulation)-branch in the Myodaiji-Area, and Ogawa, Sawada, Katagiri, and Kamiya working in the Yamate-Area.

Number of registrants and users in 2004 April to November are shown in table 1.

Users going in and out the controlled areas counted by the monitoring system are 5,046 persons in 2004 April to November. The items in each area is shown in Figure 1 and table 2. Annual changes of registrants and number of totals per year is shown in figure 2. And amounts of radioisotopes used in the CRF is shown in Table 3.

At 2004 December ,the animal radiation wastes drying system was renewed. (figure 3) This system can dry animal bodies etc. with heat, and can perform the pretreatment handed over to JRA.

	Myodaiji-Area	Yamate-Area
registrants	199	113
users	118	50

Table 1. Number of registrants and users in the 2004.

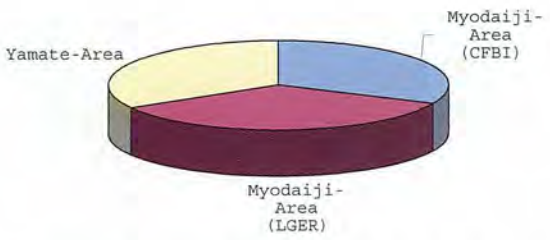

Figure 1. Percentage of users going in and out the controlled areas in 2004.

	Myodaiji-Area		Yamate-Area	total
	CFBI-branch	LGER-branch		
users	1382	1675	1459	4516
visitors	225	86	219	530
total	1607	1761	1678	5046

Table 2. The numbers of users and visitors who entered into an each controlled area in 2004

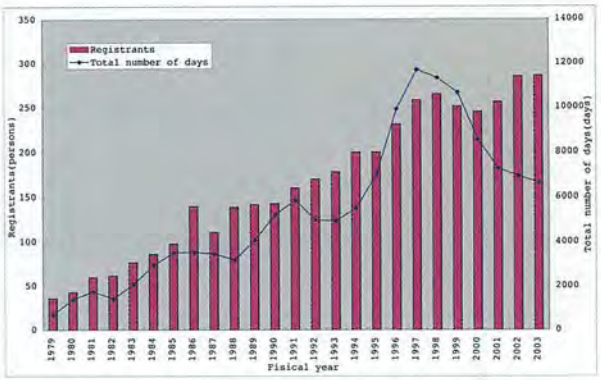

Figure 2. Annual changes of registrants and number of totals per fisical year

	Myodaiji-Area		Yamate-Area	Total
	CFBI-branch	LGER-branch		
^{125}I Received	1110.000	0.000	1110.000	2220.000
^{125}I Used	1110.000	0.000	740.000	1850.000
^{35}S Received	1217000.000	259000.000	523180.000	1999180.000
^{35}S Used	969500.000	65380.000	734161.826	1769041.826
^{32}P Received	558750.000	1147000.000	1017000.000	2722750.000
^{32}P Used	394217.000	912020.000	788340.000	2094577.000
^{14}C Received	11470.000	11100.000	0.000	22570.000
^{14}C Used	1917.060	5651.000	0.000	7568.060
^{3}H Received	74740.000	0.000	925000.000	999740.000
^{3}H Used	48148.840	0.000	747400.000	795548.840

Table 3 Balance of radioisotopes received and used (MBq)

Figure 3. the animal radiation wastes drying system (Wakaida Engneering Co. type WINDY2000)

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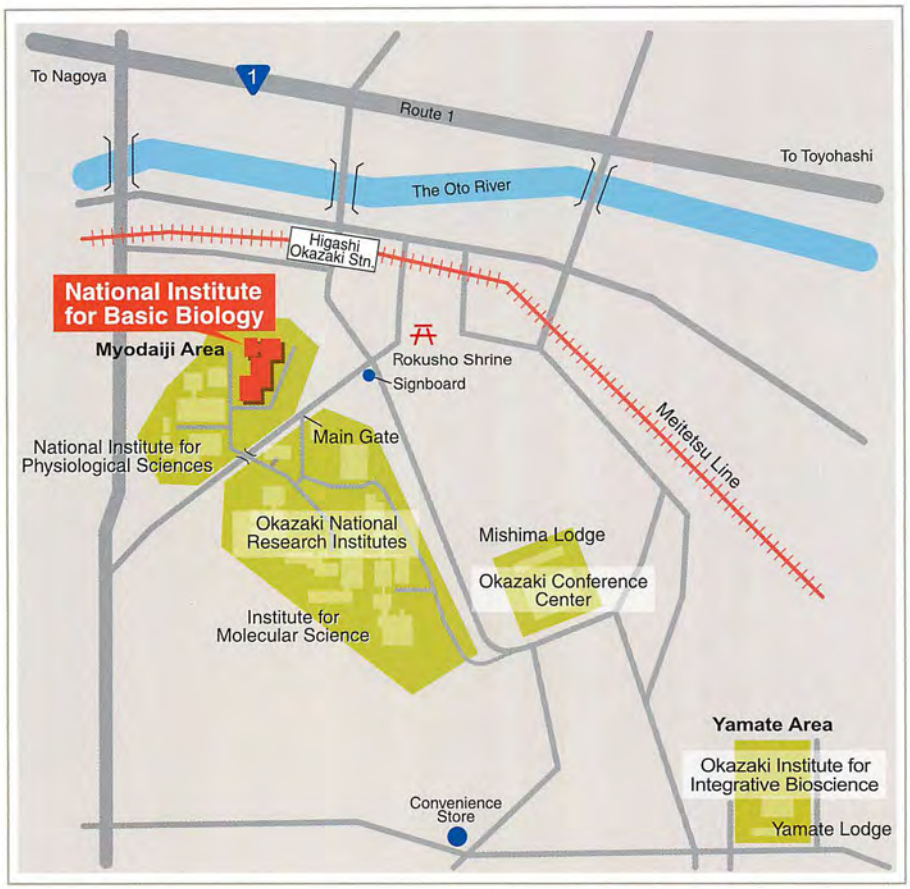

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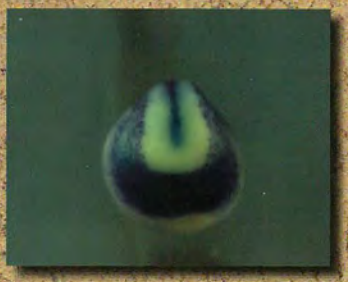

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