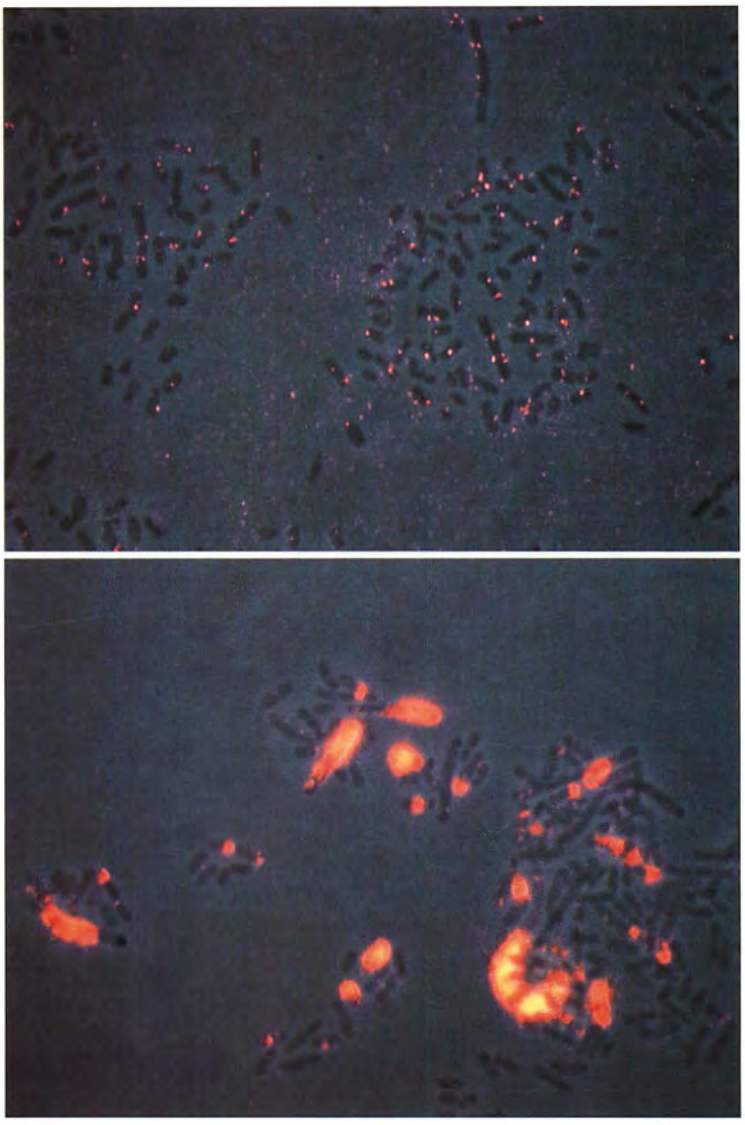
NATIONAL INSTITUTE FOR BASIC BIOLOGY

岡崎国立共同研究機構

基 礎 生 物 学 研 究 所



ANNUAL REPORT
2001

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The cover photographs are FISH images which indicate amplification of a specific DNA region, called HotA, on the genome of *Escherichia coli.* For details, see p49.

INTRODUCTION

The National Institute for Basic Biology (NIBB) is a government-supported research institute 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 the basic biology but also the modern biological sciences by conducting first-rate research on site as well as in cooperation with other universities and research organizations. Researchers at NIBB investigate cell structure and function, reproduction and development, neuronal and environmental biology, gene expression and regulation, and molecular evolution of eukaryotic organisms to elucidate the general and fundamental mechanisms underlying various biological phenomena.

In the beginning of April, 2001, Dr. KATSUKI, Motoya, the former Professor of the University of Tokyo and the adjunctive Professor of the NIBB, took over the position of the Director-General from the former Director-General Dr. MOHRI, Hideo, who retired from the NIBB due to his appointment as the President of the Okazaki National Research Institutes.

In April, Dr. KOBAYASHI, Satoru, who had been an Assistant Professor of Tsukuba University, became the new Professor of the Division of Development, Differentiation and Regulation in the Center for Integrative Bioscience (CIB) and made a joint appointment for the NIBB. Dr. TAKADA, Shinji, who is a Professor of Kyoto University, was also joint-appointed as a Professor of CIB and Kyoto University in March. He will join the CIB in April, 2002, as a Professor.

Drs. MURATA, Takashi and KINOSHITA, Noriyuki were appointed as Associate Professors in the Division of Speciation Mechanisms II in April and the Division of Morphogenesis in September, respectively.

Drs. MUKAI, Masanori and KOSHIDA, Sumito, were appointed as Research Associates in CIB in April. Drs. SAKUTA, Hiraki and TSUGANE, Kazuo were also appointed as Research Associates in NIBB in October.

Congratulatingly, Drs. SAKAMOTO, Atsushi, INAGAKI, Yoshishige and NISHIYAMA, Yoshitaka were promoted to Associate Professors of Hiroshima University, Okayama University and Ehime University, respectively. Associate Professor Dr. MAEDA Nobuaki left NIBB and became a staff scientist vice-Director (equivalent to Associate Professor) of the Tokyo Metropolitan Institute for Neuroscience in November. Three research associates left their positions in NIBB to become the equivalent positions in other institutes or overseas. In addition, we replaced 2 research associates, and 2 institute research fellows with 2 research associates and 7 institute research fellows, and appointed 1 technician. The total number of personnels working at NIBB including graduate students and post doctoral fellows has been kept at approximately 300 for several years.

As a COE of the biological research institute, NIBB is responsible for conducting research projects in cooperation with various research groups. As a part of

such cooperative activities, NIBB hosts International Conferences. In March the 45th NIBB International Conference was sponsored by the Ministry of Education, Culture, Sports, Science and Technology and Ministry of Environment entitled "Recent Progress in Endocrine Disruptor Research" (Professor T. Iguchi, organizer). The 46th NIBB International Conference entitled "Genetics and Epigenetics: The First 100 Years" (Professors S. IIda, M. Katsuki, S. Inagaki, et al., organizers) sponsored by the Ministry of Education, Culture, Sports, Science and Technology was also held in March. In addition, NIBB continues to sponsor interdisciplinary symposia and study meetings on current topics by inviting leading scientists from around the world to the Institute. NIBB also provides a training course in biological sciences for young investigators. To assess our continuing improvement, the activities and future plans of two professors who have spent 10 years at NIBB were subjected to peer review by international scholars in related fields. Prof. NISHIMURA, Mikio and Prof. HORIUCHI, Takashi were reviewed on the 10-year Evaluation by the committee organized by the former Director-General Dr. MOHRI and the results were accepted by the Advisory Council of the NIBB held in March. We always welcome any suggestions concerning the research activities of NIBB.

Finally, we performed the "Open Institute" for local society on 13th of October to show our activities and about 1,600 people joined this event.

KATSUKI, Motoya, D.Sc.
Director-General

ORGANIZATION OF THE INSTITUTE

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

Policy and Decision Making

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

Administration

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

Research

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

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

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

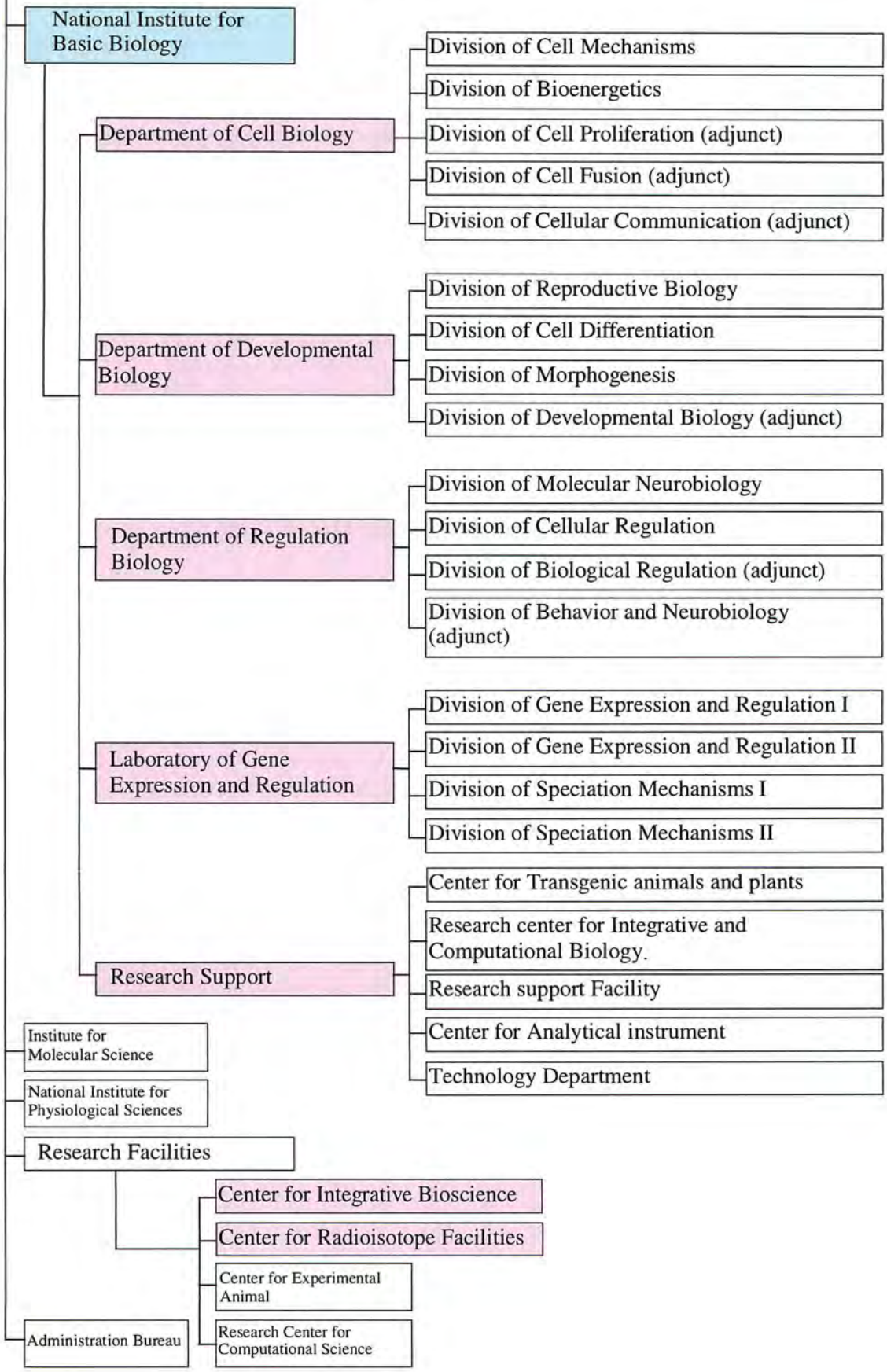
Research Support Facilities

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

Campus

The Okazaki National Research Institutes covers an area of 150,000m^2 with four principal buildings. The NIBB's main research building has a floor space of 10,930m^2. Two-thirds of the space was completed in 1982 and the remaining third in June, 1983. The buildings which 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.

Okazaki National Research Institutes



MEMBERS OF THE COUNCIL

ISHIGE, Naomichi	Director-General, National Museum of Ethnology
IWATSUKI, Kunio	Professor, The University of Air
EGUCHI, Goro	President, Kumamoto University
OHSAKI, Hitoshi	Director-General, Center for National University of Finance
OKADA, Masukichi	Vice-President, International Institute for Advanced Studies
OGAWA, Tomoko	Professor, Iwate College of Nursing
KISHIMOTO, Tadamitsu	President, Osaka University
SHIMURA, Yoshirou	Director, Biomolecular Engineering Research Institute
SUZUKI, Akinori	President, Akita Prefectural University
TAKEICHI, Masatoshi	Professor, Kyoto University
TAKEUCHI, Ikuo	President, Novartis Foundation (Japan) for the Promotion of Science
NAKAMURA, Keiko	Deputy Director-General, JT Biohistory Research Hall
HIDAKA, Toshitaka	Director-General, Research Institute for Humanity and Nature
HOSHI, Motonori	Professor, Keio University
HOTTA, Yoshiki	Director-General, National Institute of Genetics
YAMASHITA, Koujun	Dean, Nagoya University
YOSHIKAWA, Hiroshi	Advisor, JT Biohistory Research Hall
YOSHIDA, Mitsuaki	Director, Banyu Tsukuba Research Institute
YONEYAMA, Toshinao	President, Otemae University
WATANABE, Okitsugu	Director-General, National Institute of Polar Research

MEMBERS OF THE ADVISORY COMMITTEE FOR PROGRAMMING AND MANAGEMENT

AIZAWA, Shinichi	Professor, Kumamoto University
OKADA, Kiyotaka	Professor, Kyoto University
KUROSAWA, Yoshikazu	Professor, Fujita Health University
GO, Michiko	Professor, Nagoya University
KOMEDA, Yoshifumi	Professor, Hokkaido University
KONDO, Hisato	Professor, Osaka University
SASAZUKI, Takehiko	Director-General, International Medical Center of Japan Research Institute
HASUNUMA, Kohji	Professor, Yokohama City University
MACHIDA, Yasunori	Professor, Nagoya University
YAMAMOTO, Masayuki	Professor, University of Tokyo
IGUCHI, Taisen	Professor, Okazaki National Research Institutes, Center for Integrative Bioscience.
IIDA, Shigeru	Professor, Okazaki National Institute for Basic Biology
UENO, Naoto	Professor, Okazaki National Institute for Basic Biology
OHSUMI, Yoshinori	Professor, Okazaki National Institute for Basic Biology
NAGAHAMA, Yoshitaka	Professor, Okazaki National Institute for Basic Biology
NISHIMURA, Mikio	Professor, Okazaki National Institute for Basic Biology
NODA, Masaharu	Professor, Okazaki National Institute for Basic Biology
HORIUCHI, Takashi	Professor, Okazaki National Institute for Basic Biology
MURATA, Norio	Professor, Okazaki National Institute for Basic Biology
MOROHASHI, Ken-ichiro	Professor, Okazaki National Institute for Basic Biology
YAMAMORI, Tetsuo	Professor, Okazaki National Institute for Basic Biology

GRADUATE PROGRAMS

The NIBB sponsors two graduate programs.

1. Graduate University for Advanced Studies

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

The Department consists of the following Divisions and Fields:

Divisions	Fields
Molecular Cell Biology	Biomolecular Systems Cell Dynamics
Developmental Gene Expression and Regulation	Gene Expression Morphogenesis Transgenic Biology
Regulation Biology	Biological Regulation Biological Information

2. Graduate Student Training Program

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

OFFICE OF DIRECTOR

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

Mechanisms determining the outline shape of the adult lepidopteran wings

KODAMA, Ryuji

Wings of the lepidopteran insects (butterflies and moths) develop from the wing imaginal disc, which is a hollow sac made of simple epithelium. When the pupariation is completed, the wing, which was hidden inside the body wall of the larvae, is exposed on the surface of the pupa, which gradually turns into the adult wing. 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, while the internal area develops as adult wing blade. The marginal dying area is called the degeneration region and the internal area is called the differentiation region, hereafter.

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 regeneration region have two characteristics common with the apoptotic cell death in mammalian cells. These are i) the presence of apoptotic bodies, which are heavily condensed cells or their fragments engulfed by other cells or macrophages, shown by transmission electron microscopy and ii) the presence of conspicuous accumulation of fragmented DNA evidenced by the TUNEL histological staining (Kodama, R. et al., Roux's Arch. Dev. Biol. 204, 418-426, 1995).

The cells in the degeneration region are actively engulfed by the macrophages in the cavity beneath the wing epithelium. Moreover, 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. By injecting the india ink or ferritin solution to the body cavity of the pupa, we have confirmed that this adhesion is tight enough to exclude the macrophages from the differentiation region, because the injected probes was found mostly concentrated in the degeneration region when observed several minutes later (Yoshida, A. (Biohistory Research Hall) and Kodama, R., unpublished).

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 trcheoles 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 through 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.

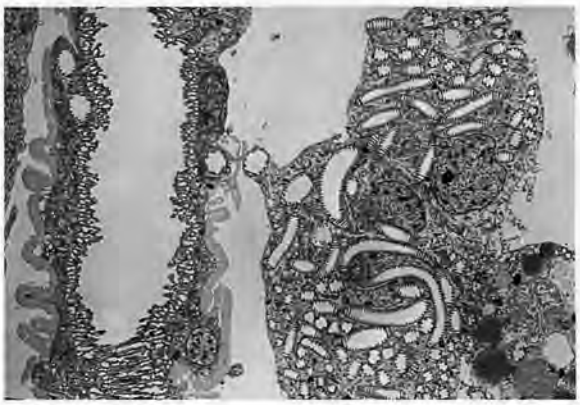


Fig.1. The tracheole cells (on the right) depart from the primary trachea (thick tube on the left) leaving behind tracheole, which was stored within the cytoplasm of the tracheole cell.

The figure depicts how the tracheoles protrude from the primary trachea at the pre-pupa stage (Fig. 1). The tracheoles are arranged with even spaces within the wing lumen and may closely related with the scale cell pattern formation.

In collaboration with other developmental biologists, a database of cellular behaviors, which are essential steps of morphogenesis of multicellular organisms, is deviced (S3-P7, 14th ICDB, Kyoto, 2001). An instance of the database is basically made up of (i) the subject cell, (ii) the input the cell receives, and (iii) the output the cell excecutes. With the aid of methodologies in the field of information science and computer science, automatization of database collection is planned. This database will be important in incorporating rapidly increasing knowledge in developmental biology in highly reusable form even by non-expert researches of this filed.

Protein palmitoylation and its role in neural developmental at embryogenesis

UENO, Kohji

Protein palmitoylase modifies specific cysteine residues of signalling proteins such as G proteins and G protein-coupled receptors with palmitate via thioester linkages. This modification is thought to be important in the regulation of signal transduction. We have previously found that protein palmitoylase is expressed in neural cells during mouse embryogenesis.

Immunocytochemical analyses revealed that growth cone-associated protein (GAP)-43 and G_o, which is one of G proteins, were mainly localized in growth cones and the cell body of cultured primary neural cells. Fig.

2 shows the localization of protein palmitoylase and GAP-43 in a neural cell. Cysteine residues in the amino terminal regions of GAP-43 and G_o are palmitoylated. Furthermore an inhibitor of protein palmitoylase reduces the axonal growth of cultured neurons. From these findings, we speculated that the localization of the palmitoylated proteins in growth cones are critical for the development of axons.

In this study, we are attempting to elucidate the mechanism that determines the localization of palmitoylated proteins in growth cones. For this analysis, we have established a method to chemically modify the amino terminal region of a synthetic GAP-43 peptide with palmitate or other fatty acids via a thioester linkage. Using these acylated peptides, we plan to analyze the mechanism that regulates the localization of palmitoylated peptide in growth cones.

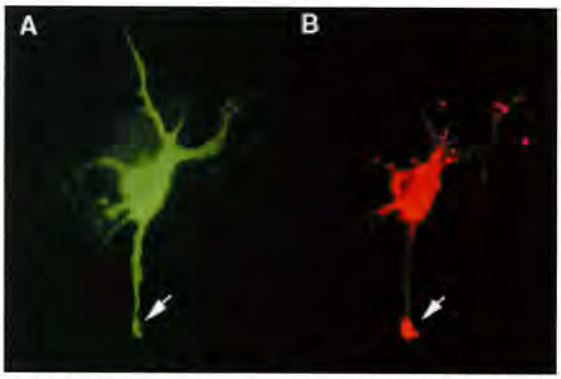


Fig. 2 Localization of protein palmitoylase and GAP-43 in a cultured primary neural cell. (A) and (B) A neuron was double-labeled with anti-protein palmitoylase and anti-GAP-43 antibodies, respectively. Protein palmitoylase (green) was widely detected in growth cones, processes, and the cell body, whereas GAP-43 (red) was mainly localized in growth cones and the cell body.

DEPARTMENT OF CELL BIOLOGY

Chairperson: OHSUMI, Yoshinori

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

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

DIVISION OF CELL MECHANISMS

Professor:	*NISHIMURA, Mikio*
Associate Professor:	*HAYASHI, Makoto*
Research Associate:	*MANO, Shoji*
Technical Staff:	*KONDO, Maki*
NIBB Research Fellow:	*SHIRAHAMA, Kanae*
Post doctoral Fellows:	*HAYASHI, Yasuko (~March 15)*
	HAYASHI, Hiroshi (~March 31)
	MITSUHASHI, Naoto (~March 31)
Graduate Students:	*NITO, Kazumasa*
	WATANABE, Etsuko
	FUKAO, Youichiro
	KAMADA, Tomoe (April1~)
	HATSUGAI, Noriyuki (April 1~)
Technical Assistants:	*KUROYANAGI, Miwa (~March 31)*
	NAKAMORI, Chihiro
	TAKEI, Rie (~April 30)
	YAGI, Mina (March 1~)
Secretaries	*UEDA, Chizuru*
	KOMORI, Akiko

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 un-dergo a transformation of function, while other organelles may degenerate. Because the dynamic transformation of organellar function (differentiation of organ-elles) 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. For example, etioplasts diffe-rentiate 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.

To investigate the roles of peroxisomal membrane proteins in the reversible conversion of glyoxysomes to leaf peroxisomes, we characterized several membrance proteins of glyoxysomes. One of them (PMP38) was identified a putative ATP/ADP carrier protein. Cell fractionation and immunocytochemical analysis using pumpkin cotyledons revealed that PMP38 is localized on peroxisomal membranes as an integral membrane protein. The amount of PMP38 in pumpkin cotyledons increased and reached the maximum protein level after 6 d in the dark but decreased thereafter. Illumination of the seedlings caused a significant decrease in the amount of the protein. These results clearly showed that the membrane protein, PMP38 in glyoxysomes changes dramatically during transformation of glyoxysomes to leaf peroxisomes, as do the other glyoxysomal enzymes, especially enzymes of the fatty acid β-oxidation cycle, that are localized in the matrix of glyoxysomes. An ascorbate peroxidase (pAPX) was also identified as one of glyoxysomal membrane proteins. Its cDNA was isolated by immunoscreening. The deduced amino acid sequence encoded by the cDNA insert does not have a peroxisomal targeting signal (PTS), suggesting that pAPX is imported by one or more PTS-independent pathways. Subcellular fractionation of 3- and 5-d-old cotyledons of pumpkin revealed that pAPX was localized not only in the glyoxysomal fraction, but also in the ER fraction. A magnesium shift experiment showed that the density of pAPX in the ER fraction did not increase in the presence of Mg^{2+}, indicating that pAPX is not localized in the rough ER. Immunocytochemical analysis using a transgenic Arabidopsis which expressed pumpkin pAPX showed that pAPX was localized on peroxisomal membranes, and also on a unknown membranous structure in green cotyledons. The overall results suggested that pAPX is transported to glyoxysomal membranes via this unknown membranous structure.

II. Peroxisomes defective mutant of Arabidopsis.

It has been suggested that the functional conversion between glyoxysomes and leaf peroxisomes is controlled by gene expression, protein translocation, and protein degradation. A genetic approach is an effective strategy toward understanding the regulatory mechanism(s) of peroxisomal function at the level of gene expression, protein translocation, and protein degradation. We isolated and characterized 2,4-dichloro-phenoxybutyric acid (2,4-DB)-resistant mutants. It has been demonstrated previously that 2,4-dichlorophe-noxybutyric acid (2,4-DB) is metabolized to produce a herbicide, 2,4-D, by the action of peroxisomal fatty acid β-oxidation in higher plants. To isolate mutants that have defects in peroxisomal fatty acid β-oxidation, we screened mutant lines of Arabidopsis seedlings for growth in the presence of toxic levels of 2,4-DB. Genetic analysis revealed that these mutants can be classified as carrying alleles at three independent loci, which we designated *ped1*, *ped2*, and *ped3*, (where *ped* stands for peroxisome defective). The *ped1* mutant lacks the 3-ketoacyl CoA thiolase, an enzyme involved in fatty acid β-oxidation during germination and subsequent seedling growth, while *At*Pex14p, the *PED2* gene product, is a peroxisomal membrane protein that determines the peroxisomal protein targeting. *PED3* gene was recently identified by

positional cloning. The phenotype of the *ped3* mutant indicated that the mutation in the *PED3* gene inhibits the activity of fatty acid β-oxidation. Ped3p, the *PED3* gene product, is a 149-kD protein that exists in peroxisomal membranes. The amino acid sequence of Ped3p had a typical characteristic for "full-size" ATP-binding cassette (ABC) transporter consisting of two transmembrane regions and two ATP-binding regions. This protein was divided into two parts, that had 32% identical amino acid sequences. Each domain showed a significant sequence similarity with peroxisomal "half" ABC transporters so far identified in mammals and yeast. Ped3p may contribute to the transport of fatty acids and their derivatives across the peroxisomal membrane. *ped1/ped3* double mutant showed severe defects on leaves and inflorescences, and was sterile (Fig. 1). The phenotype may tell us unidentified function(s) of plant peroxisomes.

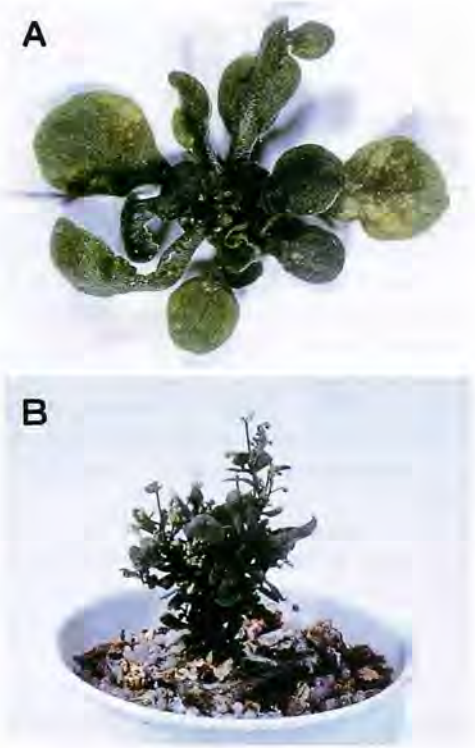


Figure 1. Phenotype of *ped1/ped3* double mutant.

ped1/ped3 double mutant showed vegetative and reproductive phenotypes. It had wavy leaves with irregular shapes (Fig. 1A). Influorescence of the double mutant was difficult to develop, but it occasionally had dwarf inflorescences with abnormal structure (Fig. 1B). Although the inflorescences had some flowers, it was sterile. These phenotypes were not found in the parents, *ped1* and *ped2*.

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

Novel vesicles designated precursor-accumulating (PAC) vesicles that accumulate large amounts of proprotein precursors of storage proteins were purified and characterized from maturing pumpkin seeds. These vesicles had diameters of 300 to 400 nm and contained an electron-dense core of storage proteins surrounded by an electron-translucent layer and were shown that the PAC vesicles mediate a transport pathway for insoluble aggregates of storage proteins directly to protein storage vacuoles. We found a novel membrane protein with molecular mass of 73 kDa, MP73, on the membrane of protein storage vacuoles of pumpkin seeds. MP73 appeared during seed maturation and disappeared rapidly after seed germination, in association with the morphological changes of the protein storage vacuoles. Immunocytochemistry and an immunoblot analysis showed the PAC vesicles accumulated proMP73, but not MP73, on the membranes. Subcellular fractionation of the pulse-labeled maturing seeds demonstrated that the proMP73 form with N-linked oligosaccharides was synthesized on the ER and then transported to protein storage vacuoles via PAC vesicles. Tunicamycin-treatment of the seeds resulted in the efficient deposition of proMP73 lacking the oligosaccharides into the PAC vesicles, but no accumulation of MP73 in vacuoles. After arrival at protein storage vacuoles, proMP73 was cleaved by the action of a vacuolar enzyme to form a 100-kD complex on the vacuolar membranes. These results show that PAC vesicles mediate delivery of not only storage proteins but also membrane proteins of protein storage vacuoles. In order to investigate the mechanism of the PAC vesicle formation, we constructed chimeric genes that encode fusion proteins consisting to both various lengths of polypeptides derived from pumpkin 2S albumin and a selectable marker enzyme, phosphinothricin acetyltransferase and expressed in Arabidopsis. A fusion protein expressed by one of the chimeric genes is accumulated as a proprotein-precursor form, and localized in novel vesicles of vegetative cells, that show distinct features that well much to the PAC vesicles. Arabidopsis mutants that defect vesicular transport of the fusion protein are now screened and characterized by using the transgenic plants.

Plants degrade cellular materials during senescence and under various stresses. The precursors of two stress-inducible cysteine proteinases, RD21 and a vacuolar processing enzyme (VPE), were specifically accumulated in ~0.5 μm diameter x ~5 μm long bodies in *Arabidopsis thaliana*. Such bodies have previously been observed in Arabidopsis but their function was not known. Because these bodies contain precursors of lytic enzymes, we propose to call them ER bodies. They are surrounded with ribosomes and thus are assumed to be directly derived from the endoplasmic reticulum. ER bodies develop specifically in the epidermal cells of healthy seedlings. These cells are easily wounded and stressed by the external environment. When the seedlings are stressed with a concentrated salt solution, leading to death of the epidermal cells, the ER bodies start to fuse with each other and with the vacuoles, thereby mediating the delivery of the precursors directly to the vacuoles. This regulated, direct pathway differs from the usual case in which proteinases

are transported constitutively from the endoplasmic reticulum to the Golgi complex and then to vacuoles, with intervention of vesicle-transport machinery, such as a vacuolar-sorting receptor or a syntaxin of the SNARE family. Thus, the ER bodies appear to be a novel proteinase-storing system that assists in cell death of the vegetative organs of higher plants.

IV. Role of molecular chaperones in organelle 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 organelle 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) and a chloroplast co-chaperonin (Cpn20) from Arabidopsis thaliana. In 2001, we characterized a third co-chaperonin. The cDNA was 603 base pairs long, encoding a protein of 139 amino acids. From a sequence analysis, the protein was predicted to have one Cpn10 domain with an amino-terminal extension that might work as a chloroplast transit peptide. This novel Cpn10 was confirmed to be localized in chloroplasts, and we refer to it as chloroplast Cpn10 (chl-Cpn10). The phylogenic tree that was generated with amino acid sequences of other co-chaperonins indicates that chl-Cpn10 is highly divergent from the others. In the GroEL-assisted protein folding assay, about 30% of the substrates were refolded with chl-Cpn10, indicating that chl-Cpn10 works as a cochaperonin. A Northern blot analysis revealed that mRNA for chl-Cpn10 is accumulated in the leaves and stems, but not in the roots. In germinating cotyledons, the accumulation of chl-Cpn10 was similar to that of chloroplastic proteins and accelerated by light. It was proposed that two kinds of co-chaperonins, Cpn20 and chl-Cpn10, work independently in the chloroplast.

Publication List:

Fukao, Y., Y. Hayashi, S. Mano, M. Hayashi and M. Nishimura (2001) Developmental analysis of a putative ATP/ADP carrier protein localized on glyoxysomal membranes during the peroxisome transition in pumpkin. *Plant Cell Physiol.* **42**: 835-841

Hayashi, M. and M. Nishimura (2002) Genetic approaches to understand plant peroxisomes. in *Plant peroxisomes*, edited by A. Baker and I. Graham, Kluwer Acad. Pub. in press

Hayashi, M., K. Nito, R. Takei-Hoshi, M. Yagi, M. Kondo, A. Suenaga, T. Yamaya and M. Nishimura (2002) Ped 3p is a peroxisomal ATP-binding cassete transporter that might supply substrates for fatty acid β-oxidation. *Plant Cell Physiol.* **43**:1-11

Hayashi, Y., M. Hayashi, H. Hayashi, I. Hara-Nishimura and M. Nishimura (2001) Direct interaction between glyoxysomes and lipid bodies in etiolated cotyledons of *Arabidopsis thaliana ped1* mutant . *Protoplasma* **218**: 83-94

Hayashi, Y., K. Yamada, T. Shimada, R. Matsushima, N. K. Nishizawa, M. Nishimura and I. Hara-Nishimura (2001) A proteinase-storing body that prepares for cell death or stresses in the epidermal cells of Arabidopsis. *Plant Cell Physiol.* **42**: 894-899

Kimura, Y., S. Matsuno, S. Tsurusaki, M. Kimura, I. Hara-Nishimura and M. Nishimura (2002) Subcellular localization of endo-β-N-acetylglucosaminidase and high-mannose type free N-glycans in plant cell. *Biochim. Biophys. Acta* in press

Koumoto, K., T. Shimada, M. Kondo, I. Hara-Nishimura and M. Nishimura. (2001) Chloroplasts have a novel Cpn10 in addition to Cpn20 as co-chaperonins in *Arabidopsis thaliana. J. Biol. Chem.* **276**: 29688-29694

Mano, S., C. Nakamori, M. Hayashi, A, Kato, M. Kondo and M. Nishimura (2002) Distribution and characterization of peroxisomes in Arabidopsis by visualization with GFP. *Plant Cell Physiol.* in press

Minamikawa, T., K. Toyooka, T. Okamoto, I. Hara-Nishimura and M. Nishimura (2001) Degradation of ribulose 1,5-bisphosphate carboxylase/oxygenase by vacuolar enzymes of senescing French bean leaves: Immunocytochemical and ultrastructural observations. *Protoplasma* **218**: 144-153

Mitsuhashi, N., Y. Hayashi, Y. Koumoto, T. Shimada, T. Fukasawa-Akada, M. Nishimura and I. Hara-Nishimura (2001) A novel membrane protein of protein bodies that is transported to protein-storage vacuoles via precursor-accumulating vesicles. *Plant Cell* **13**: 2361-2372

Nito, K., K. Yamaguchi, M. Kondo, M. Hayashi and M. Nishimura (2001) Pumpkin peroxisomal ascorbate peroxidase is localized on peroxisomal membranes and unknown membranous structures. *Plant Cell Physiol.* **42**: 20-27

Tanaka, H., H. Onouchi, M. Kondo, I. Hara-Nishimura, M. Nishimura, C. Machida and Y. Machida (2001) A subtilisin-like serine protease is required for epidermal surface formation in Arabidopsis embryos and juvenile plants. *Development* **128**: 4681-4689

Watanabe, E., T. Shimada, M. Kuroyanagi, M. Nishimura and I. Hara-Nishimura (2002) Calcium-mediated association of a putative vacuolar sorting receptor PV72 with a propeptide of 2S albumin. *J. Biol. Chem.* in press

Xu, W., K. Morita, K. Yamada, M. Kondo, M. Nishimura, H. Shioiri, M. Kojima and M. Nozue (2001) Expression and localization of a 36-kDa vacuolar protein (VP24) precursor in anthocyanin-producing sweet potato cells in suspension culture. *Plant Biotechnol.* **18**: 203-208

Yamada, K., R. Matsushima, M. Nishimura and I. Hara-Nishimura (2001) A unique cysteine protease with a granulin domain that slowly matures in the vacuoles of senescing Arabidopsis leaves. *Plant Physiol.* **127**: 1626-1634

DIVISION OF BIOENERGETICS

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

I. Background

Upon nutrient starvation, autophagic process starts as building up a membrane structure, an autophagosome, in the cytoplasm. The autophagosome sequesters cytosol and organelles nonselectively. Then it is delivered to the vacuole/lysosome, and the cytoplasmic materials inside are degraded by vacuolar/lysosomal proteases. We had discovered autophagy in a simple eukaryotic model organism, *Saccharomyces cerevisiae* and morphologically defined the whole process. We have isolated a set of autophagy-deficient mutants (*apg*), and have cloned most of the *APG* genes essential for autophagy. We are now characterizing these gene products and their homologues in mammals and higher plants.

2. Discovery of novel preautophagosomal structure

We have cloned the last *APG* genes, *APG2*, and characterized of the gene product. We drew a rough functional map of each Apg proteins this year. This is based on the finding of novel structure called preautophagosomal structure (PAS). Many Apg proteins (Apg1, Apg2, Apg5, Apg8, Apg16) are colocalized on preautphagosomal structure. Autophagy-specific phosphatidylinosítol 3-kanise complex (Apg6, Apg14, Vps34 and Vps15) are essential for organization of PAS. Apg8, ubiqitin-like protein, gathers to PAS depending on lipidation reaction and the Apg5-Apg12 conjugates. Apg5 is recruited to PAS depending on Apg16 but independent on Apg12-conjugation. Apg2 is localized on PAS via Apg1. Apg1 protein kinase function in transition from PAS to autophagosome. Thus, PAS is the organizing center of autophagosome.

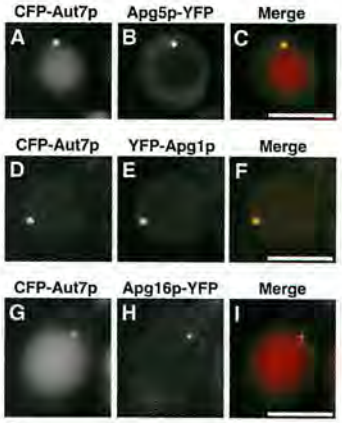

Fig.1 Discovery of preautophagosomal structure
Most Apg proteins are gathered on preautophagosmal structure. Based on this finding, functional role of each Apg protein can be discussed in detail.

3. Autophagosome formation depends on specific sets of coat proteins

We revealed that autophagosme fuses with the vacuole by NSF/SNARE meditated mechanism, which is common through endmembrane system, such as ER and Golgi. However, autophagosome formation is not dependent of NSF. Instead, the involvement the proteins of early secretory pathway are revealed. Sec12, Sec16, Sec23, Sec24 are required for autophagosome formation. Interestingly, they are not prerequisite for Cvt pathway, the similar pathway to autophagy. The findings may imply that early phase of autophagosome formation is related to secretory pathway in some way.

4. Molecular dissection of autophagosome formation

Apg12 and Apg5 are covalently attached in a manner similar to the ubiquitin conjugation system. The Apg12-Apg5 conjugation system is well conserved in mammalian cells. Part of the mammalian Apg12-Apg5 conjugate localized to the isolation membranes when autophagy proceeds, whereas most of it existed in the cytoplasm. Using GFP-tagged Apg5, we revealed that the

cup-shaped isolation membrane is developed from a small crescent-shaped compartment. Apg5 localized on the isolation membrane throughout its elongation process. Apg5 was preferentially distributed in the outer side of the membrane and detaches from it immediately before or after autophagosome formation is completed. In contrast, LC3, the mammalian homologue of Apg8, was associated with both sides of isolation membranes and autophagosomes as well as their precursors. To examine the role of Apg5, we generated Apg5-deficient ES cells. *APG5* $^{-/-}$ cells are viable but bulk protein degradation was significantly reduced. Autophagosome formation was impaired in these cells. The mutant Apg5^{K130R}, which is unable to be conjugated with Apg12, could bind to the autophagosome precursors but could not start elongation of the membranes. Thus, the covalent modification of Apg5 with Apg12 was not required for its membrane targeting but is essential for involvement of Apg5 in elongation of the isolation membranes. Intriguingly, Apg12-Apg5 was required for processing of LC3 and its targeting to the membranes. Therefore, the Apg12-Apg5 conjugate plays essential roles in isolation membrane development in co-operation with LC3. In addition, our studies provided good molecular markers, LC3 and Apg12-Apg5, for autophagic membrane at all stages and isolation membranes, respectively, which so far have been defined only by morphology.

5. Production of phosphatidylinositol 3-phosphate (PI3P) at the *trans*-Golgi network (TGN) is required for autophagy

This year, we reported that, in yeast, Vps34 PI3-kinase and Apg6 form a protein complex that plays a essential role in autophagy by producing PI3P. Then, we found in mammalian cells that the complex of the mammalian Apg6 homologue, beclin and PI3-kinase was distributed in the TGN rather than autophagosomes (Figure 2A). We propose that beclin functions as a regulatory-subunit of the PI3-kinase complex at the TGN, which supplies PI3P for autophagosome formation (Figure 2B).

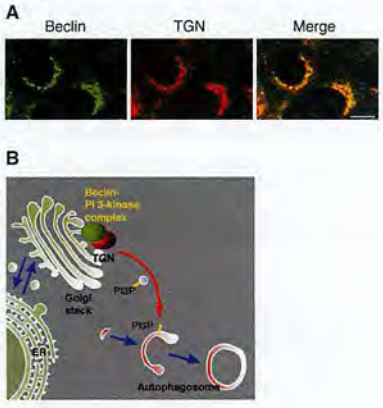

Fig.2
A. Beclin localizes in the TGN.
B. Model for beclin function. The beclin-PI3-kinase complex localized at the TGN supplies PI3P to the membranes in the autophagic pathway.

Original papers :

Ishihara, T., Hamasaki, M., Yokota, S., Suzuki, K., Kamada, Y., Kihara, A., Yoshimori, T., Noda, T., and Ohsumi, Y. (2001) Autophagosome requires specific early Sec proteins for its formation and NSF/SNARE for its fusion to the vacuole. *Mol. Biol. Cell* **12**, 3690-3702.

Kim, J., Kamada, Y., Stromhaug, P. E. Guan, J., Hefner-Gravinnk, A., Bevan, A., Scott, S. V., Ohsumi, Y., Dunn, Jr., W. A., Klionsky, D. J. (2001) Cvt9/Gsa9 function in sequestering selective cytosolic cargo destined for the vacuole. *J. Cell. Biol.* **153**, 381-396.

Kihara, A., Noda, T., Ishihara, N. and Ohsumi, Y. Two distinct Vps34 Ptdlns 3-kinase complex functionin autophagy and CPY. (2001) sorting in Saccharomyces cevisiae. *J. Cell. Biol.* **152**, 519-530.

Kihara, A., Kabeya, Y., Ohsumi, Y., and Yoshimori, T. (2001) Beclin-phosphatidylinositol 3-kinase complex functions at the trans-Golgi Network. *EMBO Report*, **2**, 330-335.

Komtatsu, M., Tanida, I., Ueno, T., Ohsumi, M., Ohsumi, Y., and Kominami, E. (2001) The C-terminal region of an Apg7p/Ctv2p is required for homodimerization and is essential for its E1-activity and E1-E2 complex-formation. *E. J. Biol. Chem.* **276**, 9846-9854.

Mizushima, N., Yamamoto, A., Hatano, M., Kobayashi, Y., Kabeya, Y., Suzuki, K., Tokuhisa, T., Ohsumi, Y and Yoshimori, T. (2001) Dissection of autophagosome formation using Apg 5-deficient mouse embryonic stem cells. *J. Cell. Biol.* **152**, 657-667.

Shintani, T., Suzuki,K., Kamada, Y., Noda, T., Ohsumi, Y. (2001)Apg2p functions in autophagosome formation on the perivacuolar structure. *J. Biol. Chem.* **276**, 30452-30460.

Suzuki, K., Kirisako, T., Kamada, Y., Mizushima, N., Noda, T. and Ohsumi, Y. (2001) The pre-autophagosomal structure organized by concerted functions of *APG* genes is essential for autophagosome formation. *EMBO. J.* **20**, 5971-5981.

Review :

Ohsumi, Y. (2001) Molecular dissection of autophagy: Two ubiquitin-lke systems. *Nature Reviews, Molecular Cell Biol*, **2**, 211-216.

DIVISION OF CELL PROLIFERATION (ADJUNCT)

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The aim of this adjunct division is to understand the basic rules by which elaborate neural circuits develop and function. With less than 10^5 neurones, and subject to powerful molecular and genetic techniques, the brain of the fruit fly *Drosophila melanogaster* is a good model system for investigating the whole of an easily accessible nervous system that shares certain of the architectural and functional features of the more complex vertebrate brains.

I. Comprehensive identification of cells in the adult brain

In spite of the hundred years of efforts using Golgi and other anatomical techniques, the circuit structure of higher-order associative regions of the brain is still essentially unresolved. The GAL4 enhancer-trap system, which is widely used for mutagenesis and gene cloning of *Drosophila*, is also a powerful tool for obtaining a vast array of transformant strains that label specific subsets of brain cells. We screened such strains from a stock of 4500 GAL4 lines made by the *NP consortium*, a joint venture of eight Japanese fly laboratories organised by us. In the first step, all the lines were crossed with the flies carrying the UAS-GFP transgene, which fluoresces only in GAL4-expressing cells. Their patterns were recorded from freshly dissected, unfixed adult brains with a high-speed confocal microscope. In June 2001, we finished making the image database of 131,406 photographs depicting the confocal sections of 3,939 GAL4 strains. From July 2001, we started making a similar database of the late larval brain using the same set of GAL4 strains.

In the second step, useful lines were selected from the database, and fixed and clealised brains were subjected to more precise serial sectioning with conventional confocal microscopes and to three-dimensional reconstruction with UNIX workstations. Although our long-term aim is to get the comprehensive overview of the fly brain neural network, at the initial stage a few brain regions are chosen for intensive study. The first target is to identify projection interneurones that connect lower-level sensory processing sites and higher-order associative regions. These fibres convey olfactory, gustatory, auditory and visual sensory information.

For visual pathways, we identified another 10 types of interneurones that project from the optic lobe to the central brain. The total number of identified neurone types of this kind now became 40. Their structures, as well as their role in the controlled movement of flies, are under investigation.

For olfactory pathways, we selected 34 strains that label various subsets of antennal lobe projection neurones, which transmit information from the first-order olfactory processing site (antennal lobes) to the second-order sites (mushroom bodies and lateral horns). We found that the terminals of these PNs form clear zonal separation in both second-order sites. We identified three zones in the mushroom bodies and nine zones in the lateral horns (Fig. 1). Projection neurones from several glomeruli, each deriving from a single glomerulus of the antennal lobe, converge at selective zones. Another type of neurones, each contributing multiple glomeruli, terminate at zones that overlap with the zones of uniglomerular neurones. The olfactory code across glomeruli is thus both distributed to, and convergent at, discrete zones of the second-order sites.

These stereotypic projection patterns were established before adult eclosion, and surgical ablation of olfactory sense organ caused no significant reorganisation. These suggest that distinct odortypic pathways are established and maintained genetically rather than in an activity dependent manner.

II. Mapping of neurotransmitters and receptors in the adult brain

To understand the function of the identified neural network, it is also important to get the information about the types of neurotransmitters and receptors used in these cells. Previously, such information is obtained by staining brain tissue with various antibodies. This approach, however, has the limitation due to the availability of good antibodies.

Taking the advantage of the completion of *Drosophila* whole genome sequence, we employed a novel approach. From the database, we first search genes that code receptors and enzymes associated to transmitter synthesis. Cells that express those genes are labelled by using in-situ RNA hybridisation. We then screen GAL4 enhancer-trap strains that label cells in the corresponding area, and perform double labelling to certify the colocalisation of GAL4 expression and in-situ label. Second year of this project, we concentrate on GABA, a major inhibitory transmitter, and are mapping the GABA-generating cells and cells that express three types of GABA receptors (Fig. 2).

III. Analysis of the cell lineage-dependent modular structures in the brain

The central brain of *Drosophila melanogaster* is produced by an average of 85 stem cells (neuroblasts) per hemisphere. We visualised the innervation patterns of the progeny of single neuroblasts in the adult brain using the FRT-GAL4 system. In most cases, cell bodies form a tightly packed cluster and their neurites fasciculate to form a single bundle to innervate a

limited number of brain regions in a stereotypic manner. These suggest that the progeny of a single neuroblast often form a lineage-dependent circuit module, which we named a "clonal unit."

To understand the mechanisms underlying this clonal clustering and fasciculation, we focused on the role of neural-specific homophilic cell adhesion molecules in the cell body layer (cortex) of the developing larval brain. DN-cadherin and Neuroglian are distributed uniformly along the border between all the neurones. FasciclinII (FasII), on the other hand, localises in several clusters of neurones, each of which looks like clonally related. Double labelling of FRT-GAL4 clones and FasII-expressing cells revealed that FasII clusters indeed correspond to clones. The distribution of FasII is limited to the cell border inside the clones. Cell surface flanking the neighbouring clones is free of FasII. Such localisation might infer that FasII would mediate cell-cell adhesion within clonal cluster.

fasII mutant clones, however, showed no remarkable defect on the formation of clonal cell clustering. Pan-neuronal ectopic expression of fasII caused little effect, either. The ectopically expressed FasII showed the same characteristic localisation pattern: it concentrates along the intraclonal cell borders but not along the interclonal cell borders.

Why doesn't ectopic FasII exist at the interclonal cell borders? One possible explanation is that there might be physical boundary that prevents direct contact of neurones between different clones. We thus examined the arrangement of glial cells in the larval brain. Double labelling of glial cells and FasII-expressing clones showed that a type of glia send extensive processes between neurones. In the outer area of the cell body layer, which is near the brain surface and houses neuroblasts and newly-generated cells, glial processes wrap only the outer surface of the clonal clusters. Processes are not observed within the cluster. Glial cells thus physically separate the clonal border in this area. Deeper in the cell body layer, which consists of old cells, thin glial processes penetrate the boundary between essentially all the neurones.

IV. Contribution to the science community

As a joint venture with German and US research groups, we maintain *Flybrain*, a web-based image database of the *Drosophila* nervous system (http://flybrain.nibb.ac.jp). Another database maintained here, mainly for Japanese-speaking fly esearchers, is *Jfly* (http://jfly.nibb.ac.jp). Archives of research-related discussions, experimental protocols, images and movies are provided.

Publication List:

Awasaki, T. and Kimura, K. (2001) Multiple function of *poxn* gene in larval PNS development and in adult appendage formation of *Drosophila, Dev. Genes. Evol.* **211:** 20-29

Kurusu, M., Awasaki, T., Masuda-Nakagawa, L. M., Kawauchi, H., Ito, K. and Furukubo-Tokunaga, K. (2002) Embryonic and larval development of the *Drosophila* mushroom bodies: Concentric layer subdivisions and the importance of fasciclin II. *Development*, in press

Verkhusha, V. V., Otsuna, H., Awasaki, T., Oda, H., Tsukita, S., and Ito, K. (2001) An enhanced mutant of red-fluorescent protein DsRed for double labelling and developmental timer of neural fiber bundle formation. *J Biol Chem* **276 (32):** 29621-29624.

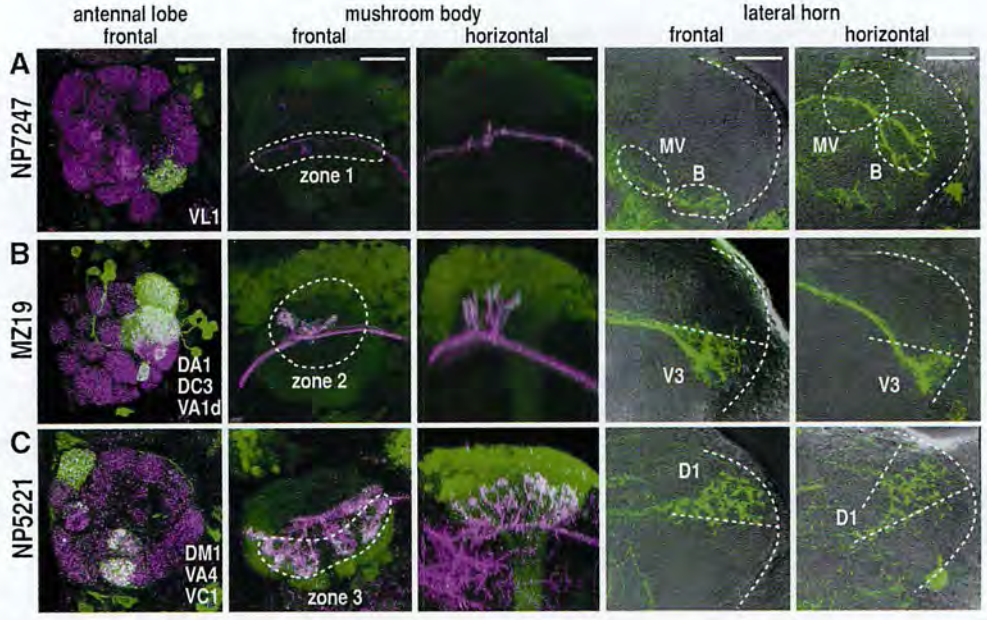

Figure 1. Zonal separation of the projection neurone terminals. The five columns show the frontal view of the antennal lobe (green: UAS-GFP, magenta: antennal lobe neuropile), frontal and horizontal views of the mushroom body calyx (magenta: UAS-GFP, green: mushroom body neuropile), and frontal and horizontal views of the lateral horn (green: UAS-GFP, grey: Nomarski-contrasted transmission). Dotted lines indicate the outer margin of the lateral horn and the border of each zone. Bar = 20 μm. A: Uniglomerular vACT neurons projecting into zone 1 of the mushroom body and zones MV and B of the lateral horn. B: Uniglomerular iACT neurons innervating zone 2 and zone V3. C: Uniglomerular iACT neurons innervating zone 3 and zone D1.

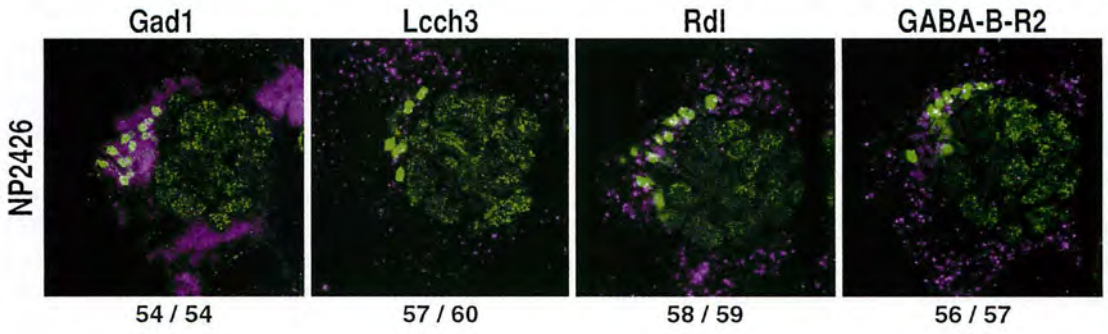

Figure 2. GABA related gene expression in the antennal lobe local inteneurones. Green: One type of local interneurones (in total about 54-60 cells) visualised with GAL4 strain NP2426 and UAS-GFP. Magenta: in situ hybridisation against GABA synthesis enzyme Gad 1 and three types of GABA receptors (Lcch3, Rdl, GABA-B-R2). Numbers below each panel show the ratio of double-positive cells, suggesting that essentially all the interneurones of this type are GABAergic and GABA-responding.

DIVISION OF CELLULAR COMMUNICATION (ADJUNCT)

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The research in this laboratory is aimed at an understanding of the molecular mechanisms that regulate the assembly and function of cytoskeletal proteins. Specifically, we are currently studying the functional properties of axonemal dynein and actin in *Chlamydomonas*, an organism ideally suited for genetic and molecular biological studies.

I. Function of Multiple Axonemal Dyneins

It is well established that the beating of cilia and flagella is based on sliding movements of outer-doublet microtubules driven by motor proteins dyneins, but how the sliding is converted into axonemal oscillatory bending movement has not been made clear. Recently, various lines of evidence have suggested that dynein is crucially important also in the sliding-bending conversion mechanism. Thus our research effort is now focused on the properties of various dyneins.

Biochemical studies by us and other laboratories have established that a single flagellar axoneme contains at least eleven kinds of dynein heavy chains in inner and outer arms. An important question is how different dynein heavy chains differ in function. To answer this question, we have been isolating and characterizing mutants that lack different kinds of axonemal dyneins. During the last ten years, we have isolated as many as 15 genetically different mutants lacking various subsets of dyneins. The isolation of these mutants greatly advanced our understanding of the function and organization of various dyneins within the axoneme, because only three mutants had been known to lack dynein heavy chains before we started mutant isolation.

The motility phenotypes of the isolated mutants have indicated that different dynein species differ in function in a fundamental manner. For example, the outer-arm heavy chains are important for flagellar beating at high frequency, whereas the inner-arm heavy chains are important for producing proper waveforms. Indirect evidence also suggests that the force generation properties differ greatly among different heavy chains. Interestingly, the axoneme can beat without some of these heavy chains, but cannot beat if certain combinations of heavy chains are lost. It appears that simultaneous presence of dyneins with different properties is necessary for the axonemal beating. Thus, it should be important to understand the mechano-chemical property of each dynein. To this end, we are currently trying to directly measure the force production in wild-type and mutant axonemes that lack various combinations of dyneins.

In addition to exploring the functional properties of individual dyneins, we have been studying the mechanism by which various dyneins are regularly arranged within the axoneme. In particular, we have concentrated our effort on understanding why outer dynein arms are attached to a particular protofilament in outer doublet microtubules, with a constant spacing of 24 nm. Such a regular arrangement of dynein should be important for the axoneme to beat with regular waveforms. Our study has indicated that a protein complex called the outer dynein arm docking complex (ODA-DC) is particularly important. We found that this complex, made up of three different protein subunits, is transported to the axonemes independently of outer arms and attaches on to the microtubule protofilament, providing the docking site for outer arms. Interestingly, the periodicity of 24 nm appears to originate from the periodicity in ODA-DC binding (Fig. 1). An attractive hypothesis is that ODA-DC has a tendency to longitudinally associate with each other and serves as a molecular ruler with a 24 nm spacing. Our study has also shown that the outer doublet microtubule contains an unidentified structure that can specify the ODA-DC binding site among different protofilaments of a single outer doublet. We are currently trying to elucidate this structure, as well as to prove the molecular ruler hypothesis.

II. Function of Actin and an Actin-related Protein in *Chlamydomonas*

The inner dynein arms are known to contain actin as a subunit. Hence the two independent motility systems of eukaryotes - the actin-based and microtubule-based motility systems - should somehow cooperate in the inner arm dynein although the function of actin in dynein arms is totally unknown at present. We have found that the mutant *ida5*, lacking four out of the seven subspecies of inner-arm dyneins, has a mutation in the actin-encoding gene. Intriguingly, *Chlamydomonas* has been known to have only a single gene of conventional actin, and the mutant *ida5* was found to express no conventional actin at all. On close inspection, the cytoplasm and axonemes of this mutant were found to contain a novel actin-like protein (NAP) which displays exceptionally low homology (64%) to conventional actin. The mutant *ida5* is deficient in the formation of the fertilization tubule and thus has a low mating efficiency. However, it displays normal cell division and grows as rapidly as wild type, possibly because NAP can substitute for actin in important cellular functions. Thus conventional actin and NAP may overlap in some, but not all, cellular functions. It is interesting to note that NAP is expressed in significant amount only in the mutant lacking actin; i.e., the expression of NAP appears to depend on the presence of actin. We are currently investigating how such regulation takes place.

What is the function of NAP in wild-type cells? Recently, we found NAP is expressed even in wild type, specifically during the re-flagellation process after flagellar amputation. The expression of NAP mRNA

precedes that of actin mRNA. Interestingly, cell's ability to re-grow flagella is greatly impaired when *ida5* is transformed with a chimeric actin gene consisting of a 5'-UTR sequence derived from the NAP gene and a sequence that codes for conventional actin. NAP and actin mRNA are expressed simultaneously in this mutant, suggesting that the prior expression of NAP is important for efficient flagellation. Phylogenetic analysis revealed that NAP homologs exist in at least three species in Volvocales, forming a distinct gene family of divergent actin. Taken together, NAP may play a specific role in the flagellation mechanism in various species of Volvocales.

Publication List:

Hayashi, M., Hirono, M., and Kamiya, R. (2001) Recovery of flagellar dynein function in *Chlamydomonas* actin/dynein-deficient mutant upon introduction of muscle actin by electroporation. *Cell Motil. Cytoskeleton* **49**, 146-153.

Inaba, K. (2001) High level of protein ubiquitination in ascidian sperm. In The biology of ascidians. (Sawada, Yokosawa, Lambert, eds,), pp.74-79.,Springer-Verlag

Itoh, A., Inaba, K., Fujinoki, M., and Morisawa, M. (2001) Motility-associated and cyclic AMP-dependent protein phosphorylation in the sperm of the chum salmon, *Oncorhynchus keta*. *Biomed. Res.* **22**, in press.

Kho, K. H., Tanimoto, S., Inaba, K., Oka, Y., and Morisawa, M. (2001) Transmembrane cell signaling for the initiation of trout sperm motility: Roles of ion channels and membrane hyperpolarization for cyclic AMP synthesis. *Zool. Sci.* **18**, 919-928.

Padma, P, Hozumi, A, Ogawa, K., and Inaba, K. (2001) Molecular cloning and characterization of a thioredoxin/NDPK related dynein intermediate chain from the ascidian, *Ciona intestinalis*. *Gene* **275**, 177-183.

Takada, S., Wilkerson, C. G., Wakabayashi, K., Kamiya, R., and Witman, G. B. (2001) The outer dynein arm docking complex: composition and characterization of a subunit (Oda1) necessary for outer arm assembly. *Mol. Biol. Cell* in press.

Wakabayashi, K., Takada, S. Witman, G. B., and Kamiya, R. (2001) Transport and arrangement of the outer-dynein-arm docking complex in the flagella of *Chlamydomonas* mutants that lack outer dynein arms. *Cell Motil. Cytoskeleton* **48**, 277-286.

Yanagisawa, H. and Kamiya, R. (2001) Association between actin and light chains in *Chlamydomonas* flagellar inner-arm dyneins. *Biochem. Biophys. Res. Commun.* **288**, 443-447.

Yoshimura, K. and Kamiya, R. (2001) The sensitivity of *Chlamydomonas* photoreceptor is optimized for the frequency of bodily rotation. *Plant Cell Physiol.* **42**, 665-672.

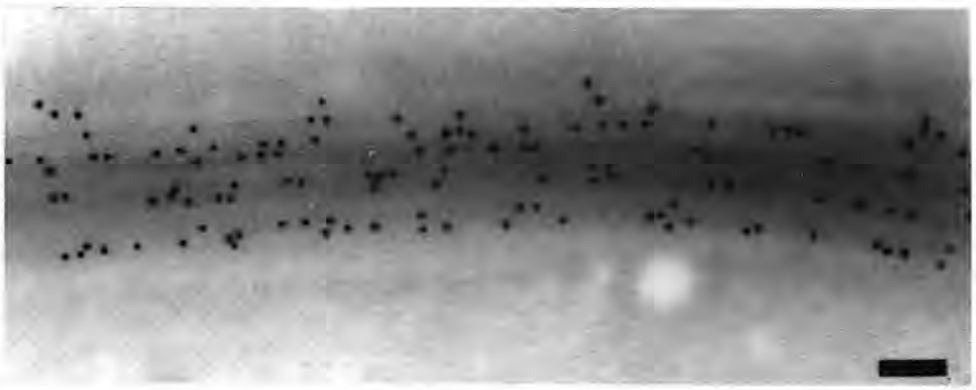

Fig. 1. Immunoelectron micrographs of an axoneme labeled with anti-ODA-DC antibody and secondary antibody conjugated with gold paticles. Note that gold particles tend to align linearly along microtubules, occasionally with an apparent periodicity of ~24 nm. Bar: 100 nm.

DIVISION OF CELL FUSION (ADJUNCT)

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Cytokinesis in animal and some primitive eukaryotic cells is achieved by the progressive contraction of the cleavage furrow. The cleavage furrow contains a contractile apparatus, called the contractile ring, which is composed of a bundle of actin filaments that lies in the furrow cortex beneath the plasma membrane. It has been established that the contractile ring contracts as the result of interaction between actin filaments and myosin. However, little is known about process of its formation, mechanism that controls its formation, protein constituents, and its ultrastructure. The goal of our research is to solve these problems and thereby clarify the molecular mechanism of cytokinesis. For this purpose, we use three kinds of cells, namely, sea urchin egg, *Xenopus* egg, and the fission yeast *Schizosaccharomyces pombe*.

S. pombe is an excellent system to investigate the changes in the actin cytoskeleton during cell cycle since F-actin patches, F-actin cables and F-actin ring are only visible structures in the cell. The F-actin ring is considered to correspond to the contractile ring in animal cells. It is formed during anaphase in this organism. We have examined process of formation of the F-actin ring by optical sectioning and 3-D reconstitution fluorescence microscopy. In wild type cells, both formation of an aster-like structure composed of F-actin cables and accumulation of F-actin cables was recognized at the medial cortex of the cell during prophase to metaphase. The formation of the aster-like structure seemed to initiate by branching of the longitudinal F-actin cables at a site near the spindle pole bodies which had been duplicated but not yet separated. A single cable extended from the aster and encircled the cell at the equator to form a primary F-actin ring during metaphase. During anaphase, the accumulated F-actin cables were linked to the primary F-actin ring, and then all of these structures seemed to be packed to form the F-actin ring. These observations suggest that formation of the aster-like structure and the accumulation of the F-actin cables at the medial region of the cell during metaphase may be required to initiate the F-actin ring formation. We further examined F-actin structures in both *cdc12* and *cdc15* early cytokinesis mutants. As a result, Cdc12 seemed to be required for the primary F-actin ring formation during prophase, while Cdc15 may be involved in both packing the F-actin cables to form the F-actin ring and rearrangement of the F-actin after anaphase. In *spg1*, *cdc7* and *sid2* septum initiation mutants, the F-actin ring seemed to be formed in order.

In large eggs which undergo unilateral cleavage, it has been proposed that localized elevation of cytosolic free calcium ions ($[Ca^{2+}]i$) at the growing end of the cleavage furrow (CF) triggers CF formation by the activation of myosin ATPase activity through the myosin light chain kinase. However, data regarding the relevance of Ca waves in CF formation is contradictory or incomplete. We have improved the method to visualize Ca wave in dividing *Xenopus* eggs: we removed the fertilization membrane and labeled the egg surface with rhodamine-wheat germ agglutinin (WGA) to visualize the growing ends of the early CF. We imaged wave type Ca signal with Calcium Green-1 dextran from the animal hemisphere of the egg. In addition to the Ca wave, which is the orchestrated Ca release from a global area of the cell, smaller classes of Ca signal have been described. These are Ca puffs, which are thought to be Ca release from 10-30 of coordinately opened Ca channels, and Ca blips, which are Ca released from single opened Ca channel. These signals were also examined during the furrow formation. Consequently, we could not detect any Ca wave, Ca puff, or Ca blip at the growing end of the CF, although we could detect two Ca waves at later stages of cytokinesis. Furthermore, we lowerd $[Ca^{2+}]i$ in the egg by injection of Ca-chelators. However, it did not affect cleavages of the egg. Therefore, we concluded that Ca signals are not involved in CF formation in *Xenopus* egg.

We also concentrate our study on function of actin-regulatory proteins, including ADF/cofilin family proteins, during cytokinesis using *Xenopus* eggs and embryos. ADF/cofilin family proteins exist in all animals and plants examined and have been shown to be essential. We found that ADF/cofilin family proteins are essential for cytokinesis. Recent studies revealed that ADF/cofilin accelerates turnover of actin filaments both *in vitro* and *in vivo*. Most recently, we found a novel actin-regulatory protein which induces disassembly of actin filaments cooperatively with ADF/cofilin. cDNA analysis revealed that this protein is a *Xenopus* homologue of yeast actin interacting protein 1 (AIP1). Thus, we designated this protein as *Xenopus* AIP1 (XAIP1). Purified XAIP1 itself binds to pure actin filaments to some extent, but it induces a rapid, drastic disassembly of actin filaments associated with cofilin. Microinjection of this protein into *Xenopus* embryos arrested development by the resulting actin cytoskeletal disorder. XAIP1 represents the first case of a protein cooperatively disassembling actin filaments with ADF/cofilin family proteins. In addition, we also identified the second protein which functions with ADF/cofilin. That was a *Xenopus* homologue of cyclase-associating protein (CAP), originally reported as an actin monomer-binding protein. Our biochemical analyses, however, suggest that XCap accelerates nucleotide exchange of actin monomer and induces actin polymerization from actin-cofilin complex at steady state.

Publication List:

Asano, Y., and Mabuchi, I. (2001). Calyculin-A, an inhibitor for protein phosphatases, induces cortical contraction in unfertilized sea urchin eggs. *Cell Motil. Cytoskele.* **48**, 245-261.

Arai, R., and Mabuchi, I. (2002). F-actin ring formation and roles of F-actin cables in the fission yeast *Schizosaccharomyces pombe*. *J. Cell Sci.* in press.

Bernstein, B. W., Painter, W. B., Chen, H., Minamide, L. S., Abe, H. and Bamburg, J. R. (2000). Intracellular pH Modulation of ADF/Cofilin Proteins. *Cell Motil. Cytoskel.* **47**, 319-336.

Motegi, F., Arai, R., and Mabuchi, I. (2001). Identification of two type V myosins in fission yeast, one of which functions in polarized cell growth and moves rapidly in the cell. *Mol. Biol. Cell* **12**, 1367-1380.

Nakano, K., Sato, K., Morimatsu, A., Ohnuma, M., and Mabuchi, I. (2001). Interactions among a fimbrin, a capping protein, and an actin-depolymerizing factor in organization of the fission yeast actin cytoskeleton *Mol. Biol. Cell* **12**, 3515-3526.

Nakano, K., Muto, T., and Mabuchi, I. (2001). Characterization of GTP-activating proteins for the Rho-family small GTPases in the fission yeast *Schizosaccharomyces pombe*. *Genes to Cells* **6**, 1031-1042.

Noguchi, T., and Mabuchi, I. (2001). Reorganization of actin cytoskeleton at the growing end of the cleavagefurrow of *Xenopus* egg during cytokinesis. *J. Cell Sci.* **114**, 401-412.

Noguchi, T., and Mabuchi, I. (2002). Localized calcium signals along the cleavage furrow are not involved in the furrow formation. *Mol. Biol. Cell* in press.

Takahashi, T., Koshimizu, U., Abe, H., Obinata, T. and Nakamura, T. (2001). Functional Involvement of *Xenopus* LIM-Kinases in Progression of Oocyte Maturation. *Dev. Biol.* **229**, 554-567.

Toya, M., Motegi, F., Nakano, K., Mabuchi, I., and Yamamoto, M. (2001). Identification and functional analysis of the gene for type I myosin in fission yeast. *Genes to Cells* **6**, 187-199.

Yamashiro, S., Kameyama, K., Kanzawa, N., Tamiya, T., Mabuchi, I., and Tsuchiya, T. (2001). The gelsolin/fragmin family protein identified in the higher plant *Mimosa pudica*. *J. Biochem.* **130**, 243-249.

Yonemura, I., and Mabuchi, I. (2001). Heterogeneity of mRNAs coding for *Caenorhabditis elegans* coronin-like proteins *Gene* **271**, 255-259.

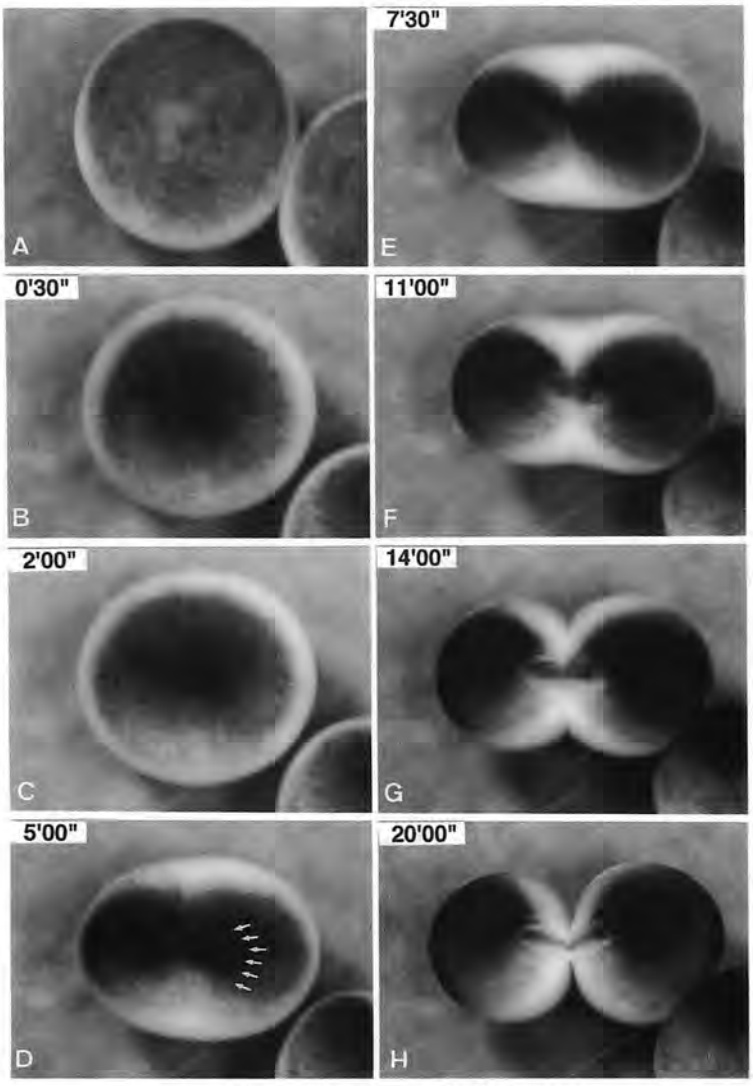

Fig. 1 First cleavage of a *Xenopus* egg.

DEPARTMENT OF DEVELOPMENTAL BIOLOGY

Chairperson: NAGAHAMA, Yoshitaka

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

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

*Closed during 2001 and will be reinitiated on new projects.

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	SUDHAKUMARI, Cheni Chery
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	SENTHILKUMARAN, Balasubramanian
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	FAN, Hai-Guang (Graduate University for Advanced Studies)
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Visiting Scientist:	*MATSUDA, Chika*
	MATSUOKA, Yoh
	CONSTEN, Dimitori

The pituitary-gonadal axis plays an important role in regulating gametogenesis in vertebrates. Gonadotropins typically act through the biosynthesis of gonadal steroid hormones which in turn mediate various stages of gametogenesis. Their effects are particularly profound in teleost fishes which provide several excellent models for investigating the basic hormonal mechanisms regulating gonadal sex differentiation and gametogenesis (spermatogenesis, sperm maturation, oocyte growth and oocyte maturation). Our research focuses on (1) the identification of steroidal mediators involved in gonadal sex differentiation and gametogenesis, and (2) the mechanisms of synthesis and action of these mediators.

I. Sex-determining gene of medaka

Although the sex-determining gene, *Sry*, has been identified in mammals, no comparable genes have been found in non-mammalian vertebrates. We used positional cloning to identify the sex-determining gene of medaka, *Oryzias latipes*. Chromosome walking using a BAC library and DNA markers derived from a congenic strain mapped the sex-determining region to a 530 kb stretch of the Y chromosome. We found a congenic XY female medaka lacking 250 kb of this region, further shortening the probable sex-determining region. Shotgun sequencing of this deleted region predicted 27 genes; however, only three were expressed in embryos during sexual differentiation. Furthermore, only one (*PG17*) of these three genes was present specifically on the Y chromosome (Fig. 1). We also found a naturally-occurring XY female. Offspring of this female inherited a Y chromosome that contained, but did not express, *PG17* suggesting an important role for *PG17* in testis development. Based on its homology

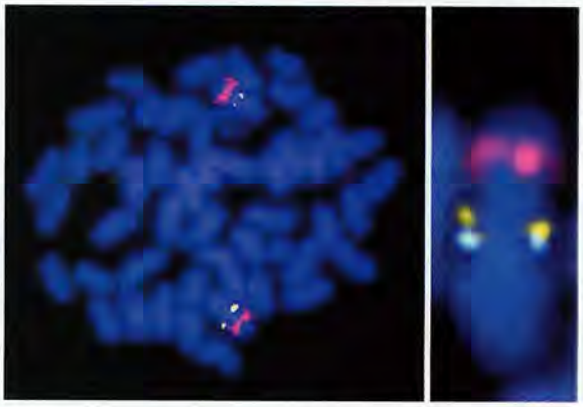

Fig. 1 Cytogenetical mapping of the sex-determining region (*SD*) of medaka. **Left,** Fluorescence *in situ* hybridization (FISH) of metaphase chromosomes. *SL2* (sex-linked marker 2, red) localizes on the short arms of the sex chromosomes, whereas a BAC clone containing *SL1* (sex-linked marker 1, yellow) localizes on the long arms of the sex chromosomes. Arrowheads indicate sex chromosomes. **Right,** FISH of one sex chromosome with three different probes (*SL2, SL1, SD*). Signals of a BAC clone containing *SD* are light blue.

with the DM gene family, Y chromosome specificity, and apparent role in testis development, *PG17* was identified as the sex-determining gene of 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. Fertilized eggs hatch after 4 days at 26℃. On the day of hatching, primordial germ cells (PGCs), are located in the outer layer of the lateral plate mesoderm around the hind gut. At 3 days post-hatching, PGCs are located in the gonadal anlagen after the formation of the coelomic cavity in the lateral plate mesoderm rather than through active migration.

The gene *vasa* encodes a DEAD (Asp-Glu-Ala-Asp) family of putative RNA helicase and is present in the germ line of several animal species. Two isoforms of *vasa* mRNA and protein are present in tilapia. One (*vas-s*) lacks a part of the N-terminal region found in the other isoform (*vas*). Both isoforms are expressed in oocytes through the embryonic stage when PGCs localize in the lateral plate mesoderm. After PGCs localization in the gonadal anlagen, vas-s expression increases and vas expression becomes undetectable. Expression of both isoforms is observed again after morphological gonadal sex differentiation, irrespective of genotypic sex. In ovary, compared to *vas* expression *vas-s* expression predominates throughout oogenesis. In testis, *vas* expression is predominant compared with *vas-s* during spermatogenesis. These results indicate that relative expression of two *vasa* isoforms is

dependent upon germ cell differentiation and sex. We have also generated medaka transgenic lines with green fluorescent protein (GFP) fluorescence controlled by the regulatory regions of the *olvas* gene in the germ cells. The intensity of GFP fluorescence increases dramatically in PGCs located in the ventrolateral region of the posterior intestine around stage 25 (the onset of brood circulation). Whole-mount *in situ* hybridization and monitoring of ectopically located cells by GFP fluorescence suggest that 1) the increase in zygotic *olvas* expression occurs after PGC specification and 2) PGCs can maintain their cell characteristics ectopically after stages 20-25. The GFP expression persists throughout the later stages in the mature ovary and testis.

In tilapia, mitosis of germ cells begins around 10 days post-hatching in genetic females, but can not be confirmed until after sex differentiation in testes of genetic males. Steroid-producing cells in ovaries, but not testes, at the undifferentiated and differentiating stages express all of the steroidogenic enzymes required for estradiol-17β biosynthesis from cholesterol. Transcripts of estrogen receptors (ER) α and β first appear in both female and male gonads of fry 5-10 days post-hatching. These results strongly suggest that endogenous estrogens act as the natural inducers of ovarian differentiation in tilapia. This hypothesis is further supported by evidence of masculinization of genetic female tilapia by inhibition of estrogen synthesis using an inhibitor of cytochrome P450 aromatase. In contrast, the ability of steroid-producing cells to synthesize steroid hormones in the testes only appears at the time of testicular differentiation. Transcripts of androgen receptors are not present in gonads of genetic males during sex differentiation. We have isolated two DM (*Doublesex/Mab-3* DNA-binding motif)-domain cDNAs from tilapia testis and ovary, named *DMRT1* and *DMO*, respectively. *DMRT1* is expressed only in Sertoli cells and *DMO* is detected only in oocytes by *in situ* hybridization. The correlation between expression of *DMRT1* and testicular differentiation of both normal XY-male and sex reversed XX-males suggest that *DMRT1* is a candidate testis-determining gene in tilapia. In contrast, abundant *DMO* expression in pre- and early vitellogenic oocytes in XX- and sex reversed XY-females indicates a relationship between *DMO* and oocyte growth.

III. Endocrine regulation of spermatogenesis

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

IV. Endocrine regulation of oocyte maturation

Meiotic maturation of fish oocytes is induced by the action of maturation-inducing hormone (MIH). 17α, 20β-dihydroxy-4-pregnen-3-one (17α,20β-DP) has been identified as the MIHs of several fish species. The interaction of two ovarian follilce cell layers, the thecal and granulosa cell layers, is required for the synthesis of 17α,20β-DP. The theal layer produces 17α-hydroxyprogesterone that is converted to 17α,20β-DP in granulosa cells by the action of 20β-hydroxysteroid dehydrogenase (20β-HSD). The preovulatory surge of LH-like gonadotropin is responsible for the rapid expression of 20β-HSD mRNA transcripts in granulosa cells during oocyte maturation. In tilapia, Northern blot and RT-PCR analyses reveal that the expression of 20β-HSD in ovarian follicles is not detectable in postvitellogenic follicles. A distinct expression is evident at the day of spawning. *In vitro* incubation of postvitellogenic follicles with hCG induced the expression of 20β-HSD mRNA within 1-2 hrs, followed by final oocyte maturation of oocytes. Actinomycin D completely blocks both hCG-induced 20β-HSD expression and final oocyte maturation, indicating the involvement and transcriptional regulation of 20β-HSD in final maturation.

17α,20β-DP induces oocyte maturation by acting on a pertussis toxin-sensitive G-protein-coupled membrane receptor. The early steps of 17α,20β-DP action involve the formation of downstream mediator of this steroid, the maturation-promoting factor or metaphase-promoting factor (MPF) consisting of cdc2 kinase and cyclin B. 17α,20β-DP induces oocytes to synthesize cyclin B which activates a preexisting 35-kDa cdc2 kinase via phosphorylation of its threonine 161 by a threonine kinase (MO15), thus producing the 34 kDa active cdc2. Polyadenylation of cyclin B mRNA is involved in 17α,20β-DP-induced initiation of cyclin B mRNA translation. The Mos/MAPK pathway is not essential for initiating goldfish oocyte maturation

despite its general function as a cytostatic factor (CSF). 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.

Publication List:

Ikeuchi, T., Todo, T., Kobayashi, T. and Nagahama, Y. (2001) Two subtypes of androgen and progestogen receptors in fish testes. *Comp. Physiol. Biochem. Part B* **129**, 449-455.

Ikeuchi, T., Todo, T., Kobayashi, T. and Nagahama, Y. A novel progestogen receptor subtype in the Japanese eel, *Anguilla japonica*. *FEBS Letters* (in press).

Imahara, J., Tokumoto, M., Nagahama, Y., Ishikawa, K. and Tokumoto, T. (2001) Reconstitution of sperm nuclei of zebrafish, *Danio rerio*, in *Xenopus* egg extracts. *Marine Biotechnology* **3**, 53-57.

Kitano, T., Takamune, K., Nagahama, Y. and Abe, S. (2001) Role of P450 aromatase in gonadal sex differentiation in Japanese flounder (*Paralichthys olivaceus*). *Environmental Sciences*, **8**, 1-11.

Kobayashi, T., Kajiura-Kobayashi, H. and Nagahama, Y. Two isoforms of *vasa* homologs in a teleost fish: their differential expression during germ cell differentiation. *Mech. Develop.* (in press).

Lee, Y.H., Du, J.L., Yueh, W.S., Lin, B . Y., Huang, J. D., Lee, C. Y., Lee, M. F., Lau, E. L., Lee, F. Y., Morrey, C., Nagahama, Y. and Chang, C. F. (2001) Sex change in the protandrous black porgy, *Acanthopagrus schlegeli*: A review in gonadal development, estradiol, estrogen receptor, aromatase activity and gonadotropin. *J. Exp. Zool.* **290**, 715-726.

Liu, S.J., Govoroun, M., D'Cotta, H., Ricordel, M.J., Lareyre, J.J., McMeel, O.M., Smith, T., Nagahama, Y. and Guiguen, Y (2001) Expression of cytochrome P450β (11β-hydroxylase) genes during gonadal sex differentiation and spermatogenesis of rainbow trout, *Oncorhynchus mykiss*. *J. Steroid Biochem. Mol. Biol.* **75**, 291-298.

Morrey, C.E., Nagahama, Y. and Grau, E. G. Terminal phase males stimulate ovarian function and inhibit sex change in the protogynous wrasse *Thalassoma duperrey*. *Zool. Sci.* (in press).

Matsuda, M. and Nagahama, Y. Positional cloning of the sex-determining region of medaka using a Y congenic starain. *Aqua Genome* (in press).

Matsuda, M., Kawato, N., Asakawa, S., Shimizu, N., Nagahama, Y., Hamaguchi, S., Sakaizumi, M. and Hori, H. (2001) Construction of a BAC library derived from the inbred Hd-rR strains of the teleost fish, *Oryzias latipes*. *Gene Genetic Systems* **76**, 61-63.

Matsuyama, M., Sasaki, A., Nakagawa, K., Kobayashi, T., Nagahama, Y. and Chuda, H. (2001) Maturation-inducing hormone of the tiger puffer, *Takifugu rubripes* (Tetraodontidae, Teleostei): biosynthesis of steroids by the ovaries and the relative effectiveness of steroid metabolites for germinal vesicle breakdown *in vitro*. *Zool. Sci.* **18**, 225-234.

Nakahata, S., Katsu, Y., Mita, K., Inoue, K., Nagahama, Y. and Yamashita, M. (2001) Biochemical identification of *Xenopus* Pumilio as a sequence-specific cyclin B1 mRNA-binding protein that physiologically interacts with a Nanos homolog, Xcat-2, and a cytoplasmic polyadenylation element-binding protein. *J. Biol. Chem.* **276**, 20945-20953.

Nakahata, S., Mita, K., Katsu, Y., Nagahama, Y. and Yamashita, M. (2001) Immunological detection and characterization of poly(A) polymerase, poly(A)-binding protein and cytoplasmic polyadenylation element-binding protein in goldfish and *Xenopus* oocytes. *Zool. Sci.* **18**, 337-343.

Nakamura, M., Kobayashi, T., Yoshiura, Y. and Nagahama, Y. Masculinizing all-female tilapia by administrating aromatase inhibitor. *Mol. Reprod. Develop.* (in press).

Oba, Y., Hirai, T., Yoshiura, Y., Kobayashi, T. and Nagahama, Y. (2001) Fish gonadotropin and thyrotropin receptors: The evolution of glycoprotein hormone receptors in vertebrates. *Comp. Physiol. Biochem. Part B* **129**, 441-448.

Ohta, K., Rhaman, M.A., Chuda, H., Yoshikuni, M., Nagahama, Y. and Matsuyama, M. Maturation-inducing hormone and it's membrane receptor in gonads of Japanese yellowtail, *Seriola quenqueradiata*. *Fisheries Science* (in press).

Tanaka, M., Kinosita, M. and Nagahama, Y. (2001). Establishment of medaka (*Oryzias latipes*) transgenic lines with the expression of GFP fluorescence exclusively in germline cells: a useful model to monitor germline cells in a live vertebrate. *Proc. Natl. Acad. Sci. USA* **98**, 2544-2549.

Tanaka, M., Nakajin, S., Kobayashi, D., Fukada, S., Guan, G. J., Todo, T., Senthilkumaran, B. and Nagahama, Y. . Teleost ovarian carbonyl reductase-like 20β-hydroxysteroid dehydrogenase: Potential role in the production of maturation-inducing hormone in final maturation of oocytes. *Biol. Reprod.* (in press).

Tokumoto, M., Nagahama, Y. and Tokumoto, T. Molecular cloning of cDNA encoding a polypeptide chain elongation factor 1α from goldfish (*Carassius auratus*). *DNA Sequence* (in press).

Tokumoto, M., Nagahama, Y. and Tokumoto, T. A major substrate for MPF: cDNA cloning and expression of polypeptide chain elongation factor 1γ from goldfish (*Carassius auratus*). DNA Sequence (in press).

Yamaguchi, A. and Nagahama, Y. Structure and protein compositions of the nuclear matirix prepared from germinal vesicles of goldfish (*Carassius auratus*) oocytes. *Mol. Reprod. Develop.* (in press).

Yamaguchi, A. and Nagahama, Y. Somatic lamins in germinal vesicles of goldfish vitellogenic oocytes. *Cell Structure and Function* (in press).

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Cell and tissue differentiation proceeds systematically based on orchestrated expressions of sets of genes. The expressions commence successively along with the passage of time. As the consequence, a single fertilised egg develops into a variety of tissues and organs, which consist of specialised cells in terms of their structures and functions. Accordingly, it is reasonable to assume that investigation of the mechanisms underlying the cell and tissue-specific gene expression at a molecular level is essential for understanding molecular frameworks for genetic cascades proceeding along with cell and tissue differentiation. Based on the concept above, our division of Cell Differentiation has focussed on sex differentiation of the gonads and differentiation of the steroidogneic tissues form the aspect of functions of tissue-specific transcription factors and growth factors.

Several transcription factors are involved in the process of gonadal differentiation. Some of these factors, such as *SRY*, *WT-1*, *DAX-1*, and *SOX-9* have been identified as the responsible genes for various human diseases that display structural and functional defects in tissues including the gonads. The essential functions of other transcription factors such as *Ad4BP/SF-1*, *Emx-2*, *M33*, and *Lhx-9* were identified by phenotypes of their gene disrupted mice. In addition, the expression profiles with respect to their distribution and sexual dimorphism strongly suggest the functional significance at the early stage of gonadal differentiation. However, it remains to be clarified how the transcription factors above regulate their target genes and how the genes encoding the transcription factors are regulated. When considering a gene regulatory cascade that supports differentiation of the gonadal tissues, studies of the above two directions are quite important. Based on this background, we investigated mainly the functions of Ad4BP/SF-1 and Dax-1, and the mechanism of gene regulation encoding these factors.

I. Gene regulatory cascade in the steroidogenic tissue differentiation

When a differentiation process of a tissue is considered, it is possible to assume that certain genes encoding transcription factors are involved in a gene regulatory cascade as the critical components. As the component in the cascades required for the steroidogenic adrenocortical and gonadal differentiation, Ad4BP/SF-1 is locates at the upstream of tissue-specific genes, including the steroidogenic *CYP* genes, and should locate at the downstream of other transcription factors regulating *Ad4BP/SF-1* gene. When considering that the cascade flows from upstream to downstream along with the tissue differentiation and moreover Ad4BP/SF-1 is an essential transcription factor in the adrenocortical and gonadal cascade, identification of the components functioning with Ad4BP/SF-1 and regulating Ad4BP/SF-1 gene transcription is essential for fully understanding the molecular mechanisms underlying the tissue differentiation. Thus, some of the members in this division have investigated gene regulation of *Ad4BP/SF-1* and *Dax-1*, both of which are quite important for the tissue differentiation.

Based on the aspect above, the regulatory region of the *Ad4BP/SF-1* gene has been investigated *in vivo* by making transgenic mice. A genomic DNA fragment longer than 480 kb containing four genes including *Ad4BP/SF-1* has been examined if they have tissues-specific enhancer element. Our survey for the genomic DNA revealed that particular regions in the Ad4BP/SF-1 gene are responsible for the gene expression specific for the adrenal cortex and ventromedial hypothalamic nucleus.

Dax-1 is another transcription factor of our interest, which is also implicated in the steroidogenic tissue differentiation. Our previous study revealed that the factor acts as a suppressor against Ad4BP/SF-1. However, regulation of the suppressive effect has remained to be clarified at the molecular level. We recently uncovered the function of particular sequences, LXXLL motifs, located at the amino terminal half of Dax-1. When Dax-1 functions as the suppressor, the amino acid sequences in the repeats are essential for a protein-protein interaction with target nuclear receptors, and thereby the transcription mediated by the nuclear receptors are largely inhibited. The LXXLL motif was

originally identified in coactivators as a motif essential for interaction with nuclear receptors. Thus, it is highly likely that the LXXLL motifs in Dax-1 compete with those of the coactivators for interaction with nuclear receptors such as Ad4BP/SF-1 (Fig 1). Although it remains unclear how the inhibitory activity is regulated in a variety of physiological conditions, interaction through the LXXLL motifs should be a crucial step for the functional regulation of the transcription facotors.

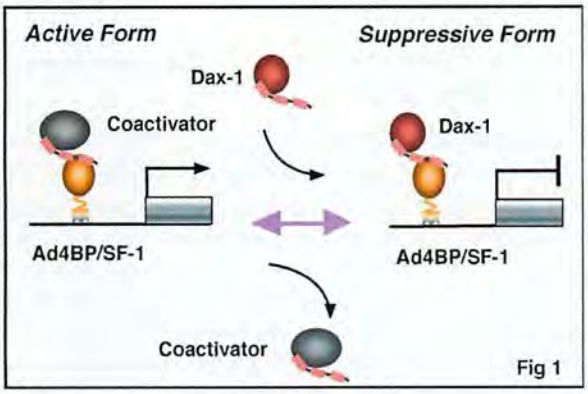

Fig 1

In addition to these transcription factors, it has been accepted that Sox-9, Wt-1, Emx-2, and GATA-4 are implicated in gonad differentiation through regulating transcription of gene essential for gonadal structures and functions. Although the essential functions of these transcription factors have been elucidated through symptoms of the genetic disorders and/or the phenotypes of the gene disrupted mice, characterization of their transcriptional regulation has not yet been performed enough. Therefore, we started to investigate it through characterizing proteins interacting with the transcription factors. In order to isolate the interacting factors, yeast two-hybrid screening has been performed using a cDNA library constructed with mRNA prepared from mouse fetal gonads. Extensive screening resulted in isolation of molecules including coactivators, other type of transcription factors, proteins carrying protein modification activities, and proteins carrying domains capable of signal transduction. *In situ* hybridization analyses with these particular molecules revealed that some of them are expressed in the developing gonads, and some of them showed sex dependent expression. For investigating their biological significance, gene-disruption study has been performed.

II. Wnt4 signal for the gonad sex differentiation

As reported previously, Ad4BP/SF-1 is an indispensable component for *Dax-1* gene transcription. In fact, multiple binding sites recognized by Ad4BP/SF-1 at the upstream region of *Dax-1* gene are necessary for transcriptional activation. The *in vitro* observation using reporter gene assays was confirmed subsequently by an *in vivo* study using *Ad4BP/SF-1* gene disrupted mice, which lacked Dax-1 expression in the developing genital ridge. Although these results strongly indicated that *Ad4BP/SF-1* gene is genetically located upstream from the *Dax-1* gene, their expression profiles in terms of distribution and sexual dimorphism do not necessarily agree with our findings. In this regard, it should be noticed that a recent gene disruption study indicated implication of Wnt4 in gonadal sex differentiation. Normally, steroidogenic *3β-HSD* gene and *MIS* gene are expressed in the developing fetal gonads of males but not females. Interestingly, however, the expression was detected in the fetal ovary of the gene-disrupted mice, suggesting that the Wnt4 represses *3β-HSD* and *MIS* gene transcription in the fetal ovaries of the wild type. If considering that some of Wnt signals activate downstream gene transcription through stabilization of β-catenine, it is unlikely that the signal represses the *3β-HSD* and *MIS* gene transcription.

To provide a rational explanation, we hypothesized that Wnt4 expressed in the developing gonad upregulates a suppressor molecule and thereby downregulates *3β-HSD* and *MIS* gene transcription. Since transcription of both genes is regulated in a positive fashion by Ad4BP/SF-1, it was reasonable to assume that Dax-1 plays a role as the suppressor. To confirm this assumption, we examined if β-catenin activates the Dax-1 gene transcription. As the result, *Dax-1* gene transcription was activated in the presence of β-catenin in a dose-dependent manner. Interestingly the action of β-catenin is further upregulated in the presence of Ad4BP/SF-1, indicating that the two factors, β-catenin and Ad4BP/SF-1, synergistically activate the *Dax-1* gene transcription. Showing a good correlation, interaction between Ad4BP/SF-1 and β-catenin was confirmed with protein-pull down and yeast tow-hybrid analyses.

The mechanisms of *Dax-1* gene regulation governing its sexually dimorphic and spatial characteristics are summarized in Fig. 2. As described previously, it is difficult to explain the whole regulatory mechanism of the *Dax-1* gene transcription by Ad4BP/SF-1 alone. For instance, Ad4BP/SF-1 is expressed in the male developing gonads more abundantly than in the female. Nevertheless, the amount of Dax-1 in the female developing gonad is higher than that in the male gonad. In the case of Wnt4 expression in the developing gonads and mesonephros, *in situ* examination revealed that the amount expressed in the female tissues is higher than in the male. With respect to the distribution of Dax-1, strong signals were detected in the gonadal regions facing the mesonephros although such expression domain was not observed in the case of Ad4BP/SF-1. In such inconsistent distribution, it is interesting to note that the expression of Wnt4 in the gonads was more abundant at the region proximal to than that distal to mesonephros. Therefore, to understand the mechanism underlying Dax-1 expression, we propose that Ad4BP/SF-1 plays basal and fundamental roles and that the Wnt4 signal modulates the transcription mediated by Ad4BP/SF-1. Although the regulation above is likely to function in the sexually differentiating gonads

of both sexes, the mechanisms of other transcription factors such as Sox-9 and Emx-2 are not fully understood. In addition, it should be noted that other growth factors as well as other forms of Wnt molecules are expressed in the developing gonads and mesonephros. Further studies of the functional relationship between growth factors and transcription factors should identify the fine and sophisticated mechanisms underlying the sex differentiation of the gonads.

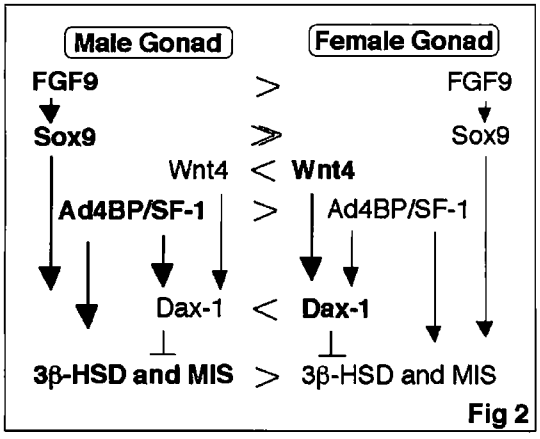

Fig 2

Publication List:

Asoy, R., Mellgren, G., Morohashi, K., Lund, J. Activation of cAMP-dependent Protein Kinase increases the protein level of Steroidogenic Factor-1. *Endocrinol.* in press.

Ikeda, Y., Takeda, Y., Shikayama, T., Mukai, T., Hisano, S., Morohashi, K. (2001) Comparative localization of Dax-1 and Ad4BP/SF-1 during development of the hypothalamic-pituitary-gonadal axis implies their closely related and distinct functions. *Develop. Dynam.* **220**, 363-376.

Shibata, H., Ikeda, Y., Mukai, T., Morohashi, K., Kurihara, I., Ando, T., Suzuki, T., Kobayashi, S., Murai, M., Saito, I., Saruta, T. (2001) Expression profiles of COUP-TF, DAX-1 and SF-1 in the human adrenal gland and adrenocortical tumors: Possible implications in steroidogenesis. *Mol. Genet. Metab.* **74**, 206-216.

Suzuki, T., Mizusaki, H., Kawabe, K., Kasahara, M., Yoshioka, H., Morohashi, K. Concerted Regulation of Gonadal Differentiation by Transcription Factors and Growth Factors. In 'The Genetics and Biology of Sex Determination' John Wiley & Sons Ltd. in press.

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

I. Regulation of growth factor diffusion in pattern formation

During early development, cells receive positional information from neighboring cells to form tissue patterns in initially uniform germ layers. Ligands of the transforming growth factor (TGF-β) superfamily are known to participate in this pattern formation. In particular, activin has been shown to act as a long-range dorsalizing signal to establish a concentration gradient in *Xenopus*. In contrast, the action of BMP-2 and BMP-4, another members of the family, appear to influence and induce ventral fates only where they are expressed. This raises a question as to how the action of BMPs is tightly restricted within and around the cells that produce them. We have demonstrated that a basic core of only three amino acids in the N-terminal region of BMP-4 is required for its restriction to the non-neural ectoderm corresponding to its expression domain. Our results also suggest that heparan sulfate proteoglycans bind to this basic core and thus play a role in trapping BMP-4. We propose that restriction of BMP diffusion *in vivo* through the interaction with extracellular environment is critical for the precise definition of non-neural ectoderm during early embryogenesis.

II. 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.

The Wnt family of secretory glycoprotein is one of the major families of developmentally important signaling molecules and plays important roles in embryonic induction, generation of cell polarity, and specification of cell fate. Wnt proteins bind Frizzled (Fz) receptors, a membrane protein with seven transmembrane domains. So far, the Wnt/Fz signaling cascade has been shown to branch into at least three pathways. These are the canonical pathway, the planar cell polarity (PCP) pathway, and the pathway through protein kinase C (PKC). The canonical pathway is the best-characterized signaling cascade which involves Dishevelled (Dsh/Dvl) and β-catenin and contributes to establish the dorsoventral axis in *Xenopus* embryos. The PCP pathway is essential for cell polarization and cell movement during gastrulation. This pathway seems to

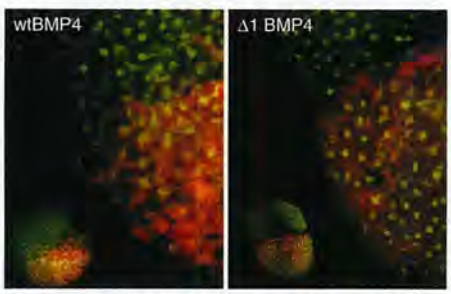

Figure 1. Diffusion rate of BMP is drastically modified by N-terminally localized basic amino acid core. The photographs show that wtBMP-4 (left) acts almost cell-autonomously and induces Smad1 phosphorylation only in the adjacent cells, whereas Δ1 BMP-4 (right), a mutated BMP-4 from which N-terminally localized core of basic amino acids was deleted acquired a long range effect affecting over many diameters of cells and induced their Smad1 phosphorylation. Green fluorescence indicates nuclear translocation of phosphorylated Smad1 representing the BMP signal and cells stained with red fluorescence are recipient animal cap cells that received BMP signal from left-upper animal cap cells conjugated to them.

involve Dsh/Dvl and c-Jun N-terminal kinase (JNK). Although these signaling pathways have been investigated extensively, it is still unknown exactly how the Fz receptors generate signals to regulate gastrulation cell movement. Thus, we screened for proteins which interact with the cytoplasmic domains of Fz using the yeast two-hybrid assay. So far, we have cloned two *Xenopus* genes. One encodes a protein which contains two PDZ domains. This gene is expressed ubiquitously during gastrulation, and later in the central nervous system. Our preliminary results suggest its role in the Fz signaling, particularly in the PCP pathway. (a) It binds Fz-7, an important receptor in the PCP signaling pathway in *Xenopus* embryos, more strongly than other Fzs. (b) Overexpression of a predicted dominant-negative mutant, causes severe gastrulation defect, a typical phenotype seen by the inhibition of the PCP pathway. These results suggest that this protein may be an essential component for gastrulation cell movement acting in the PCP pathway.

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

Drosophila is one of the ideal model organisms to dissect signal transduction pathway by genetic methods. We have carried out dominant suppressor screening for two transgenic mutant flies expressed constitutively activated DPP/BMP type-I receptors in wing imaginal discs. We isolated 19 suppressor mutants, *Suppressor of constitutively activated Dpp singnaling* (*Scad*). Alleles of *punt*, *Mad*, *shn* and *dCrebA* were found in isolated Scad mutants. We now focus to study a novel locus *Scad67*. *Scad67* encodes a protein with a SP-RING motif. SP-RING motif was originally found in PIAS-family proteins including *Drosophila* PIAS homolog Su(var)2-10/Zimp. Recent studies have shown that PIAS-family proteins function as a E3-SUMO ligase and enhance SUMO conjugation against specific substrates. *Scad67* is an evolutionary conserved molecule and we found two *Scad67* homologous genes in human and also in other vertebrates. Homozygous *Scad67* mutants show embryonic to pupal stage lethality. The most severe zygotic mutant shows embryonic head structure and segmentation defects. Detailed Scad67 function analysis especially in the SUMO mediated biological processes and also in DPP signalling are ongoing.

We also have been interested in the *in vivo* function of the TGF-β activated kinase-1 (TAK1) in *Drosophila*. We succeed to isolate *dTAK1* null mutant in collaborated with B. Lemaitre's group (CNRS, France). Interestingly, *dTAK1* mutation does shows morphological and viability defects. However, we observed an impairment of antibacterial peptide gene expression in *dTAK1* mutants. Genetic studies of dTAK1 suggested an evolutionary conserved role for TAK1 in the control of rel/NF-κB dependent innate immune responses.

IV. Brachyury downstream notochord differentiation in the ascidian embryo

Ascidians, urochordates, are one of the three chordate groups, and the ascidian tadpole is thought to represent the most simplified and primitive chordate body plan. It contains a notochord, which is a defining characteristic of chordate embryo composed of only 40 cells. To understand the morphogenesis in this simple system, we have focused on a gene, *Brachyury*, which is known to play an important role in the notochord development. In ascidian, *Brachyury* is expressed exclusively in the notochord and the misexpression of the *Brachyury* gene (*Ci-Bra*) of *Ciona intestinalis* is sufficient to transform endoderm into notochord. This gene encodes a sequence-specific activator that contains a T-box DNA-binding domain, and in vertebrates, it is initially expressed throughout the presumptive mesoderm and gradually restricted to the developing notochord and tailbud. The phenotype of the *Brachyury* mutants in mice and zebrafish revealed that this gene is essential for notochord differentiation. Our goal is to elucidate the down stream pathway of this important gene in ascidian in order to set the stage for understanding not only the formation and function of the notochord but how this important structure has evolved. We conducted the subtractive hybridization screens to identify potential *Brachyury* target genes that are induced upon *Ci-Bra* overexpression. Out of 501 independent cDNA clones that were induced cDNAs, 38 were specifically expressed in notochord cells (Fig. 2). We characterized 20 of them by determining the complete nucleotide sequences and *in situ* hybridization analyses which show the spatial and temporal expression patterns of the cDNAs. These potential *Ci-Bra* downstream genes appear to encode a broad spectrum of divergent proteins associated with notochord formation and function.

V. TGF-β family in nematode

We have previously shown that DBL-1, a member of TGF-β superfamily regulates body length in *C. elegans*. To understand molecular mechanism of body length regulation by DBL-1, we examined tissue-specific requirement of a DBL-1 receptor SMA-6 by rescuing *sma-6* mutation with tissue specific expression of the receptor. We found that hypodermal expression of SMA-6 is sufficient to rescue the *sma* phenotype, suggesting that hypodermis is the most critical target tissue of DBL-1 to regulate body length. Next we screened for target genes regulated by DBL-1 in hypodermal cells, and identified a gene yk298h6 whose disruption by dsRNAi resulted in long worm. yk298h6 was later found to be identical with *lon-1*, a gene encoding a type II membrane protein belonging to the PR family conserved from plant to human. LON-1 appears to suppress hypodermal polyploidization and thus negatively regulate body length.

Publication List

Dong, W., Ogawa, S., Tsukiyama, S., Okuhara, S. Niiyama, M., Ueno, N., Peterson, R. E. and Hiraga, T.(2002) 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin toxicity in the zebrafish embryo: altered regional blood flow and impaired lower jaw development. Teraoka, H., *Toxicological Sciences* **65**,192-199.

Hanazawa, M., Mochii, M. Ueno, N., Kohara, Y. and Iino, Y. (2001) Use of cDNA subtraction and RNA interference screens in combination reveals genes required for germ-line development in *Caenohabditis elegans*. *Proc. Natl. Acad. Sci. USA* **98**, 8686-8691.

Ishizuya-Oka, A., Ueda, S,, Amano, T., Shimizu, K., Suzuki, K., Ueno, N. and Yoshizato, K. (2001) Thyroid-hormone-dependent and fibroblast-specific expression of BMP-4 correlates with adult epithelial development during amphibian intestinal remodeling. *Cell Tissue Res.* **303**, 187-195.

Itoh, K., Udagawa, N., Katagiri, T., Iemura, S., Ueno, N., Yasuda, H., Higashio, K., Quinn, J. M., Gillespie, M. T., Martin, T. J., Suda, T., Takahashi, N. (2001) Bone morphogenetic protein 2 stimulates osteoclast differentiation and survival supported by receptor activator of nuclear factor-kappab ligand. *Endocrinology.* **142**, 3656-3662.

Kurata T., Nakabayashi, J., Yamamoto, T.S., Mochii, M. and Ueno, N. (2001) Visualization of endogenous BMP signaling during Xenopus development. *Differentiation* **67**, 33-40.

Mitani, Y., Takahashi, H. and Satoh, N. (2001) Regulation of the muscle-specific expression and function of an ascidian T-box gene, As-T2. *Development* **128**, 3717-3728.

Morita, K., Shimizu, M., Shibuya, H. and Ueno, N. (2001) A Daf-1-binding protein BRA-1 is a negative regulator of Daf-7 TGF-β signaling. *Proc. Natl. Acad. Sci. USA* **98**, 6284-6288.

Ohkawara, B., Iemura, S., ten Dijke, P. and Ueno, N. (2002) Action range of BMP is defined by its N-terminal basic amino acid core. *Curr. Biol*, **12**, 205-209.

Sakuta, H., Suzuki, R., Takahashi, H., Kato, A., Shintani, T., Iemura, S.-i, Yamamoto, T. S., Ueno, N. and Noda, M. (2001) Ventroptin: a BMP-4 antagonist expressed in a double-gradient pattern in the retina. *Science* **293**, 111-115.

Sugawara, K. Morita, K., Ueno, N. and Shibuya, H. (2001) BIP, a BRAM-interacting protein involved in TGF-beta signalling, regulates body length in *Caenorhabditis elegans*. *Genes Cells* **6**, 599-606.

Takahashi, H. and Satoh, N. (2001) Trunk lateral cell-specific genes of ascidian Halocynthia roretzi. *Zool. Sci.* **18**, 361-366.

Vidal, S. S., Khush, R. S., Leulier, F., Tzou, P., Nakamura, M. and Lemaitre, B. (2001) Mutations in the *Drosophila* dTAK1 gene reveal a conserved function for MAPKKKs in the control of rel/NF-kappaB-dependent innate immune responses. *Genes. Dev.* **15**, 1900-12.

Yamamoto, T. S.,Takagi, C., Hyodo, A. C. and Ueno, N.(2001) Suppression of head formation by Xmsx-1through the inhibition of intracellualr nodal signaling. *Development* **128**, 2769-2779.

Yoshida, S., Morita, K., Mochii, M. and Ueno, N. (2001) Hypodermal expression of *Caenorhabditis elegans* TGF-β type I receptor SMA-6 is essential for the growth and maintenance of body length. *Dev. Biol.* **240**, 32-45.

DEPARTMENT OF REGULATION BIOLOGY

Chairperson: NODA, Masaharu

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

The department is composed of two regular divisions and two adjunct divisions. The study of this department is focused on molecular mechanisms for the development of central nervous systems in vertebrates, and also on molecular mechanisms for the response of plants toward external environments, such as light, temperature and salinity

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We have been studying the molecular and cellular mechanisms underlying the development and functioning of the vertebrate central nervous system. The scope of our interests encompasses regional specification in the retina, neuronal differentiation, cellular migration, path-finding and target recognition of axons, formation and refinement of specific synapses, and also various functions of the matured brain.

I. Molecular mechanism of the retinotectal projection

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

Since 1992, we have been devoting our efforts to searching for topographic molecules which show asymmetrical distribution in the embryonic chick retina. In the first-round screening using a cDNA subtractive hybridization technique, we identified two winged-helix transcriptional regulators, CBF-1 and CBF-2, expressed in the nasal and temporal retina, respectively. Furthermore, our misexpression experiments using a retroviral vector showed that these two transcription factors determine the regional specificity of the retinal ganglion cells, namely, the directed axonal projections to the appropriate tectal targets along the anteroposterior axis. To further search for topographic molecules in the embryonic retina, we next performed a large-scale screening using a new cDNA display system called Restriction Landmark cDNA Scanning (RLCS). With the assistance of a computer image-processing software, we successfully identified 33 molecules along the nasotemporal axis and 20 molecules along the dorsoventral axis, with various asymmetrical expression patterns in the developing retina. We have elucidated the primary structures of all these cDNA clones and

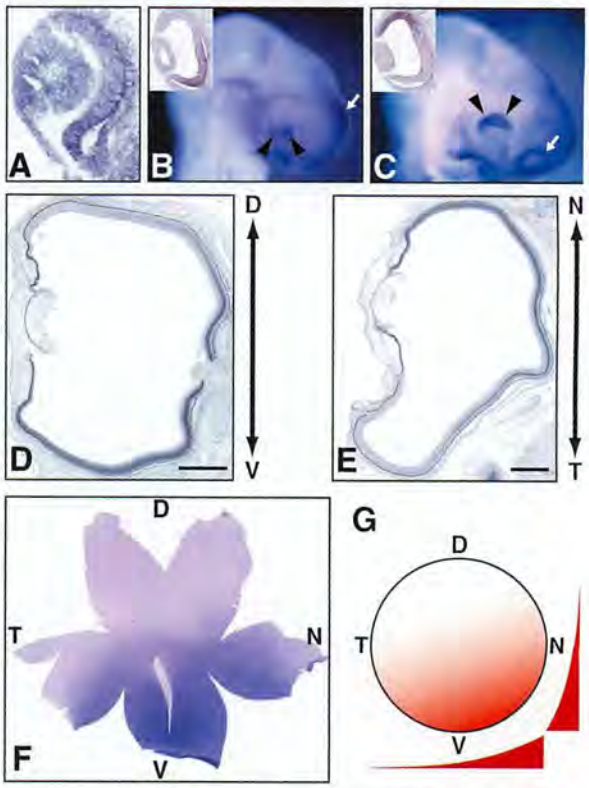

Fig.1 Expression patterns of *Ventroptin* mRNA during development
(A) Coronal section *in situ* hybridization of the eye at stage 14.. (B, C) Whole mount *in situ* hybridization of E3 (stage 16-17) chick embryos for *Ventroptin* (B) and *BMP-4* (C). Insets show results in coronal sections of the E3 eyes. *Ventroptin* was expressed in the ventral retina (arrowheads in B) and the forebrain (arrow). *BMP-4* was expressed in the dorsal retina (arrowheads in C) and the periphery of the nasal pit (arrow). (D) Coronal section *in situ* hybridization at E8. *Ventroptin* is expressed in a ventral high-dorsal low gradient. (E) Horizontal section at E8. *Ventroptin* is expressed in a nasal high-temporal low gradient. (F) Flat mount *in situ* hybridization of E8 retina. (G) Schematic drawing of *Ventroptin* expression in the retina. Double-gradient expression is represented by the density-gradient of color. Scale bars; 600 μm (D and E). N, T, D and V indicate nasal, temporal, dorsal and ventral, respectively (D-G).

examined their expression patterns during development. These included many novel molecules together with the known molecules: transcription factors (CBF-2, COUP-TFII, etc.), receptor proteins (EphA3, EphB3, etc.), secretory factors, intracellular proteins, and so on.

Among them, we identified a novel retinoic acid (RA)-generating enzyme, RALDH-3, which is specifically expressed in the ventral region of the retina, together with a dorsal-specific enzyme RALDH-1. Furthermore, we recently identified a novel secretory protein, Ventroptin, which has BMP-4 neutralizing activity (Fig. 1). *Ventroptin* is expressed in the retina with a ventral high-dorsal low gradient at early stages. This expression pattern is complementary to that of *BMP-4*. At later stage (E6-8), a nasal high-temporal low gradient expression pattern is also detected. *Ventroptin* thus shows a double-gradient expression profile along the dorsoventral and the anteroposterior axes. Misexpression of *Ventroptin* altered expression patterns of several topographic genes: *BMP-4*, *Tbx5* and *cVax* along the dorsoventral axis and *ephrin A2* along the anteroposterior axis. Consistently, in these embryos, projection of the retinal ganglion cell axons to the tectum was also changed along the both axes (Fig. 2). The topographic retinotectal projection along the dorsoventral and the anteroposterior axes thus appears to be controlled not separately but in a highly concerted manner by Ventroptin.

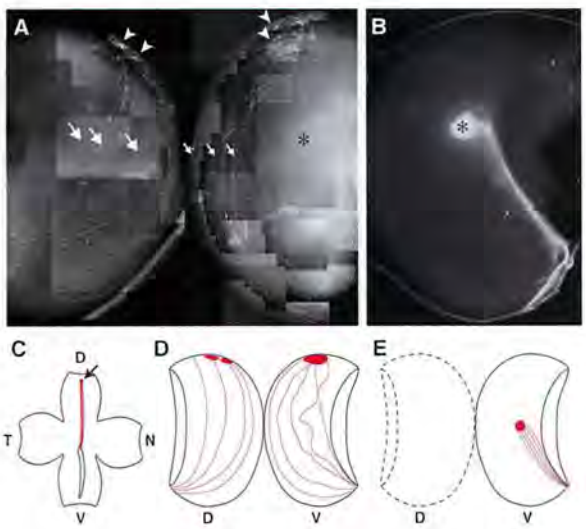

Fig. 2 Retinotectal projection at E18-19 after *in ovo* electroporation
(A) A typical projection pattern in the *Ventroptin* misexpressed embryo. A number of the dorsal axons entered the dorsal tectum (large arrows) and all the axons extended to the posterior end of the tectum (arrowhead). (B) A typical projection pattern in the control embryo. The dorsal axons form a tight terminal zone (*) in the middle of the ventral tectum. (C) The arrow indicates the position of the DiI lavel in the dorsal periphery of the right retina. (D and E) Schematic drawings of (A) and (B), respectively. Anterior is down and posterior is up.

Currently, with respect to the identified topographic molecules, we are conducting over- and misexpression experiments using viral vectors and *in ovo* electroporation to elucidate their molecular functions. We expect that our studies will lead to elucidation of the molecular mechanism underlying the retinal patterning and topographic retinotectal projection, and ultimately to uncovering the basic principles for establishing complicated but extremely precise neural networks.

II. Functional roles of protein tyrosine phosphatase ζ and γ

Protein tyrosine phosphorylation plays crucial roles in various aspects of brain development and brain functions. In 1994, we found that PTPζ/RPTPβ, a nervous system-rich RPTP, is expressed as a chondroitin sulfate proteoglycan in the brain. The extracellular region of PTPζ consists of a carbonic anhydrase-like domain, a fibronectin-type III-like domain and a serine-glycine-rich region, which is considered to be the chondroitin sulfate attachment region. There exist three splice variants of this molecule: a full-length transmembrane form (PTPζ-A); a short transmembrane form (PTPζ-B); and a soluble form (PTPζ-S), which is also known as 6B4 proteoglycan/phosphacan. PTPζ is expressed from the early developmental stage to the adulthood. This suggests that this gene plays variegated roles in the brain development and brain function.

We began by searching for ligand molecules of this receptor. We found in 1996 that PTPζ binds pleiotrophin/HB-GAM and midkine, closely related heparin-binding growth factors which share many biological activities. The chondroitin sulfate portion of PTPζ is essential for the high affinity binding (Kd = ~0.25 nM) to these growth factors, and removal of chondroitin sulfate chains results in a marked decrease of binding affinity (Kd = ~13 nM). We further revealed that chondroitin sulfate interacts with Arg^{78} in Cluster I, one of the two heparin-binding sites in the C-terminal half domain of midkine. This is the first demonstration that chondroitin sulfate plays an important regulatory role in growth factor signaling.

Next, we examined the roles of pleiotrophin/midkine-PTPζ interaction in neuronal migration using the glass fiber assay and Boyden chamber cell migration assay. Pleiotrophin and midkine on the substratum stimulated migration of neurons in these assays. Experiments using various midkine mutants with various affinities for PTPζ indicated that the strength of binding affinities and the neuronal migration-inducing activities are highly correlated. These results suggest that PTPζ is involved in migration as a neuronal receptor for pleiotrophin and midkine.

In order to reveal the intracellular signaling mechanism of PTPζ, we performed yeast two-hybrid screening using the intracellular region of PTPζ as bait. We found in 1999 that PTPζ interacts with PSD-95/SAP90 family members, SAP102, PSD-95/SAP90 and

SAP97/hDlg, which are concentrated in the central synapses mediating protein-protein interactions to form large synaptic macromolecular complexes. Here, the C-terminus of PTPζ binds to PSD-95/SAP90 proteins through the second PDZ domain. This suggests that PTPζ is involved in the regulation of synaptic function. However, PSD-95/SAP90 family members are not likely to be the substrate for PTPζ because this family members are not tyrosine-phosphorylated.

To identify the substrate molecules of PTPζ, we have recently developed the yeast substrate-trapping system. This system is based on the yeast two-hybrid system with two essential modifications: conditional expression of v-src to tyrosine-phosphorylate the prey proteins and screening using a substrate-trap mutant of PTPζ as bait. Using this system, we successfully isolated a number of candidate clones for substrate molecules or interacting molecules (Fig. 3). Among them, we first identified GIT1/Cat-1 as a PTPζ substrate. It is known that Cat-1 regulates Pak, a serine threonine kinase which serves as a target for the small GTP-binding proteins, Cdc42 and Rac, and is implicated in a wide range of cellular events including the cell adhesion and cell morphological change. Pleiotrophin, PTPζ and GIT1/Cat-1 might regulate the neuronal migration and neurite extension by controlling the Pak signaling pathway. We are continuing efforts to characterize the other candidate clones.

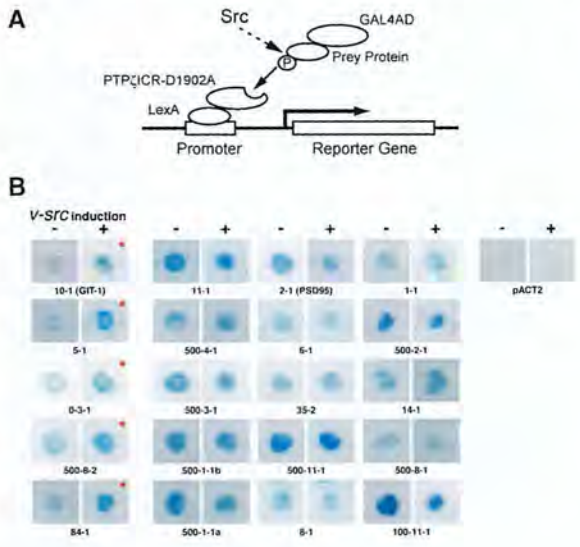

Fig. 3 The yeast substrate-trapping system
(A) In the absence of methionine, prey proteins could be phosphorylated by the induced v-src, and trapped by the bait of the whole intracelluar domain with an Asp1902Ala mutation (PTPζ ICR-D1902A). In the presence of 1 mM methionine, when *v-src* is not induced, only the standard two-hybrid bindings occur. The complex formation leads to activation of transcription of the reporter gene, *LacZ*. (B) β-Galactosidase filter-lift assay of the isolated clones. Colonies with red asterisks showed an increase in blue-color development upon induction of *v-src*. These are candidates for the substrate molecules. The other clones, mostly containing PDZ domains, showed no difference in the color development by *v-src* induction. pACT is a negative control.

To further study the physiological function of PTPζ *in vivo*, we generated PTPζ-deficient mice in which the PTPζ gene was replaced with the LacZ gene in 1997. By investigating the expression of LacZ in heterozygous mutant mice, we demonstrated that neurons as well as astrocytes express PTPζ in the central nervous system. We are currently studying the phenotype of PTPζ-deficient mice biochemically, anatomically, physiologically and ethologically, and have already found abnormalities in behavior, learning and memory, etc.

III. Physiological roles of Na_x sodium channel

Human $Na_v2.1$, mouse $Na_v2.3$ and rat NaG/SCL11 were cloned as a subfamily of voltage-gated sodium channel (NaCh). We found that these genes are species orthologs. This channel molecule is divergent from the previously cloned NaChs ($Na_v1.1$-1.9) including the regions involved in activation, inactivation and ion selectivity. In the end of 2000, it was renamed Na_x as a novel enigmatic member of sodium channels.

To clarify the physiological function and cells expressing Na_x sodium channels *in vivo*, we generated knock-out mice in which Na_x channel gene was replaced with the *LacZ* or *neo* gene by gene targeting. Analysis of the targeted mice allowed us to identify Na_x-producing cells by examining the lacZ expression. Besides in the lung, heart, dorsal root ganglia and Schwann cells in the peripheral nervous system, Na_x was expressed in neurons and ependymal cells in restricted areas of the central nervous system, particularly in the circumventricular organs that are involved in body-fluid homeostasis (see Fig. 1 in the part of Center for Transgenic Animals and Plants). The null mutant mice showed markedly elevated *c-fos* expression in neurons in the subfornical organ and organum vasculosum laminae terminalis compared with wild-type animals. This suggests that these neurons are in a hyperactive state in the Na_x-deficient mice. Moreover, the null mutants showed abnormal intakes of hypertonic saline. These findings suggest that the Na_x sodium channel plays an important role in the central sensing of the body-fluid sodium level, and in regulation of salt intake behavior. We are currently examining differences in the electrophysiological property between the cells derived from the Na_x-null mutant mouse and wild-type mouse to gain insight into the channel property of Na_x.

Publication List:

Kawachi, K., Fujikawa, A., Maeda, N. and Noda, M. (2001) Identification of GIT1/Cat-1 as a substrate molecule of protein tyrosine phosphatase ζ/β by the yeast substrate-trapping system. *Proc. Natl. Acad. Sci. USA* **98**, 6593-6598.

Qi, M., Ikematsu, S., Maeda, N., Ichihara-Tanaka, K., Sakuma, S., Noda, M., Muramatsu, T. and Kadomatsu, K. (2001) Haptotactic migration by midkine: Invovement of protein-tyrosine phosphatase ζ, mitogen-acti-vated protein kinase and phosphatidylinositol 3-kinase. *J.Biol. Chem.*, **276**, 15868-15875.

Sakuta, H., Suzuki, R., Takahashi, H., Kato, A., Shintani, T., Iemura, S., Yamamoto, T.S., Ueno, N. and Noda, M. (2001) Ventroptin: A BMP-4 antagonist expressed in a double-gradient pattern in the retina. *Science* **293**, 111-115.

Shintani, T., Maeda, N. and Noda, M. (2001) Receptor-like protein tyrosine phosphatase γ (RPTPγ), but not PTPζ/RPTPβ, inhibits NGF-induced neurite outgrowth in PC12D cells. *Dev. Neurosci.*, **23**, 55-69.

Sugawara, T., Tsurubuchi, Y., Agarwala, K. L., Ito, M. Fukuma, G., Mazaki-Miyazaki, E., Nagafuji, H., Noda, M., Imoto, K., Wada, K., Mitsudome, A., Kaneko, S., Montal, M., Nagata, K., Hirose, S. and Yamakawa, K. (2001) A missense mutation of the Na^+ channel α_{II} subunit gene $Na_v1.2$ in a patient with febrile and afebril seizures causes channel dysfunction. *Proc. Natl. Acad. Sci. USA* **98**, 6384-6389.

Thomaidou, D., Coquillat, D., Meintanis, S., Noda, M., Rougon, G. and Matsas, R. (2001) Soluble forms of NCAM and F3 neuronal cell adhesion molecules promote Schwann cell migration: identification of protein tyrosine phosphatase ζ/β as the putative F3 receptors on Schwann cells. *J. Neurochem.* **78**, 767-778.

Zubair, M., Watanabe, E., Fukada, M. and Noda, M. (2002) Genetic labeling of specific axonal pathways in the mouse central nervous system. *Eur. J. Neurosci.*, in press.

Abstracts:

Fujikawa, A., Watanabe, E., Sakaguchi, G., Katsuura, G., Hattori, S., Song, W.J., Noda, M. Dopmainergic dysfunction in the mice lacking the receptor tyrosine phosphatase ζ/RPTPβ gene. *ibid.*

Hiyama, T.Y., Watanabe, E., Yoshida, S., Noda, M. Na_x channel is involved in monitoring extracelular sodium concentration. *ibid.*

Kawachi, H., Fujikawa, A., Maeda, N. Noda, M. Identification of GIT1/Cat-1 as a substrate molecule of protein tyrosine phosphatase ζ/β by the yeast substrate-trapping system. *ibid.*

Sakuta, H., Suzuki, R., Takahashi, H., Kato, A., Shintani, T., Iemura, S., Yamamoto,T.S., Ueno, N., Noda, M. Ventroptin: A novel BMP-4 antagonist expressed in a double-gradient pattern in the retina. In the abstracts of the 31th Society for Neuroscience of North America. 2001.

Suzuki, R., Shintani, T., Sakuta, H., Kato, A., Ohkawara, T., Osumi, N., Noda, M. Novel retinoid dehydrogenases expressed in the retina. *ibid.*

Watanabe, E., Fujikawa, A., Matsunaga, H., Yasoshima, Y., Sako, N., Yamamoto, T., Noda, M. Na_x channel is involved in control of salt intake behavior in CNS. *ibid*

DIVISION OF CELLULAR REGULATION

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The major thrust of the research efforts of this division is directed towards understanding the signal transduction mechanisms operating in plants and microorganisms with respect to perception of various kinds of stress that arise due to changes in environmental conditions, with particular emphasis on extreme temperatures and salinity. In addition, techniques are being developed for transformation of plants so that they gain the ability to acclimate to and tolerate such stress conditions. In 2001, significant progress was made in the following areas using cyanobacteria as a model system.

I. Regulation of gene expression in *Synechocystis* subjected to salt and hyperosmotic stress

Salt stress and hyperosmotic stress are very important environmental factors that severely limit the growth and viability of plants and microorganisms. However, these kinds of stress have been used without proper discrimination in a number of investigations. In this study, these effects were separately investigated on the cytoplasmic volume and gene expression in *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*).

Hyperosmotic stress due to 0.5 M sorbitol decreased the cytoplasmic volume by 70% whereas salt stress due to 0.5 M NaCl decreased it by 30% and the effect was only transient. Furthermore, DNA microarray analysis indicated that salt stress strongly induced the expression of genes for some ribosomal proteins and a number of genes for proteins of unknown function. In contrast, hyperosmotic stress strongly induced the expression of the genes for proteins involved in maintenance of cell wall structure (Fig. 1). However, it was also observed that the expression of certain genes was induced by both salt stress and hyperosmotic stress and this category included the genes for heat-shock proteins, the genes for the enzymes that catalyze the synthesis of a compatible solute, glucosylglycerol, and in addition a number of genes for proteins of unknown function (Fig. 1A). Our findings suggest that *Synechocystis* is capable of discriminating salt stress and hyperosmotic stress as different stimuli [Kanesaki, Suzuki, Allakhverdiev, Mikami and Murata, *Biochem. Biophys. Res. Commun.* (2002) **290**, 339-348]. However, it does not preclude the possibility that mechanisms common to the responses to each form of stress might also exist. Both salt stress and hyperosmotic stress also repressed the expression of certain genes specifically, whereas certain other genes like the genes included in the synthesis of phycobilisome and photosystem I (PSI) subunits were repressed by both (Fig. 1B).

II. Identification of Mn^{2+} sensor and signal transduction pathway

Manganese (Mn) is an essential metal in all organisms. It functions as a cofactor or as a prosthetic group in

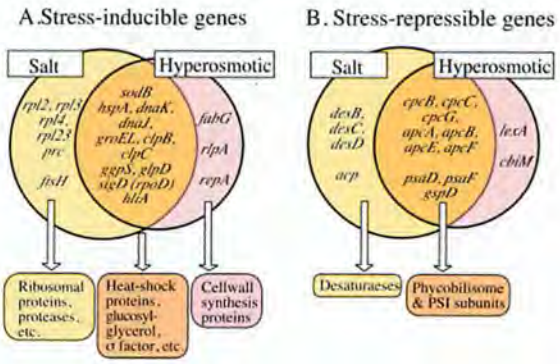

Figure 1. Categorization of salt and hyperosmotic stress-regulated genes in *Synechocystis*.

various enzymes. In particular in the photosynthetic autotrophs which perform oxygenic photosynthesis, four Mn atoms constitute the catalytic center of the oxygen-evolving machinery in a pocket that is formed by the D1 and D2 subunits of photosystem II. Therefore, transport of Mn^{2+} ions into the cell is crucial for the survival of organisms.

It is known that the *mntCAB* operon that encodes an ABC-type translocater of Mn^{2+} ions is inducibly expressed in *Synechocystis*, when the external supply of Mn^{2+} is limiting. However, the signal transduction mechanism by which cyanobacterial cells recognize the deficiency of Mn^{2+} ions and induce the downstream expression of the *mntCAB* operon for the translocater of Mn^{2+} ions remained to be understood. With this in view a knock-out library of histidine kinases and response regulators of *Synechocystis* was systematically analyzed by DNA microarray technique. The analysis revealed that mutants of a histidine kinase, ManS, and a response regulator, ManR, exhibited highly induced expression of the *mntCAB* operon under Mn^{2+}-repleted conditions, suggesting that ManS and ManR constitute the Mn^{2+}-ion sensor and response regulator, respectively, of the signal-transducing pathway in *Synechocystis*. In the presence of Mn^{2+}, ManS produces a signal to activate ManR and the activated ManR represses the expression of the *mntCAB* operon, whereas, under Mn^{2+}-depleted conditions, ManS does not generate the signal and therefore ManR exists in its inactive form as a consequence of which the expression of *mntCAB* operon remains active (Fig. 2). This is the first report on the identification of the functional components of a two-component signal-transduction system by the application of systematic genomics in combination with DNA microarray analysis.

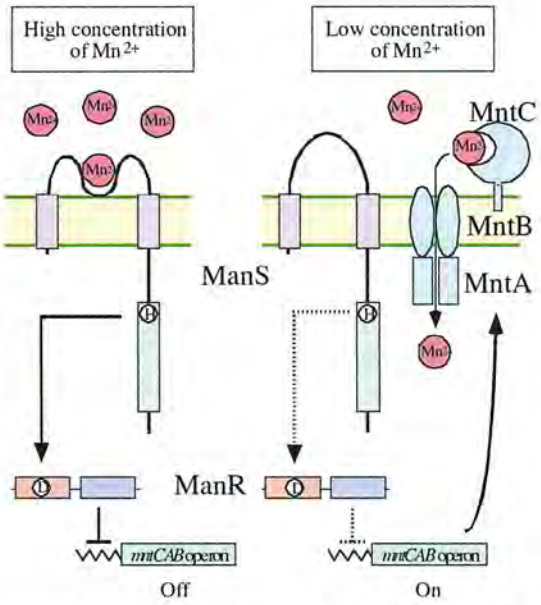

Figure 2. A hypothetical model for the regulation of the *mntCAB* operon in *Synechocystis*.
Two membrane-spanning domains and a histidine kinase domain in ManS (pink and green boxes) and a receiver domain and a DNA-binding domain in ManR (red and purple boxes) were predicted from the deduced amino-acid sequence. "H" and "D" represent histidine and aspartate residues that might be involved in the phospho-relay.

III. Membrane rigidification enhances the cold-induced expression of heat-shock genes

Changes in the ambient temperature affect the physical properties of membranes. To obtain insights into the role of membranes in the mechanism of cold signal perception, we have used a mutant of *Synechocystis*, in which the *desA* gene for the Δ12 desaturase and the *desD* gene for the Δ6 desaturase are both inactive as a result of targeted mutagenesis. Cells of the *desA*$^-$*desD*$^-$ mutant synthesizes only a saturated C16 fatty acid and a monounsaturated C18 fatty acid, regardless of the growth temperature, whereas wild-type cells synthesize di-unsaturated and tri-unsaturated C18 fatty acids in addition to the monounsaturated C18 fatty acid [Tasaka *et al.* (1996) EMBO J. **15**, 6416-6425].

In the present study, Fourier transform infrared (FTIR) spectrometry revealed that the *desA*$^-$*desD*$^-$ mutation rigidified the plasma membrane of *Synechocystis* 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 certain heat-shock genes, namely, the *hspA*, *htpG* and *dnaK2* genes, was markedly enhanced by the membrane rigidification. The cold inducibility of cold-shock genes, such as the *crh* gene for an RNA helicase and the *rbp1* gene for an RNA-binding protein, was not further enhanced by the rigidification. Northern blotting confirmed the results of DNA microarray analysis. Our findings suggest that the expression of these heat-shock genes upon cold shock might be regulated by changes in the physical properties of the plasma membrane, supporting the hypothesis that rigidification of membrane lipids is a primary signal for cold sensing.

IV. Tolerance of photosynthetic machinery to light stress depends on its ability to repair light-induced photo-damage

Photosynthetic machinery is sensitive to various kinds of environmental stress and, in particular, the photosystem II complex (PSII) is very sensitive to light stress. To overcome this stress photosynthetic organisms have the ability to rapidly repair the PSII from the light-induced damage. We have developed a method to separately measure the damage and repair processes of PSII in *Synechocystis*, and have examined the effect of various kinds of stress on the damage and repair processes. We have found that the rate of photodamage is proportional to

light intensity and this proportionality is unaffected by stress conditions such as oxidative stress, salt stress and cold stress. In contrast, the rate of repair was fast at low intensities of light (and reached the maximum level at relatively low intensities of light), and this rate of repair is depressed by stress conditions. These observations lead us to conclude that photodamage to PSII depends solely on the light intensity, whereas repair of PSII is dependent on the site of regulation by various kinds of environmental stress which are termed "Repair-inhibitory stress".

V. Inhibition of the translational machinery by oxidative stress

Absorption of excess light energy by the photosynthetic machinery results in the generation of reactive oxygen species (ROS), such as H_2O_2, from the photosystem complexes. We investigated the effects *in vivo* of ROS to clarify the nature of the damage caused by such excess light energy to the photosynthetic machinery in *Synechocystis*. ROS apparently augments the photodamage to photosystem II (PSII) by inhibiting the repair of the damaged PSII and not by accelerating the photodamage directly. This conclusion was confirmed by the effects of the mutation of genes for H_2O_2-scavenging enzymes on the repair of PSII. Pulse labelling experiments revealed that ROS inhibited the synthesis of proteins *de novo*, in particular, that of the D1 protein, a reaction center protein of PSII. Northern blotting revealed that the accumulation of transcripts for the D1 protein was not significantly affected by ROS. Thus, ROS might influence the outcome of photodamage primarily via an effect on translation (Fig. 3).

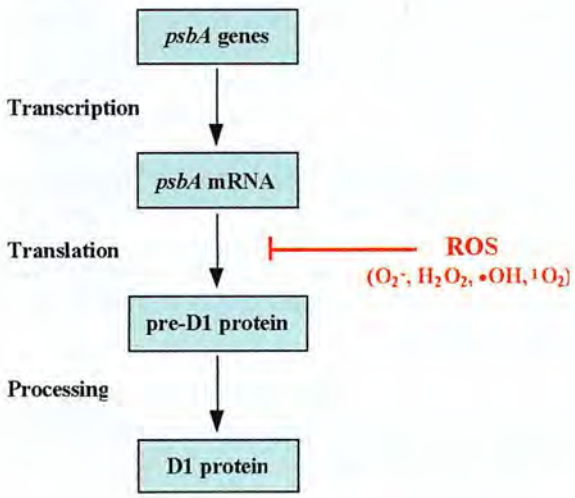

Figure 3. A hypothetical scheme for the inhibition of the D1 protein synthesis *de novo* by oxidative stress.

List of publication:

(1) Original articles

Allakhverdiev, S.I., Kinoshita, M., Inaba, M., Suzuki, I., and Murata, N. (2001) Unsaturated fatty acids in membrane lipids protect the photosynthetic machinery against salt-induced damage in *Synechococcus*. *Plant Physiol.*, **125**, 1842-1853.

Dilley, R.A., Nishiyama, Y., Gombos, Z., and Murata, N. (2001) Bioenergetics responses of *Synechocystis* 6803 fatty acid desaturase mutants at low temperature. *J. Bioenerg. Biomemb.*, **33**,135-141.

Hirani, T.A., Suzuki, I., Murata, N., Hayashi, H., and Eaton-Rye, J.J. (2001) Characterization of a two-component signal transduction system involved in the induction of alkaline phosphatase under phosphate-limiting conditions in *Synechocystis* sp. PCC 6803. *Plant Mol. Biol.*, **45**, 133-144.

Inaba, M., Sakamoto, A., and Murata, N. (2001) Functional expression in *Escherichia coli* of low-affinity and high-affinity Na^+/H^+ antiporters of *Synechocystis*. *J. Bacteriol.*, **183**, 1376-1384.

Nishiyama, Y., Yamamoto, H., Allakhverdiev, S.I., Inaba, M., Yokota, A., and Murata, N. (2001) Oxidative stress inhibits the repair of photodamage to the photosynthetic machinery. *EMBO J.*, **20**, 5587-5594.

Suzuki, I., Kanesaki, Y., Mikami, K., Kanehisa, M., and Murata, N., (2001) Cold-regulated genes under control of the cold sensor Hik33 in *Synechocystis*. *Mol. Microbiol.*, **40**, 235-244.

Turnbull, A.P., Rafferty, J.B., Sedelnikova, S.E., Slabas, A.R., Schierer, T.F., Kroon, J.T.M., Simon, J.W., Fawcett, T., Nishida, I., Murata, N., and Rice, D.W. (2001) Analysis of the structure, substrate specificity, and mechanism of squash glycerol-3-phosphate (1)-acyltransferase. *Structure*, **9**, 1-20.

(2) Review articles

Sakamoto, A., and Murata, N. (2001) The use of choline oxidase, a glycine betaine-synthesizing enzyme, to create stress resistant transgenic plants. *Plant Physiol.* (Update), **125**, 180-188.

Suzuki, I., Los, D.A., and Murata, N. (2001) Perception and transduction of low-temperature signals to induce desaturation of fatty acids. *Biochem. Soc. Trans.*, **28**, 628-630.

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Plants use light as an environmental factor which controls their development as well as their other physiological phenomena. Phytochrome and blue light receptors, such as cryptochrome and phototropin (phot), are the main photoreceptors for plant photomorphogenesis. The goal of our research is to clarify the signal transduction pathways of photomorphogenesis. 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 not only because the gametophytes are very sensitive to light, but also because the organization of the cells is very simple. We also use *Arabidopsis* mutants to clarify the genes regulating chloroplast photo-relocation movement.

I. Cloning and characterization of blue-light photoreceptors

We have described many blue-light induced photomorphological responses in gametophytes of the fern *Adiantum capillus-veneris*. As the first step in understanding the molecular mechanisms of these blue-light responses, we are cloning and sequencing the genes of blue light receptors, and are studying intracellular distributions of the gene products and their function in *Adiantum* and *Physcomitrella*

1- 1 Cryptochromes

Cryptochrome functions were studied using *CRY1a* and *CRY1b* double mutant of *Physcomitrella patens*. It was revealed that blue light cryptochrome signals regulate many steps in moss development including induction of side branch of protonemata, and regulation of gametophyte induction and its development. In addition, the disruption of cryptochrome altered auxin response, including auxin-inducible genes. Cryptochrome disruptants were more sensitive to external auxin than wild type in a blue light-specific manner, suggesting that cryptochrome light signals repress auxin signals to control plant development.

1- 2 Phototropin

Phototropin (phot1) is another blue light photoreceptor isolated recently in higher plants, and is a flavin binding protein with light sensitive protein kinase activity. A cDNA of *Adiantum* PHOT2, a homologue of phototropin has been sequenced. Photocycle of FMN binding LOV domains of the phot1 and phot2 expressed in *E. coli* were studied.

II. Chloroplast relocation movement

2- 1 *Arabidopsis*

Chloroplasts accumulate at the cell surface under weak light and escape from the cell surface to the anticlinal wall under strong light to optimize photosynthesis. The mechanism of chloroplast relocation, however, is not known. We screened several mutants from T-DNA tagging lines as well as EMS lines of *Arabidopsis*. By mutant analysis, we found last year that phot1 and phot2 were photoreceptors of chloroplast relocation movement induced by blue light. This year, we clarified that the phot1 and phot2 were also the photoreceptors redundantly working on stomatal opening in *Arabidopsis*, in collaboration with the Prof. Shimazaki's group of Kyushu University. Gene analysis of mutants defective in chloroplast accumulation response are also under way.

2- 2 *Adiantum*

Adiantum phytochrome3 (PHY3) is a unique kimeric protein with a phytochrome structure in the N-terminal half and a phototropin structure in the C-terminal half. PHY3 gene analysis of EMS-induced rap (red light-induced aphototropic) mutants of *Adiantum* which do not show phototropic response and chloroplast photorelocation movement under red light revealed that five *rap* mutant lines tested have a mutation in the PHY3 gene. Moreover, over expression of PHY3 genes in a *rap* mutant rescued the red light-induced chloroplast movement, indicating that phy3 is the photoreceptor of this phenomenon. PHY3 is not yet known whether it works as a blue light photoreceptor.

Adiantum mutants which do not show chloroplast avoidance movement under strong blue light were isolated. Gene analysis of these mutants revealed that they have a mutation in *Adiantum* phot2 gene, suggesting that *Adiantum* also use phot2 as a photoreceptor of blue light-induced avoidance response as in the case of *Arabidopsis*.

List of publication:

Briggs, W.R. et al (2001) The phototropin family of photoreceptors. *Plant Cell* **13**:993-997.

Imaizumi, T. et al Cryptochrome light signals control development to suppress auxin sensitivity in the moss *Physcomitrella patens*. *Plant Cell* in press.

Kagawa, T. et al (2001) Arabidopsis NPL1: A phototropin homolog controlling the chloroplast high-light avoidance response. *Science* **291**: 2138-2141.

Kinoshita, T. et al (2001) phot1 and phot2 mediate blue light regulation of stomatal opening. *Nature* **414**: 656-660.

Sakai, T. et al (2001) Arabidopsis nph1 and npl1: Blue-light receptors that mediate both phototropism and chloroplast relocation. *Proc. Natl. Acad. Sci. USA* **98**:6969-6974.

Schultz, T. F. et al (2001) A role for LKP2 in the circadian clock of Arabidopsis. *Plant Cell* **13**:2659-2670.

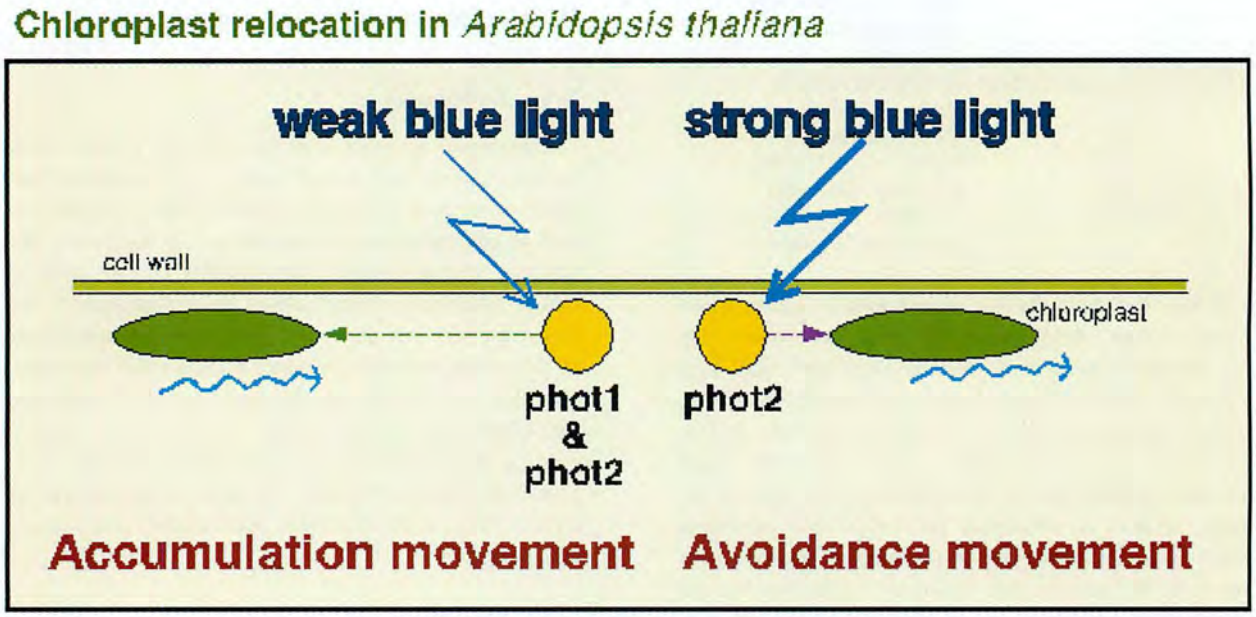

Figure 1 A scheme of chloroplast photorelocation movement in *Arabidopsis thaliana*. Strong blue light is absorbed by phototropin 2 (phot2) and chloroplasts move out from the light (avoidance movement) (Kagawa et al 2001). Under weak blue light condition, the blue light is absorbed by phot1 and phot2, and chloroplasts move towards the light irradiated area (accumulation movement) (Sakai et al 2001). The signal from photoreceptors to chloroplasts is not yet known.

DIVISION OF BEHAVIOR AND NEUROBIOLOGY (ADJUNCT)

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The vertebrate nervous system contains a large number of neurons that are connected to each other by specific axonal projections. We are interested in how the complex but precise neuronal network, which is indispensable for functioning of the nervous system, is constructed during development. The goal of our research in this division is to elucidate the cellular and molecular mechanisms underlying the neuronal network formation in vertebrates, with current focuses on the mechanisms of axon guidance and neuronal migration.

I. Mechanism of Axonal Guidance

During development of the nervous system, growing axons are guided through specific pathways to correct targets. Our research interest focuses on the cellular and molecular mechanisms of axon guidance in the vertebrate nervous system. We are particularly interested in how guidance mechanisms operating in different phases of axonal growth are assembled to generate the complex but precise axonal wiring patterns in the brain. To address these issues, we are first trying to understand axonal wiring patterns during development with reference to the structural organiza-tion of the brain. Using whole-mounted preparations of embryonic rat brains, we will reveal whole axonal trajectories by labeling with neuronal tracers, such as DiI, or by immunohistochemical staining with antibodies against molecular markers expressed in a subset of neurons. We will next explore the axonal guidance mechanisms generating these wiring patterns. Following questions will be addressed. 1) What structures along the pathway have key roles in axonal guidance? 2) What guidance cues exist in these structures? Are these attractive cues or repulsive cues? Are these short-range cues or long-range cues? 3) What molecules are responsible for these cues? 4) How do multiple guidance mechanisms work in concert to generate specific wiring patterns? To answer these questions, we use neuroanatomical techniques, in vitro culture techniques including dissociated cell, explant and whole-mount cultures, and biochemical and molecular biological techniques.

II. Mechanism of Neuronal Migration

A variety of neurons migrate from their birthplace to the position where they finally settle. Neuronal migration in the vertebrate central nervous system occurs both along the radial axis and along the tangential axis of the neural tube. Another interest of our research focuses on the cellular and molecular mechanisms underlying the radial and tangential neuronal migration. In many regions of the central nervous system, neurons migrate radially from the ventricular zone where they are born toward the pial surface. We are investigating the mechanisms of radial migration, using neurons in the neocortex as a model system. In addition to the radial migration, some neurons in the brain, such as interneurons in the forebrain, move tangentially for a long distance. We are also investigating how tangentially migrating neurons are guided for a long distance through specific routes to the final positions, using neurons that migrate from the rhombic lip at the dorsal rim of the hindbrain, such as cerebellar granule cells and precerebellar neurons.

To address these issues, we developed in vitro culture systems that reconstruct migratory events occurring in vivo. We labeled migrating neurons with green fluorescent protein (GFP) by transplantation of small piece of explant taken from a transgenic rat expressing GFP or by introducing GFP cDNA into limited regions of the brain by electroporation. Slices of the brain or flat-mounted brain preparation are then cultured on permeable membrane filters. These culture systems enable us to analyze migration pattern of neurons in real time (Fig. 1). Using these in vitro culture systems, we are currently investigating the cellular and molecular mechanisms of neuronal migration.

Publication List:

Imai, T., Tokunaga, A., Yoshida, T., Hashimoto, M., Mikoshiba, K., Weinmaster, G., Nakafuku, M., and Okano, H. (2001). The neural RNA-binding protein Musashi1 translationally regulates mammalian numb gene expression by interacting with its mRNA. *Mol. Cell.Biol.* **21**, 3888-900.

Mizuguchi, R., Sugimori, M., Takebayashi, H., Kosako, H., Nagao, M., Yoshida, S., Nabeshima, Y., Shimamura, K., and Nakafuku, M. (2001). Combinatorial roles of olig2 and neurogenin2 in the coordinated induction of pan-neuronal and subtype-specific properties of motoneurons. *Neuron* **31**, 757-71.

Nakashima, K., Takizawa, T., Ochiai, W., Yanagisawa, M., Hisatsune, T., Nakafuku, M., Miyazono, K., Kishimoto, T., Kageyama, R., and Taga, T. (2001). BMP2-mediated alteration in the developmental pathway of fetal mouse brain cells from neurogenesis to astrocytogenesis. *Proc. Natl. Acad. Sci. USA* **98**, 5868-73.

Otsuka, T., Murakami, F., and Song, W. J. (2001). Excitatory postsynaptic potentials trigger a plateau potential in rat subthalamic neurons at hyperpolarized states. *J. Neurophysiol.* **86**, 1816-25.

Sawamoto, K., Nakao, N., Kobayashi, K., Matsushita,

N., Takahashi, H., Kakishita, K., Yamamoto, A., Yoshizaki, T., Terashima, T., Murakami, F., Itakura, T., and Okano, H. (2001). Visualization, direct isolation, and transplantation of midbrain dopaminergic neurons. *Proc. Natl. Acad. Sci. USA* **98**, 6423-8.

Shirasaki, R., and Murakami, F. (2001). Crossing the floor plate triggers sharp turning of commissural axons. *Develop. Biol.* **236**, 99-108.

Tashiro, Y., Miyahara, M., Shirasaki, R., Okabe, M., Heizmann, C. W., and Murakami, F. (2001). Local nonpermissive and oriented permissive cues guide vestibular axons to the cerebellum. *Development* **128**, 973-81.

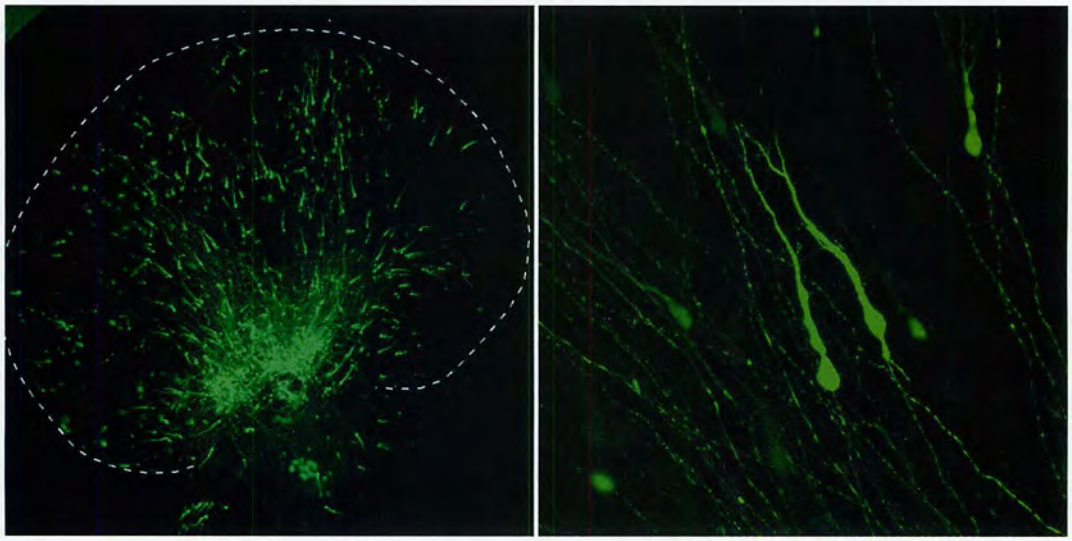

Figure 1. Migrating neocortical neurons visualized with green fluorescent protein (GFP) in vitro.
(Left) Slice culture of rat neocortex that was introduced with GFP cDNA into the ventricular zone by electroporation. A broken line shows the outer margin of the slice. GFP-positive neurons were moving radially from the ventricular zone (bottom) toward the pial surface (top).
(Right) High power view of labeled migrating neurons. Leading processes of neurons extended toward the pial surface.

LABORATORY OF GENE EXPRESSION AND REGULATION

Chairperson: IIDA, Shigeru

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

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

DIVISION OF GENE EXPRESSION AND REGULATION I

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	PARK, Kyeung-Il
Postdoctoral Fellow:	*URAWA, Hiroko*
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	KIKUCHI, Yasumasa[8)]
	SAITOH, Miho[9)]
	Matthew William Hahn[4)]
Technical Assistant:	*MORITA, Yasumasa*

* *until April 30, 2001*
** *until Marchl 31, 2001*
1) *from Chinese Academy of Sciences*
2) *from University of California*
3) *from Vrije University*
4) *from Yeungnam University*
5) *from Duke University*
6) *from Kasetsart University*
7) *Graduate University for Advanced Studies*
8) *from University of Shizuoka*
9) *from Toho University*

The main interest of the group is in understanding the biology of the dynamic genome, namely, genome organization and reorganization and its impact on gene expression and regulation. Although there are many elements affecting organization and reorganization of the genomes, we are currently focused on mobile genetic elements in general and plant transposable controlling elements in particular. Since plant transposable elements are known to be associated with both genetic and epigenetic gene regulations, we are characterizing various aspects of genetic and epigenetic gene regulations. In addition, we are also undertaking reverse genetic approaches in order to elucidate the nature of dynamic genome in plants.

I. Spontaneous mutants in the Japanese morning glory.

The Japanese morning glory (*Ipomoea nil* or *Pharbitis nil*), displaying blue flowers, is believed to be originated from southeast Asia and has an extensive history of genetic and physiological studies. The plant had been introduced into Japan from China in about 8th century as a medicinal herb, seeds of which were utilized as a laxative, and has become a traditional horticultural plant in Japan since around 17th century. A number of its spontaneous mutants related to the color and shape of the flowers and leaves have been isolated, and about 10% of these mutants carry mutable alleles conferring variegated phenotypes. All of the mutants available are spontaneous mutants and most of them were isolated more than 60 years ago. Several lines of evidence indicate that an *En/Spm*-related transposable element *Tpn1* and its relatives, which we termed *Tpn1*-family elements, are a common spontaneous mutagen in the plant. Indeed, we have succeeded to identify three of these mutable alleles for flower pigmentation, *flecked, speckled* and *purple-mutable* (*pr-m*), which are caused by integration of *Tpn1*-related elements, *Tpn1, Tpn2* and *Tpn4*, respectively. All of them are non-autonomous elements and their transposition is mediated by a *Tpn1*-related autonomous element which appears to be subjected to egigenetic regulations. Due to epigenetic inactivation of the autonomous element, rare excision of these non-autonomous elements could occur and such lines displayed apparently stable mutant flowers. In accordance with this notion, an apparent stable *r-1* allele conferring white flowers is also caused by insertion of a non-autonomous *Tpn1*-family element, *Tpn3*, into the *CHS-D* gene encoding a chalcone synthase for anthocyanin biosynthesis.

II. Spontaneous mutants having *Tpn1*-related transposons inserted into the gene encoding anthocyanidin synthase in the Japanese morning glory.

The anthocyanidin synthase (ANS) catalyzes 2-oxoglutarate-dependent oxidation of leucoanthocyanidins to yield anthocyanidins in the anthocyanin biosynthesis pathway. The *ANS* gene was first isolated from maize and snapdragon, mutants of which are deficient in pigmentation: the *A2* mutations caused by insertion of transposons in maize control coloration in the aleurone layer of the kernel and a large deletion mutation including the *candi* gene in snapdragon confers completely acyanic flowers. Out of 23 mutants of the Japanese morning glory displaying white flowers examined, three were found to carry an identical insertion of a 6.6-kb *Tpn1*-related transposon, *Tpn8*, at the promoter of the *ANS* gene (Fig. 1). In addition, we also characterized a mutable line *Shibori-chidori* exhibiting white flowers with red sectors, obtained from a red flower line *Beni-chidori* about 3 years ago, and found that the mutable allele was caused by integration of a 6.4-kb transposon, *Tpn9*, into the *ANS* gene. Interestingly, *Tpn9* is almost identical to the *Tpn1* sequence found at the mutable *flecked* allele. To our knowledge, the *ANS* mutations characterized here are the first *ANS* insertion mutations, including the mutable allele, that affect flower pigmentation. Since the mutable line *Shibori-chidori* was isolated as a spontaneous mutant very recently, *Tpn1*-related elements still act as active spontaneous mutagens that would generate new interesting traits in the Japanese morning glory.

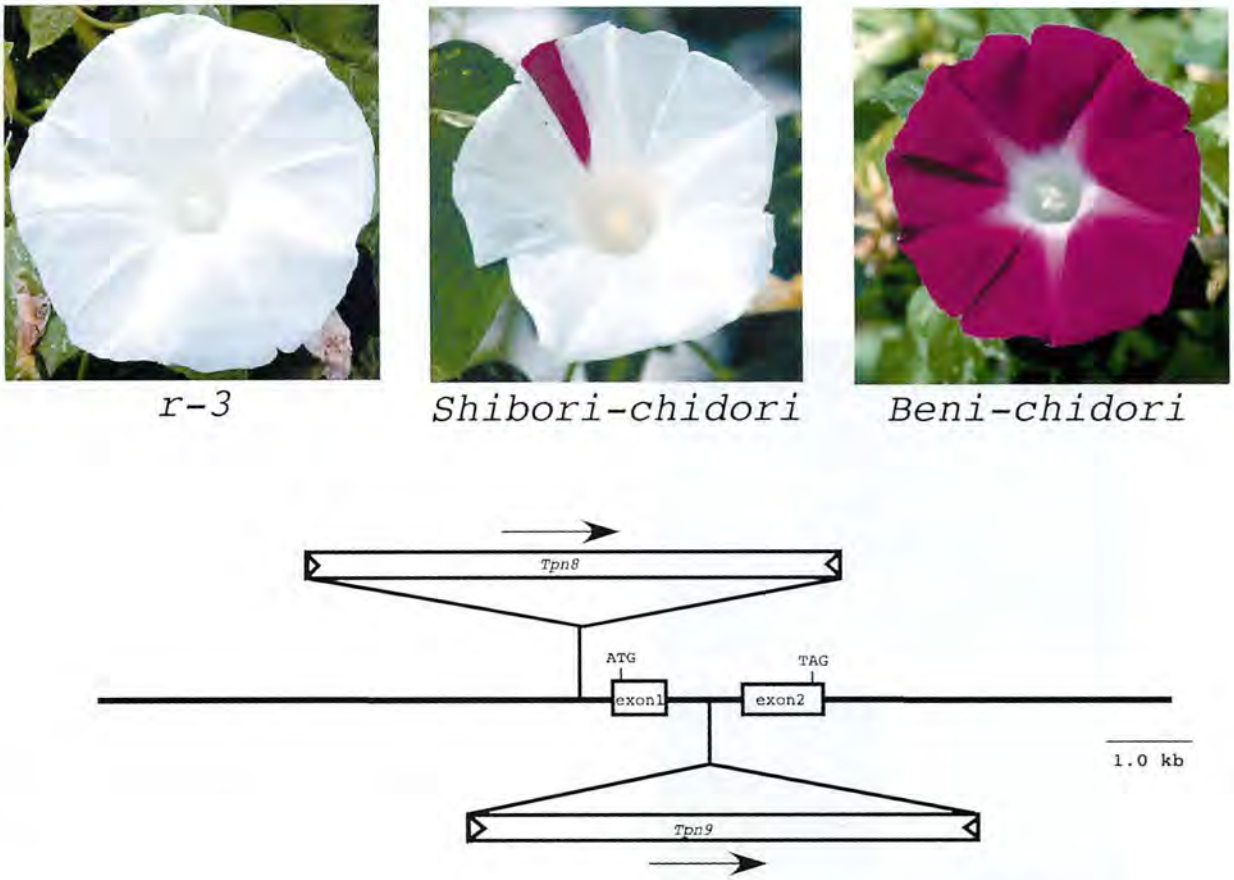

Fig. 1. Two mutant alleles in the *ANS* gene of the Japanese morning glory.
The mutants *r-3* and *Shibori-chidori* have *Tpn8* and *Tpn9* inserted into the *ANS* gene, respectively.

III. The transposable element *Tip100* found at the mutable *flaked* allele for flower variegation of the common morning glory is an autonomous element.

The mutable *flaked* line of the common morning glory (*Ipomoea purpurea* or *Pharbitis purpurea*) displays white flowers with colored flakes, and the *flaked* mutation is caused by the insertion of a transposable element *Tip100* into the *CHS-D* gene for anthocyanin biosynthesis. The 3.9 kb *Tip100* element belonging to the *Ac/Ds* family contains an open reading frame encoding a polypeptide of 808 amino acids. The frequency and timing of the flower variegation vary in different *flaked* lines and a genetic element termed *Modulator* has been postulated to affect the variegation pattern. Since the pattern of the flower variegation is determined by the frequency and timing of the excision of *Tip100* from the *CHS-D* gene, we examined whether *Tip100* is an autonomous element capable of transposition in heterologous tobacco plants. The intact *Tip100* element was able to excise from its original position in an introduced vector and to reinsert into new sites in the tobacco genome, whereas its internal deletion derivative was not. Based on these results, we concluded that *Tip100* is an autonomous element.

IV. Targeted gene disruption by homologous recombination in rice.

The modification of targeted chromosomal genes through homologous recombination is a powerful tool of reverse genetics for characterizing gene functions. In higher plants, however, a transgene is integrated randomly into the genome by illegitimate recombination even the introduced sequence contains targeted homologous region. Although generation of a single transgenic *Arabidopsis* line having a targeted gene disrupted was reported, the procedure was far from a common practice in higher flowering plants. Rice is an important staple food for more than half of the world's population and has become a model monocotyledonous plant because of accumulating information from rice genome projects as well as efficient transformation. To develop a reproducible and reliable procedure for targeted gene disruption by homologous recombination in rice, we tried to improve the following parameters: efficiency of *Agrobacterium*-mediated transformation, utilization of a strong positive-negative selection, and PCR amplification for screening and detecting a long junction fragment produced by homologous recombination. Subsequently, we conducted detailed Southern blot analysis to confirm the occurrence of the expected

precise homologous recombination. We chose the single locus gene *Waxy* encoding granule-bound starch synthase, a key enzyme in amylose synthesis, in rice as a model gene to be targeted, because its mutant is of agronomic importance and because its phenotype in endosperm and pollen can be easily monitored by simple iodine staining. A transgenic rice plant having a targeted gene disrupted by a transgene (Fig. 2) was isolated, which appears to be a heterozygote with only one copy of the integrated transgene in its geneome.

Fig. 2. Regeneration of a targeted callus through multiple shoots, a plantlet and a fertile transgenic plant from the targeted callus.
More than 50 shoots can be usually obtained from a targeted callus.

Publication List:

Fukada-Tanaka, S., Inagaki, Y., Yamaguchi T. and Iida, S. (2001) Simplified transposon display (STD): a new procedure for isolation of a gene tagged by a transposable element belonging to the *Tpn1* family in the Japanese morning glory. *Plant Biotech.* **18,** 143-149.

Hoshino, A., Johzuka-Hisatomi Y. and Iida, S. (2001) Gene duplication and mobile genetic elements in the morning glories. *Gene* **265,** 1-10.

Ishikawa, N., Johzuka-Hisatomi, Y., Sugita, K., Ebinuma H. and Iida, S. The transposon *Tip100* from the common morning glory is an autonomous element that can transpose in tobacco plants. *Mol. Gen. Genet.* (in press).

Kamiunten, H., Inoue, S., Yakabe Y. and Iida, S. Characterization of ISPsy2 and ISPsy3, newly identified insertion sequences in *Pseudomonas syringae* pv. *Eriobotryae*. Gen. Plant Pathol. (in press).

Terada, R., Ignacimuthu, S., Bauer, P., Schultze, M., Kondorosi, A., Potrykus I. and Sautter, C. (2001) Expression of early nodulin promoter gene in transgenic rice. *Curr. Sci.* **81**, 270-276.

Toki, K., Saito, N., Iida, S., Hoshino, A., Shigihara A. and Honda, T. (2001) Acylated pelargonidin 3-sophoroside-5-glucosides from the flowers of the Japanese morning glory cultivar 'Violet'. *Heterocycles* **55,** 1241–1248.

Toki, K., Saito, N., Iida, S., Hoshino, A., Shigihara A. and Honda, T. A novel acylated pelargonidin 3-sophoroside-5-glucosides from greyish-purple flowers of the Japanese morning glory. *Heterocycles* **55,** 2261–2267.

Yamaguchi, T., Fukada-Tanaka, S., Inagaki, Y., Saito, N., Yonekura-Sakakibara, K., Tanaka, Y., Kusumi T. and Iida, S. (2001) Genes encoding the vacuolar Na^+/H^+ exchanger and flower coloration. *Plant Cell Physiol.* **42,** 451-461.

DIVISION OF GENE EXPRESSION AND REGULATION II

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Graduate Student:	*SERIZAWA, Naomi*
	WATANABE, Takaaki
Visiting Fellows:	*UJIIE, Yoshifumi*

** to Department of Biological Chemistry, University of California-Irvine, California (Feb. 1, 1999- Jan. 15, 2002)*
** to Division of Gene Expression and Regulation 1 (Apr. 1, 2001)*

The genomes of higher organisms contain significant amounts of repetitive sequences, which in general, are unstable. At present, neither the physiological function(s) of repeated sequences nor the mechanism controlling the instability is fully understood. To clarify these aspects, we are pursuing the following themes using *E. coli*, *S. cerevisiae* and plants: (1) An amplification mechanism of repeated sequences or genes, especially rRNA repeated genes, (2) a mechanism of replication fork block-dependent recombination, a key reaction that increases or decreases the number of repeats, and (3) development of *in vivo* artificial gene amplification systems. Functional and structural analyses of the *E. coli* genome are also being carried out. In 2001, work on the following three subjects has advanced our knowledge of the dynamics and structure of the genome.

I. Amplification of Hot DNA segments in *Escherichia coli*

In yeast, about 150 copies of rDNA are located at a specific locus on chromosome XII. A replication fork-blocking site, called a replication fork barrier (*RFB*), is located in each rDNA unit. To block the replication fork at *RFB*, another *trans*-acting factor, named Fob1 protein, is required. Our recent work indicated that amplification of rDNA repeats requires a DNA replication fork-blocking event. Furthermore, Fob1 protein is essential for rDNA region specific homologous recombination and the production of circular rDNA molecules, as well as rDNA amplification. Such fork blocking dependent recombination was first identified in recombinational hotspot (Hot) in *E. coli*. The eight kinds of Hot DNAs (HotA-H) were identified using an *E. coli rnhA*$^-$ mutant. Among these, enhanced recombination of three kinds of Hot DNAs (HotA, B and C) was dependent on fork blocking events at *Ter* sites. Then, we examined whether *E. coli* HotA DNAs are amplified when circular DNA (HotA plus a drug-resistance DNA segment) is inserted into the homologous region on the chromosome of an *E. coli rnhA*$^-$ mutant. The resulting HotA DNA transformants were analyzed using pulsed field gel electrophoresis, fluorescence *in situ* hybridization, and DNA microarrays. The following results were obtained. (1) HotA DNA is amplified by about 40-fold on average. (2) While 90% of the cells contain 6-10 copies of HotA DNA, the remaining 10% of cells have as many as several hundred HotA copies (Fig. 1). (3) Amplification is detected in all other Hot DNAs, among which HotB and HotG DNAs are amplified to the same level as HotA. Furthermore, HotL DNA, which is activated by blocking the clockwise *oriC*-starting replication fork at the artificially inserted *TerL* site in the fork-blocked strain with a *rnh*$^+$ background, is also amplified.

II. Yeast RNA polymerase I enhancer is dispensable for both transcription of the chromosomal rDNA gene and cell growth, and its apparent transcription enhancement from ectopic promoters requires Fob1 protein.

Previously we developed a system in which various deletions can be introduced into each rDNA repeat in *S. cerevisiae*. Each rDNA repeat consists of the 35S rRNA gene, the NTS1 spacer, the 5S rRNA gene, and the NTS2 spacer. The *FOB1* gene was previously shown to be required for replication fork block (RFB) activity at the *RFB* site in NTS1, for recombination hot spot (*HOT1*) activity, and for rDNA amplification and contraction. We have constructed a strain in which the majority of the rDNA repeats are deleted, leaving two copies of rDNA covering the 5S-NTS2-35S region and a single intact NTS1. Growth of the strain is supported by a helper plasmid, carrying, in addition to the 5S rRNA gene, the 35S rRNA coding regions fused to the *GAL7* promoter. This strain carries a *fob1* mutation, and an extensive expansion of chromosomal rDNA repeats was demonstrated by introducing the missing *FOB1* gene by transformation. The budding yeast 35S rDNA transcription enhancer, which is located at the end of the 35S rRNA gene within the rDNA repeats, has been shown to greatly stimulate rDNA transcription in an ectopic reporter system. By removing the single enhancer region remaining in the single NTS1 in the two rDNA copy strain, followed by rDNA amplification, we constructed a yeast strain which was deleted of all of the enhancers from the rDNA repeated genes. We found that this strain did not show any defect in growth or rRNA synthesis. This result suggests that transcription activity measured in the ectopic site is not exactly parallel to that in the rDNA cluster.

Ectopic transcription has an effect on recombination. *HOT1* DNA enhances recombination at the nearby site when it inserted into a non-rDNA region. *HOT1* DNA consist two non-contiguous DNA fragments, called E and I, which are the enhancer and initiator of 35S rDNA transcription, respectively. Thus, it was speculated that *HOT1* recombinational enhancement was caused by transcription from the *HOT1* region. Later it was proven

that a 35S rDNA specific transcription enzyme (PolI) is required for *HOT1* recombination. On the other hand, we had previously expected that the fork blocking event is also involved in *HOT1* recombination, because the E element contains an *RFB* site ("enhancer" described above is the same as E except for *RFB*). Actually, we found that the *FOB1* gene is also required for *HOT1* activity. However, another group recently reported that *HOT1* activity is *RFB* orientation independent, suggesting that the fork blocking event is not responsible for *HOT1* enhancing. We, therefore, examined the effect of the *fob1* mutation on *HOT1* transcription. The results showed that the mutation abolishes transcription from *HOT1* DNA. This means that while Fob1 is a fork blocking protein in the rDNA cluster, it acts as transcription factor as well in the ectopic *HOT1* system. Analogously, PolI is essential for *HOT1* recombination, but it is never required for homologous recombination in the rDNA cluster. The *HOT1* activation mechanism cannot explain any mechanism working in the rDNA cluster.

III. *E. coli* genomic structure and function

We completed the whole genomic sequence of *E. coli* W3110, which is a derivative of a common ancestor of MG1655, whose entire genomic sequence was sequenced by the US team. The two strains were separated during or right after World War II. Currently, we are comparing their sequences to understand the micro-evolution of these strains.

In order to identify the minimal set of genes required for the duplication of a single cell and to elucidate the function of the genes, we initiated a team project in Japan, headed by Mori (NAIST). For the past two years, our project has been supported by CREST from JST and is being carried out by the following groups: (1) resources, (2) informatics, (3) database, and (4) functional analysis. Until now we have had adequate results, publishing in more than fifty papers and some of the resources established are now available worldwide. In the Annual Bio-training Course held in our Institute this year, our laboratory organized an experimental course entitled "DNA chip: focused on its data analysis" which was strongly supported by team members, belonging to project group (1).

Publication list:

Johzuka, K., and Horiuchi, T. (2002) Replication fork-block protein (Fob1) acts as a rDNA region specific recombinator in *S. cerevisiae*. *Genes Cells* (in press)

Kobayashi, T., Nomura, M., and Horiuchi, T. (2001) Identification of DNA cis-elements essential for expansion of ribosomal DNA repeats in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **21**, 136-147.

Urawa, H., Hidaka, M., Ishiguro, S., Okada, K., and Horiuchi T. (2001) Enhanced homologous recombination caused by the non-transcribed spacer of the rDNA in *Arabidopsis*. *Mol. Genet. Genomics* **266**, 546-555.

Wai, H., Johzuka, K., Vu, L., Eliason, K., Kobayashi, T., Horiuchi, T., and Nomura, M. (2001) Yeast RNA polymerase I enhancer is dispensable for growth and its apparent transcription enhancement for ectopic promoters Fob1 protein implicated in replication and recombination of rDNA. *Mol. Cell. Biol.* **21**, 5541-5553.

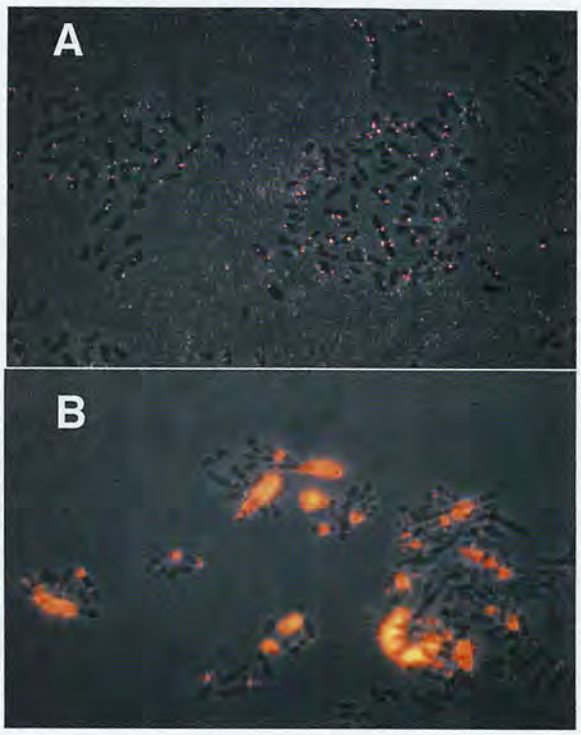

Figure 1. Amplification of HotA DNA in individual *E. coli* cells.

A. FISH image of a parental strain (KHG571) with –HotA– structure. B. FISH image of the HotA-*tet* DNA transformed KHG571 strain with –HotA–*tet*–HotA– structure. These photos show that while most cells of KHG571 have one or two red fluorescence foci, each corresponding to a single copy of HotA DNA, about 10% of cells of the transformed KHG571 have very large, bright signals, each corresponding to about 350 copies (more than half a *E. coli* genome size) on average.

DIVISION OF SPECIATION MECHANISMS I

Professor:	*YAMAMORI, Tetsuo*
Research Associate:	*KOMINE, Yuriko*
	WATAKABE, Akiya
	KITSUKAWA, Takashi
Technical Staff:	*UTSUMI, Hideko*
	SAWADA, Kaoru
NIBB Postdoctoral Fellow:	*Vigot, Rejan*
Graduate student:	*TOCHITANI, Shiro (until March)*
	HATA, Katsusuke
	KOMATSU, Yusuke
	SAKATA, Shuzo (kyoto University)
Visiting scientist:	*SHIRAI, Yoshinori (until June)*

Our research goal is to understand mechanisms underlying evolution of the nervous system. In order to approach this question, we are studying the genes that are expressed in specific areas of the primate neocortex. Using differential display method, we have obtained genes that showed marked differences within the primate neocortex.

Our second approach is to understand informational processing in the brain underlying learning behaviors with gene expression techniques. Here, we report our findings in the year of 2001.

I. Genes expressed in specific areas of the neocortex

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

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

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

i) One gene, designated *occ1*, is specifically expressed in the occipital cortex, particularly in V1 area, in the primate brain. Furthermore, the expression of occ1 turned out to be activity dependent, because, in the monocularly deprive–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).

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. We are currently examining the detailed expression pattern of the gene.

3) We have also further isolated several area specific genes with RLCS (Restriction Landmark cDNA Scanning).

II. Gene expression under audio-visual discrimination task

We studied gene expression of c-Fos under audio-visual discrimination tasks in collaboration with professor Yoshio Sakurai (Kyoto University). 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.

III CNTF is specifically expressed in the developing pineal glands

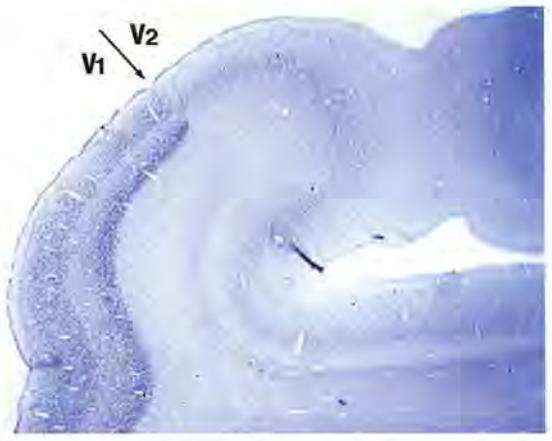

Fig. 1 Expression pattern of occ1 in the visual cortex.
In situ hybridization pattern of *occ1* in the primate visual cortex. occ1 is markedly expressed in the layer IVcβ and moderately in the layers of II, III and IVα in area V1. The boundary between V1 and V2 is shown by an arrow.

CNTF, a member of the IL-6 family, attracts quite attentions of developmental neuroscientists because it shows various effects on neurons and glial cells. The CNTF knockout mice, however, only indicates moderate motor neuron deficiency in the adult, but no apparent phenotype in the development. In order to explore the function of the IL-6 family, we extensively examined the expression of members of the family and their receptors and found the specific expression of CNTF in the embryonic pineal glands and eyes. Since sympathetic neurons are known to innervate pineal glands and be responsive to CNTF, our observation suggests that CNTF plays a role when sympathetic neurons innervate pineal glands.

Publication list:

Original Articles

Fujiwara, T., Yamamori, T. and Akagawa, K. Suppression of transmitter release by Tat HPC-1/syntaxin 1A fusion protein. (2001) *Biochim. Biophys. Acta* **1539**, 225-232.

Hanazawa, A., Mikamai, A., Angelika, P.S., Takenaka, O., Goto, S., Onishi, A., Koike, S., Yamamoi, T., Kato, k., Kondo, A., Suryobroto, B., Farajallah, A., Komatsu, H. (2001) Electroretinogram analysis of relative spectral sensitivity in genetically identified dichromatic macaques. *Proc. Natl Acad. Sci. USA.*, **98**, 8124-8127.

Hata, K., Arakai, M. and Yamamori, T. CNTF is specifically expressed in the developing rat pineal gland and eyes. *NeuroReport* (in press)

Karachot, L., Shirai, Y., Vigot, R., Yamamori, T. and Ito, M. Induction of long-term depression in cerebellar Purkinje cells requires a rapidly turned over protein. (2001) *J. Neurophysiol.* **86**, 280-289.

Onishi, A., Koike, S., Ida-Hosonuma, M., Imai, H., Shichida, Y., Takenaka, O., Hanazawa, A., Komatsu, H., Mikamai, A., Goto., S., Suryobroto, B., Farajallah, A., Varavudhi, P., Ekavhibata, C., Kitahara, K. and Yamamori, T. Variations in long and middle-wavelength-sensitive opsin loci in crab-eating monkeys. *Vision Res.* (in press)

Sakata, S., Kitsukawa, T., Kaneko, T., Yamamori, T. and Sakurai, Y. Task-dependent and cell-type-specific Fos enhancement in rat sensory cortices during audio-visual discrimination. *Eur. J. Neuroscience* (in press)

Tochitani, S., Liang, F., Watakabe, A., Hashikawa, T., and Yamamori, T. (2001) occl is preferentially expressed in the primary visual cortex in an activity-dependent manner: a pattern of gene expression related to the cytoarchitectonic area in adult macaue neocortex. *Eur. J. Neurosci.* **13**, 297-307.

Vigot, R., Batini, C., Kado, R.T. and Yamamori. T. Synaptic LTD *in vivo* recorded on the rat cerebellar cortex. *Arch. Ital. Biol.* (in press)

Watakabe A. , Sugai T., Nakaya N., Wakabayashi K., Takahashi H., Yamamori T. and Nawa H. (2001) Similarity and Variation in Gene Expression Among Human Cerebral Cortical Subregions Revealed By DNA Macroarrays: Technical Consideration of RNA Expression Profiling from Postmortem Samples. *Mol. Brain Res.*, **88**, 74-82.

Watakabe, A., Fujita, H., Hayashi, M. and Yamamori T. (2001) GDF7, a BMP/TGF beta family member, is enriched in the primary motor area of monkey neocortex. *J. Neurochem.*, **76**, 1455-1464.

DIVISION OF SPECIATION MECHANISMS II

Professor:	*HASEBE, Mitsuyasu*
Associate Professor	*MURATA, Takashi*
Assistant Professor:	*FUJITA, Tomomichi*
Technical Staff:	*SUMIKAWA, Naomi*
NIBB Research Fellow:	*MISHIMA, Misako*
	HIWATASHI, Yuji (Oct. 1-)
JSPS Research Fellow:	*NISHIYAMA, Tomoaki*
Graduate Students:	*HIWATASHI, Yuji (-Sept. 30)*
	KOBAYASHI-ARAKAWA, Satoko
	SAKAKIBARA, Keiko
	SANO, Ryosuke (-March 31)
	SAKAGUCHI, Hisako (Shinshu Univ.)
Technical Assistant:	*TANIKAWA, Yukiko*
	UMEDA, Masae (-Dec. 28)
	BITOH, Yoshimi
	NARUSE, Mayumi
	AOKI, Etsuko (April 1-)
	WATANABE, Kyoko (July 11-)
	YANO, Kana (Nov. 19-)
Secretary:	*KABEYA, Kazuko*
Visiting Scientists:	*Jo Ann Banks [1] (-July 15)*
	George Ratherford [1] (-July 15)
	Jean-Pierre Zrÿd [2] (Sept. 24-)

[1] *from Purdue University, West Lafayette, IN, USA*
[2] *from Université de Lausanne, Lausanne, Switzerland*

All living organisms evolved from a common ancestor more than 3.5 billion years ago, and accumulated mutations on their genomes caused the present biodiversity. The traces of evolutionary processes are found in the genomes of extant organisms. By comparing the genomes of different organisms, we can infer (1) the phylogenetic relationships of extant organisms and (2) the genetic changes having caused the evolution of morphology and development. The inferred phylogenetic relationships give important insights on problems in various fields of evolutionary biology, and our group focuses on biogeography, evolution of morphological traits, and systematics in wide range of taxa. On the evolution of morphology and development, we aim to explore genetic changes led the evolution of plant body plan. We selected *Arabidopsis* (angiosperm), *Gnetum* (gymnosperm), *Ginkgo* (gymnosperm), *Ceratopteris* (pteridophyte), *Physcomitrella* (bryophyte), and some green algae as models to compare the gene functions involved in development of the reproductive organs and shoot apical meristem of land plants.

I. Evolution of reproductive organs in land plants

A flower is the most complex reproductive organ in land plants and composed of sepals, petals, stamens, and gynoecium. Female haploid reproductive cells are covered with a sporangium (nucellus) and two integuments, and further enclosed in a gynoecium. Male haploid reproductive cells (pollens) are covered with a sporangium (pollen sack). On the other hand, gymnosperms and ferns have simpler reproductive organs than angiosperms and lack sepals and petals. Female sporangia (nucellus) of gymnosperms are covered with only one integument. Sporangia of ferns have no integuments and are naked on the abaxial side of a leaf.

The development of floral organs is mainly regulated by A-, B-, C-function genes, which are members of the MADS-box gene family. These genes are transcription factors containing the conserved MADS and K domains. MADS-box genes of angiosperms are divided into more than 10 groups based on the gene tree. The *LEAFY* gene is the positive regulator of the MADS-box genes in flower primordia.

What kind of changes of the MADS-box genes caused the evolution of the complex reproductive organs in the flowering plant lineage ? Comparisons of MADS-box and *LFY* genes in vascular plants suggest that the following sequential changes occurred in the evolution of reproductive organs. (1) Plant-type MADS-box genes with both MADS and K domains were established. (2) The number of MADS-box genes increased, and the three ancestral MADS-box genes that later generate A-, B-, C-functions genes were likely originated before the divergence of ferns and seed plants. (3) Specifically expressed MADS-box genes in reproductive organs evolved from generally expressed ones in the seed plant lineage. (4) The ancestral gene of the AG group of MADS-box genes acquired the C-function before the divergence of extant gymnosperms and angiosperms. (5) The gene duplication that formed the AP3 and PI groups in MADS-box genes occurred before the diversification of extant gymnosperms and angiosperms. (6) The ancestral gene of angiosperm A-function gene was lost in extant gymnosperm lineage. (7) *LFY* gene became positively regulate MADS-box genes after the divergence of ferns and seed plants, because the fern *LFY* gene does not directly regulate MADS-box genes (Himi et al. 2001). The *FLO/LFY* gene phylogenetic tree indicates that both duplication and loss of *FLO/LFY* homologs occurred during the course of vascular plant evolution. The inductive pathway from the *LFY* gene to the MADS-box genes already existed in the common ancestor of angiosperms and gymnosperms, because overexpression of *Gnetum LFY* homolog in transgenic *Arabidopsis* promoted a conversion of a shoot meristem to a floral primordium and the *Arabidopsis LFY* null mutant, *lfy-26,* with a malformed flower, was complemented by overexpression of *Gnetum LFY* gene. (8) Spatial and temporal patterns of A-, B-, C-function gene expression were established in the angiosperm lineage (Shindo et al. 2001).

II. Homology of reproductive organs in seed plants

The morphological variation among reproductive organs of extant seed plants makes assessment of organ homology difficult. Comparisons of expression patterns of homeotic genes that control organ development will yield new information about the homology of organs to assess inferences deduced from previous mor-

phological studies. To provide insights into the evolution of reproductive organs in seed plants, a *Gnetum LFY* homolog was cloned and its expression patters were compared to the conifer *LFY* homolog. The comparison suggests that the *Gnetum* collar and ovule are homologous with the conifer bract and ovule-ovuliferous scale complex, respectively. This inference is concordant with our previous comparisons of expression patters of orthologous MADS-box genes between *Gnetum* and conifers (Shindo et al. 2001).

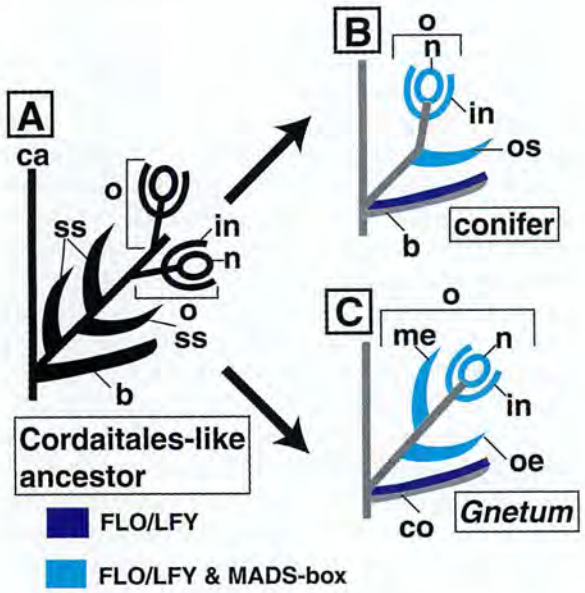

Figure 1. Expression patterns of gymnosperm *FLO/LFY* homologs and MADS-box genes are shown schematically in a scenario of the evolution of ovule-bearing structures in conifers and *Gnetum* from a Cordaitales-like prototype. A, Fertile shoot subtended by a bract in the Cordaitales-like ancestor. B, Ovuliferous structure of a conifer. C, Ovuliferous structure of *Gnetum*. The ovuliferous scale of conifers should correspond to several sterile scales of the Cordaites-like ancestor based on fossil records. Outer and middle envelopes likely correspond to two sterile scales of the Cordaites-like ancestor, because these envelopes develop from a pair of opposite primordia. b, bract; ca, cone axis; co, collar; in, integument; me, middle envelope; n, nucellus; o, ovule; oe, outer envelope; os, ovuliferous scale; ss, sterile scale.

III-I. Evolution of vegetative organs

The ancestor of land plants was primarily haploid. The only diploid cell was the zygote, which immediately underwent meiosis. It is believed that early during land plant evolution, zygotic meiosis was delayed and a multicellular diploid sporophytic generation became interpolated into the life cycle. In the early stages of land plant evolution, sporophytes are epiphytic to gametophytes, as observed in extant bryophytes. During the course of evolution, both generations started to grow independently at the stage of pteridophytes. Finally gametophytes became much reduced and epiphytic to sporophytes in seed plant lineage. Molecular mechanisms of development in a diploid generation have been well studied in some model angiosperms, but we have scarce information on those in a gametophyte generation. For example, mosses have leaf- and stem-like organs in their haploid generation, but it is completely unknown whether similar genes involved in angiosperm leaf and stem development are used in the gametophytic generation of mosses or not. To understand the evolution of body plans in diploid and haploid generation at the molecular level, we focus on the comparison of molecular mechanisms governing shoot development between Arabidopsis and the moss *Physcomitrella patens*. *P. patens* is known by its high rate of homologous recombination and suitable for analyze gene functions using the gene targeting, and should be a good model lower land plants.

III-II. Characterization of homeobox genes in the moss *Physcomitrella patens*

Homeobox genes encode transcription factors involved in many aspects of developmental processes including shoot development in angiosperms. The homeodomain-leucine zipper (HD-Zip) genes, which are characterized by the presence of both a homeodomain and a leucine zipper motif, form a clade within the homeobox superfamily and previously reported only from vascular plants. We isolated 10 HD-Zip genes from *P. patens* (*Pphb1-10* genes). Based on a phylogenetic analysis of the 10 *Pphb* genes and previously reported vascular plant HD-Zip genes, all the *Pphb* genes except *Pphb3* belong to three of the four HD-Zip subfamilies (HD-Zip I, II, and III), indicating that these subfamilies originated before the divergence of the vascular plant and moss lineages. *Pphb3* is sister to HD-Zip II subfamily, and has some distinctive characteristics, including the difference of $\mathbf{a}_1$ and $\mathbf{d}_1$ sites of its leucine zipper motif, which are well conserved in each HD-Zip subfamily. Comparison of the genetic divergence of representative HD-Zip I and II genes showed that the evolutionary rate of HD-Zip I genes was faster than HD-Zip II genes (Sakakibara et al. 2001).

The moss homologs of *SHOOTMERISTEMLESS* and *ZWILLE* genes, which are involved in *Arabidopsis* shoot development, have been cloned and their characterization is in progress.

III-III. Establishment of enhancer and gene trap lines in the moss *Physcomitrella patens*

We also established enhancer and gene trap lines and tagged mutant libraries of *P. patens* to clone genes involved in the leafy shoot development in haploid generation (Hiwatashi et al. 2001). Elements for gene-trap and enhancer-trap systems were constructed using the *uidA* reporter gene with either a splice acceptor or a minimal promoter, respectively. Through a high rate of transformation conferred by a method utilizing homologous recombination, 235 gene-trap and 1073

enhancer-trap lines were obtained from 5637 and 3726 transgenic lines, respectively. Expression patterns of these trap lines in the moss gametophyte varied. The candidate gene trapped in a gene-trap line YH209, which shows rhizoid-specific expression, was obtained by 5' and 3' RACE. This gene was named *PpGLU*, and forms a clade with plant acidic alpha-glucosidase genes. Thus, these gene-trap and enhancer-trap systems should prove useful to identify tissue- and cell-specific genes in *Physcomitrella*.

Publication List:

Fujita, T., Ikeda, M., Kusano, S., Yamazaki, S., Ito, S., Obayashi, M., Yanagi, K. (2001) Amino acid substitution analyses of the DNA contact region, two amphipathic a-helices and a recognition-helix-like helix outside the dimeric b-barrel of Epstein-Barr virus nuclear antigen 1. *Intervirology*. **44**: 271-282.

Himi, S., Sano, R., Nishiyama, T., Tanahashi, T., Kato, M., Ueda, K. and Hasebe, M. (2001) Evolution of MADS-box gene induced by *FLO/LFY* genes. *J. Mol. Evol.* **53**: 387-393.

Hiwatashi, Y., Nishiyama, T., Fujita, T. and Hasebe, M. (2001) Establishment of gene-trap and enhancer-trap systems in the moss *Physcomitrella patens*. *Plant J.* **28**: 1-14.

Kurumatani, M., Yagi, K., Murata, T., Tezuka, M., Mander, L. N., Nishiyama, M. and Yamane, H. (2001) Isolation and identification of antheridiogens in the ferns, Lygodium microphyllum and Lygodium reticulatum. *Biosci. Biotechnol. Biochem.* **65**: 2311-2314.

Sakakibara, K., Nishiyama, T., Kato, M. and Hasebe, M. (2001) Isolation of Homeodomain-Leucine Zipper Genes from the Moss *Physcomitrella patens* and the Evolution of Homeodomain-Leucine Zipper Genes in Land Plants. *Mol. Biol. Evol.* **18**: 491-502.

Shindo, S., Sakakibara, K., Sano, R., Ueda, K. and Hasebe, M. (2001) Characterizatin of a *FLORICAULA/LEAFY* homologue of *Gnetum parvifolium*, and its implications for the evolution of reproductive organs in seed plants. *Int. J. Plant Sci.* **162**: 1199-1209.

Yoshihara, S., Geng, X. X., Okamoto, S., Yura, K., Murata, T., Go, M., Ohmori, M. and Ikeuchi, M. (2001) Mutational analysis of genes involved in pilus structure, motility and transformation competency in the unicellular motile cyanobacterium *Synechocystis sp.* PCC 6803. *Plant Cell Physiol.* **42**: 63-73.

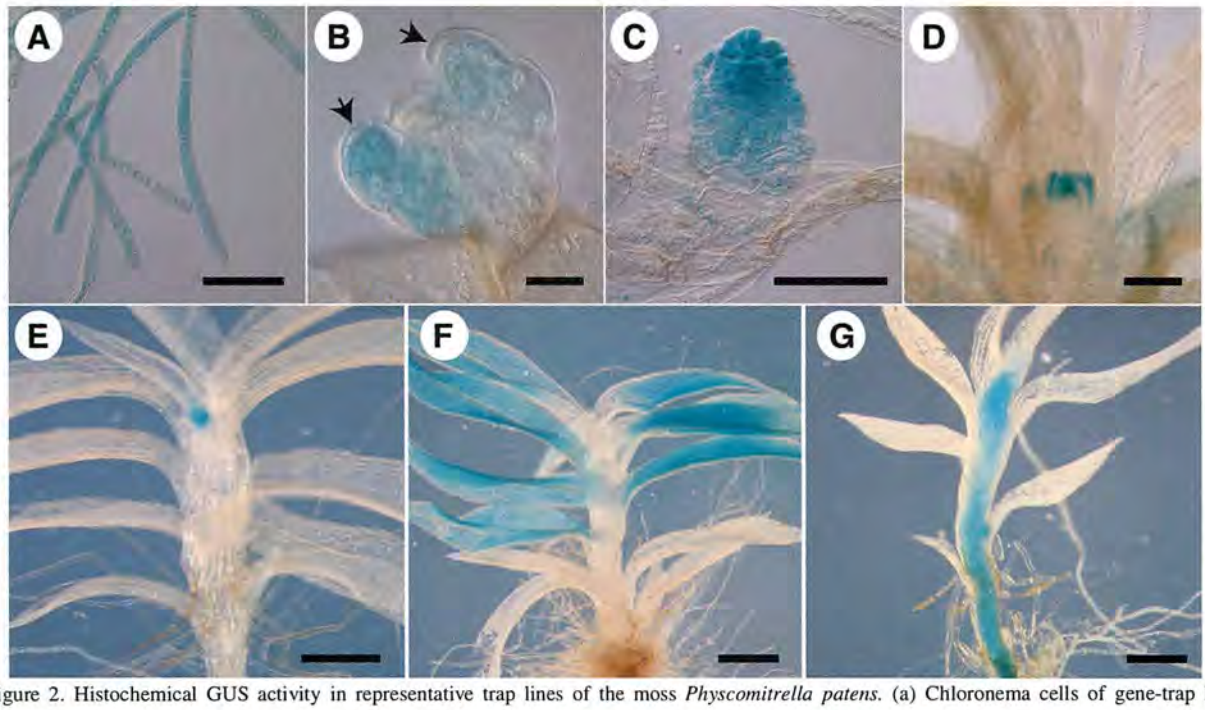

Figure 2. Histochemical GUS activity in representative trap lines of the moss *Physcomitrella patens*. (a) Chloronema cells of gene-trap line YH261. (b) A bud of enhancer-trap line ET77. Leaf-primordial cells (arrows) are predominantly stained. (c) A bud of gene-trap line YH727. An apical cell (arrow) and its surrounding cells are stained. (d) Apical portion of a gametophore in gene-trap line YH8. Axillary hairs that differentiate at the adaxial base of the leaf are stained. (e) A gametophore of enhancer-trap line ET63. The apical portion of the gametophore is predominantly stained. (f) A gametophore of gene-trap line YH560. Leaves are stained. (g) A gametophore of enhancer-trap line ET21. A stem is stained. LinesYH261, YH727, YH8, and YH560 were generated using HI-GT, lines ET63 and ET21 were generated using HI-ET, and line ET77 was generated using NHI-ET. Bars in (a), (c), and (d) = 100 μm, in (b) = 20 μm, and in (e), (f), and (g) = 300 μm.

RESEARCH SUPPORT

CENTER FOR TRANSGENIC ANIMALS AND PLANTS
RESEARCH CENTER FOR INTEGRATIVE
AND COMPUTATIONAL BIOLOGY
RESEARCH SUPPORT FACILITY
THE CENTER FOR ANALYTICAL INSTRUMENTS
TECHNOLOGY DEPARTMENT

CENTER FOR
TRANSGENIC ANIMALS AND PLANTS

Head: *NODA, Masaharu*
Associate Professor: *WATANABE, Eiji*
Supporting Staff: *YASUDA, Mie*
TOZAKI, Ayako
SHIBATA, Mariko (Jun, 2001~)
EGUSA, Chizu (Sep, 2001~)
NARUSE, Aki (Jan, 2002~)
YAGI, Eri (Feb, 2002~)

I. Research supporting activity

NIBB Center for Transgenic Animals and Plants was established in April 1998 to support researches using transgenic and gene targeting techniques in NIBB. We are now planning on the construction of the center building.

The expected activities of the Center are as follows:

1. Provision of information, materials and techniques to researchers.
2. Equipment of various instruments to analyze mutant animals and plants.
3. Development of novel techniques related to transgenic and gene targeting technology.

II. Academic activity

We are studying the functional role of Na_x sodium channel in collaboration with Division of Molecular Neurobiology. Na_x has belonged to 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, Na_x 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 Na_x *in vivo*, the Na_x-deficient mice were generated by gene targeting and the physiological phenotypes have been examined. It was 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. Details of this study are described in the part of Division of Molecular Neurobiology.

Publication List

Zubair, M., Watanabe, E., Fukada, M. and Noda, M. (2002) Genetic labeling of specific axonal pathways in the mouse central nervous system. Eur J Neurosci, in press.

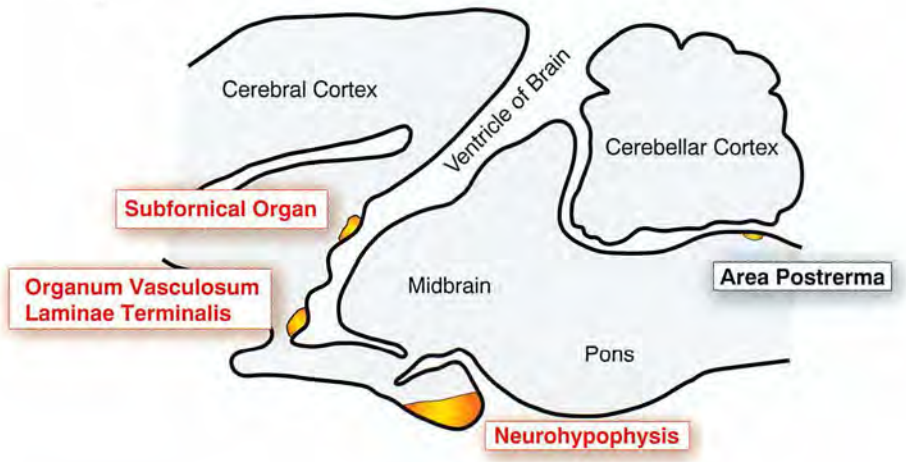

Fig. 1 Na_x sodium channel is a candidate molecule for the sodium-level sensor in the brain.

The schematic representation indicates a sagittal plane of the mouse brain at midline level. The boxed regions are the circumventricular organs, which are tissues situated outside of the blood-brain barrier and surrounded the ventricle of brain, and a site convenient for monitoring the levels of various substances in both plasma and CSF. Na_x sodium channel is expressed in specialized neurons and ependymal cells of the circumventricular organs as shown in red. We also found abnormal salt ingestion in the Na_x-deficient mice.

RESEARCH CENTER FOR INTEGRATIVE AND COMPUTATIONAL BIOLOGY

(Staff of the Center are in the process of selection.)

This center aims to integrate biology and bioinformatics through elucidation of various biological phenomena at the molecular level employing the following: (1) Computational analysis using accumulating data related to biological information; (2) analysis using mathematically model and simulations; (3) comprehensive functional analysis of genes, genomes and proteins by new technology and application of novel concepts.

RESEARCH SUPPORT FACILITY

Head of Facility:	*MURATA, Norio*
Associate Professor:	*WATANABE, Masakatsu*
Research Associates:	*HAMADA, Yoshio (Tissue and Cell Culture)*
	UCHIYAMA, Ikuo (Computer)
Technical Staff:	*HIGASHI, Sho-ichi (Large Spectrograph)*
	MIWA, Tomoki (Computer)
	NANBA, Chieko (Plant Culture, Farm, Plant Cell)
	NISHIDE, Hiroyo (Computer)
	HATTORI, Nobuko (Large Spectrograph. ~ April 3, 2001)
	ITO, Makiko (Large Spectrograph)
	KAMIYA, Yasuko (Tissue and Cell Culture. ~ February 28 2001)
	KATAGIRI, Izumi (Tissue and Cell Culture. March 1, 2001 ~)
	HARADA, Miyuki (Computer. July 16, 2001 ~)
	MAKIHARA, Nobuko (Computer. April 1, 2001 ~)
	SUZUKI, Keiko (Plant Culture, Farm, Plant Cell)

I. Facilities

1. *The Large Spectrograph Laboratory*

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

A tunable two-wavelength CW laser irradiation system is also available as a complementary light source to OLS to be used in irradiation experiments which specifically require ultra-high fluence rates as well as ultra-high spectral-, time-and spatial resolutions. It is composed of a high-power Ar-ion laser (Coherent, Innova 20) (336.6-528.7 nm, 20W output), two CW dye lasers (Coherent, CR599-01) (420-930nm, 250-1000mW output), A/O modulators (up to 40MHz) to chop the laser beam, a beam expander, and a tracking microbeam irradiator (up to 200 $\mu m\ s^{-1}$ in tracking speed, down to 2 μm in beam diameter) with an infrared phase-contrast observation system.

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 3000), data visualization terminal and molecular simulation terminal (both are SGI Octanes). Some personal computers and color/monochrome printers are also equipped. On this system, we provide various biological databases and data retrieval/analysis programs, and support large-scale data analysis and database construction for the Institute members.

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.

7. *Laboratory of Stress-Resistant Plants*

This laboratory was found to study transgenic plants with respect to tolerance toward various environmental stresses. It is located in the Agricultural Experimental Station of Nagoya University (30 km from National In-

stitute for basic Biology). The laboratory provides a variety of growth chambers that precisely control the conditions of plant growth and dacilities for molecular biological and physiological enaluations of transgenic plants.

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

II. Research Activities

1. *Faculty*

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

(1) Photobiology: Photoreceptive and signal transduction mechanisms of phototaxis of unicellular algae are studied action spectroscopically (Watanabe 1995, In CRC Handbook of Organic Photochemistry and Photobiology) by measuring computerized-videomiceographs of the motile behavior of the cells at the cellular and subcellular levels. Photo-receptive and signal transduction mechanisms of algal gene expression were also studied by action spectroscopy.

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

(3) Computational Biology: Comparative genomics is a useful approach to find clues to understanding complex and diverse biological systems from rapidly growing genome database. We have constructed a database system for comparative analysis of many of microbial genomes ever sequenced and are developing new computational techniques for large-scale genome sequence comparison. Especially, we are developing a method for orthologous grouping among multiple genomes, which is a crucial step for comparative genomics. In addition to splitting fusion genes into orthologous domains, we are also enhancing the grouping algorithm to incorporate the information of gene arrangement on the genome.

In parallel, we have developed a tool to incorporate various sequence features such as G+C contents, codon usage bias and locations of repetitive elements into the genome comparison. By this approach, we made detailed comparison of closely related microbial genomes to investigate the genomic polymorphisms or evolutionary changes in collaboration with Dr. I. Kobayashi's group (Univ. Tokyo), including comparative genome analysis of two strains of *Staphylococcus aureus.*

2. *Cooperative Research Program for the Okazaki Large Spectrograph*

The NIBB Cooperative Research Program for the Use of the OLS supports about 30 projects every year conducted by visiting scientists including foreign scientists as well as those in the Institute.

Action spectroscopical studies for various regulatory and damaging actions of light on living organisms, biological molecules, and organic molecules have been conducted (Watanabe, 1995, In "CRC Handbook of Organic Photochemistry and Photobiology". pp, 1276 - 1288).

Publication List:

I. *Faculty*

Kondou, Y., Nakazawa, M., Higashi, S., Watanabe, M. and Manabe, K. (2001) Equal-quantum action spectra indicate fluence-rate-selective action of multiple photoreceptors for photomovement of the thermophilic cyanobacterium *Synechococcus elongatus. Photochem. Photobiol.* **73**, 90-95.

Kumano, K., Chiba, S., Shimizu, K., Yamagata, T., Hosoya, N., Saito, T., Takahashi, T., Hamada, Y., Hirai, H. (2001) Notch1 inhibits differentiation of hematopoietic cells by sustaining GATA-2 expression. *Blood* **98**: 3238-9.

Kuroda, M., Ohta, T., Uchiyama, I., Baba, T., Yuzawa, H., Kobayashi, I., Cui, L, Oguchi, A., Aoki, K., Nagai, Y., Lian, J., Ito, T., Kanamori, M., Matsumaru, H., Maruyama, A., Murakami, H., Hosoyama, A., Mizutani-Ui, Y., Takahashi, N.K., Sawano, T., Inoue, R., Kaito, C, Sekimizu, K., Hirakawa, H., Kuhara, S, Goto, S., Yabuzaki, J., Kanehisa, M., Yamashita, A., Oshima, K., Furuya, K., Yoshino, C., Shiba, T., Hattori, M., Ogasawara, N., Hayashi, H., Hiramatsu, K. (2001) Whole genome sequencing of meticillin-resistant *Staphylococcus aureus*, *The Lancet* **357**, 1225-1240.

Lenci, F. and Watanabe, M. (2001) Photomovements and light sensing systems. In "Photobiology in the 21st Century" (eds. T. Coohil, D.-P. Häder, D. Valenceno) Valdemaer Press

Nakamura, S., Mikamori, M., Hiramatsu, M., Eura, S., and Watanabe, M. (2001) Specutacular fluorescence emission in sea urchin larvae. *Zool. Sci.* **18**, 807-810.

Nakamura, S., Ogihara, H., Jinbo, K., Tateishi, M., Takahashi, T., Yoshimura, K., Kubota, M., Watanabe, M.,

and Nakamura, S. (2001) *Chalamydomonas reinhardtii* Dangeard (Chlamydomonadales, Chlorophyceae) mutant with multiple eyespots. *Phycol. Res.* **49**, 115-122.

Negishi, T., Nagaoka, C., Hayatsu, H., Suzuki, K., Hara, T., Kubota, M., Watanabe, M., and Hieda, K. (2001) Somatic-cell mutation induced by UVA and monochromatic UV radiation in repair-proficient and -deficient *Drosophila melanogaster*. *Photochem. Photobiol.* **73**, 493-498.

Watanabe, M. and Erata, M. (2001) Yellow-light sensing phototaxis in cryptomonad algae. In "Photomovement" (eds. D.-P. Häder and M. Lebert) pp. 343-373. Elsevier

II. *Cooperative Research Program for the Okazaki Large Spectrograph*

Hasegawa, E., Sawada, K., Akamatsu, T., Fujita, K. and Tamai, M. (2001) Fish behaviour control by the optical fiber optomotor reaction device. *Bull. Nat. Res. Inst. Fish. Eng.* **22**, 27-33.

Inada, C., Kudo, T., Yoshizumi, K., Takatori, K., Hedge, A. (2001) Effect of light irradiation wavelength on the inactivation of *Penicillium*. *Bokin Bobai.* **12**, 757-762.

Kakiuchi, Y., Takahashi,T., Murakami, A. and Ueda, T. (2001) Light irradiation induces fragmentation of the plasmodium, a novel photomorphogenesis in the true slime mold *Physarum polycephalum*: action spectra and evidence for involvement of the phytochrome. *Photochem Photobiol.* **73**, 324-329.

Kondou, Y., Nakazawa, M., Higashi, S., Watanabe, M. and Manabe, K. (2001) Equal-quantum action spectra indicate fluence-rate-selective action of multiple photoreceptors for photomovement of the thermophilic cyanobacterium *Synechococcus elongatus*. *Photochem. Photobiol.* **73**, 90-95.

Negishi, T., Nagaoka, C., Hayatsu, H., Suzuki, K.,Hara, T., Kubota, M., Watanabe, M. and Hieda, K. (2001) Somatic-cell mutation induced by UVA and monochromatic UV radiation in repair-proficient and deficient *Drosophila melanogaster*. *Photochem. Photobiol.* **73**, 493-498.

Yoshizumi, K., Kudo, T., Imaizumi, A., Fujinami, N.(2001) Action spectrum of light fastness of carthamin dyed on silk fabric under the irradiation of UV and visible light. *SENI GAKKAISHI* 57 No.9

THE CENTER FOR ANALYTICAL INSTRUMENTS

(managed by NIBB)

Head of Facility: *MOROHASHI, Ken-ichirou*
Technical Staffs: *OHSAWA, Sonoko*
MORI, Tomoko
MAKINO, Yumiko
TAKAMI, Shigemi
NAKAMURA, Takanori
Technical Assistant: *MORIBE, Hatsumi*

The Center serves for amino acid sequence analysis, and chemical syntheses of peptides and nucleotids to support researchers in NIBB and NIPS. Newly installed instrument in 2001 is MALDI/TOF-MS. Instruments of the Center can be used by researchers outside the Institute upon proposal.

Figure 1. MALDI/TOF-MS.

Figure 2. Biomek 2000 Laboratory Automation System.

Representative instruments are listed below.

Protein Sequencers (ABI Procise 494, 473A)
Amino Acid Analyzer (Hitachi L8500A)
Peptide Synthesizers (ABI 433A, 432A)
Plasmid Isolation Systems (Kurabo PI-100Σ, PI-50)
Automatic Nucleic Acid Isolation System (Kurabo NA-2000)
DNA Sequencers (ABI 377, 373S, 310)
DNA/RNA Synthesizers (ABI 394, 392)
Thermal Cyclers (Perkin Elmer PJ-9600, Takara TP-300)
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)
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, Simadzu RF-5000)
Spectrophotometers (Hitachi 330, 557, Varian Cary 5G, Perkin Elmer Lambda-Bio)
Microplate Luminometer (Berthold MicroLumat LB 96P)
Time-resolved Fluorescence Microplate Reader (Pharmacia DELFIA Research)
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 BAS2000)
Fluorescence Bio Imaging Analyzer (Takara FMBIO)
Electrophoresis Imaging Systems (BIOIMAGE)
Microscopes (Carl Zeiss Axiophot, Axiovert)

TECHNOLOGY DEPARTMENT

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Common Facility Group

Chief: FURUKAWA, Kazuhiko

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NANBA, Chieko (Subunit Chief
NISHIDE, Hiroyo
ITOH, Makiko (Technical Assistant)
SUZUKI, Keiko (Technical Assistant)
KATAGIRI, Izumi (Technical Assistant)
MAKIHARA, Nobuko (Technical Assistant)
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TOSAKI, Ayako (Technical Assistant)
SHIBATA, Mariko (Technical Assistant)

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MAKINO, Yumiko
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NAKAMURA, Takanori
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(MORI, Tomoko)
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OKA, Sanae

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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 enligtement and education increase their capability in technical area. Each technical staff is proceeded to the fixed division usually and they support the various research with their special biological and biophysical techniques.

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

CENTER FOR INTEGRATIVE BIOSCIENCE
(jointly managed by NIBB)

Interim Head: NAGAYAMA, Kuniaki

DEPARTMENT OF DEVELOPMENT, DIFFERENTIATION AND REGENERATION I
DEPARTMENT OF DEVELOPMENT, DIFFERENTIATION AND REGENERATION II
DEPARTMENT OF BIO-ENVIRONMENTAL RESEARCH I
DEPARTMENT OF BIO-ENVIRONMENTAL RESEARCH II
DEPARTMENT OF BIO-ENVIRONMENTAL RESEARCH III

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

DEPARTMENT OF DEVELOPMENT, DIFFERENTIATION AND REGENERATION I

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Post doctoral fellows:	*AMIKURA, Reiko*
JSPS Postdoctral Fellow:	*SHIGENOBU, Shuji*
Graduate Students:	*HAYASHI, Yoshiki*
	UNNAI, Kohei [1]
	KITADATE, Yu [1]
Secretary:	*SUZUKI, Masako*
Technical Assistant:	*UEDA, Yoshiko*
	TSUCHIYA, Naomi

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

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

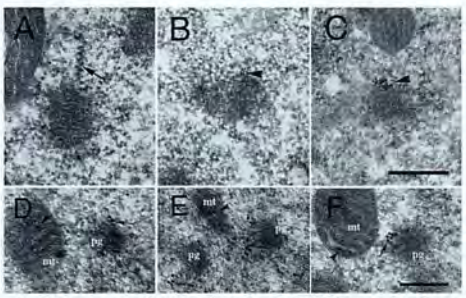


Fig. 1. Presence of mitochondrial rRNAs and ribosomal proteins in the polar granule polysomes at stage 2. (A) An electron micrograph showing well developed polysomes (arrow) on the surface of polar granules. (B and C) Electron micrographs of sections hybridized with probes for mtlrRNA (B) and mtsrRNA (C) (arrowheads). (Scale bar=0.25 μm.) (D–G) Distribution of S12-EGFP (D), L7/L12-EGFP (E), L7/L12-HA (F). Signals were detected at the periphery of polar granules (arrows). mt, mitochondria; pg, polar granules. Scale bar= 0.2 μm.

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.

Since both mtlrRNA and mtsrRNA are major components of ribosomes within mitochondria, we speculated that these rRNAs function to form ribosomes on the polar granules. We reported that mtlrRNA and

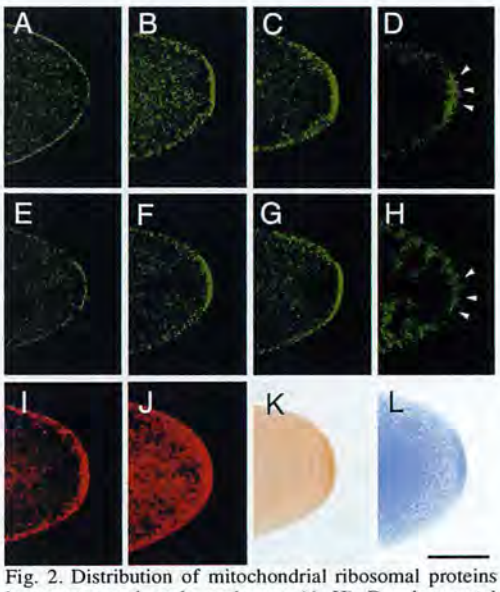


Fig. 2. Distribution of mitochondrial ribosomal proteins in oocytes and early embryos. (A–H) Developmental changes in the distribution of S12-EGFP (A–D) and L7/L12-EGFP (E–H) detected by EGFP fluorescence. (Aand E) Mature oocytes, (B and F) stage-1 embryos, (C and G) stage-2 embryos, and (Dand H) stage-3 embryos. (I–L) Immunohistochemical detection of the Gal4-induced S12-EGFP (I) and L7/L12-EGFP (J) by an anti-GFP antibody and L7/L12-HA by an anti-HA antibody (K). (L) A wild-type embryo stained with anti-S12 antibody. Scale bar= 50 μm.

mtsrRNA are both localized in the polysomes formed on the surface of polar granules during a short period (embryonic stage 2) prior to pole cell formation (Fig. 1). Furthermore, mitochondrial ribosomal proteins (S12 and L7/L12) are enriched in germ plasm (Fig. 2). They are present in the polysomes on the polar granules as well as in mitochondria. In the polysomes around polar granules are there smaller ribosomes, of which size is almost identical to that of mitochondrial ribosomes but is smaller than that of cytosolic ones. We conclude that mitochondrial rRNAs form mitochondrial-type of ribosomes on polar granules, cooperating with mitochondrial ribosomal proteins.

II. Role of Nanos protein in pole cell differentiation

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

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

Nos represses translation of mRNAs with discrete RNA sequences called Nos response elements (NREs). In the pathway leading to posterior somatic patterning, Nos acts together with unlocalized Pumilio (Pum) protein to repress translation of maternal *hunchback* (*hb*) mRNA. This translational repression is mediated by binding of Pum to NREs in the 3'-untranslated region (UTR) of *hb* mRNA. In pole cells, Nos also acts with Pum to regulate germline-specific events. Pum, like Nos, is required in pole cells for their migration to the gonads and their mitotic quiescence. We have reported that a regulatory target for Nos-dependent translational repression in pole cells is maternal *cyclin B* mRNA which contains an NRE-like sequence within its 3'-UTR. Nos, cooperating with Pum, inhibits mitosis of pole cells by repressing translation of maternal *cyclin B* mRNA. In contrast, pole cell migration and gene expression in pole cells are independent of the translational repression of *cyclin B*, suggesting the existence of another target mRNA for Nos-dependent translational repression in pole cells.

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

Publication List:

Amikura, R., K. Hanyu, M. Kashikawa and S. Kobayashi (2001) Tudor protein is essential for the localization of mitochondrial ribosomal RNAs in polar granules in germ plasm of *Drosophila* embryos. *Mech. Dev.* **107**, 97-104.

Amikura, R., M. Kashikawa, A. Nakamura and S. Kobayashi (2001) Presence of mitochondrial-type ribosomes outside mitochondria in germ plasm of *Drosophila* embryos. *Proc. Natl. Acad. Sci. USA.* **98**, 9133-9138.

Kashikawa, M., R. Amikura and S. Kobayashi (2001) Mitochondrial small ribosomal RNA is a component of germinal granules in *Xenopus* embryos. *Mech. Dev.*, **101**: 71-77.

Nakamura, A., R. Amikura, K. Hanyu and S. Kobayashi (2001) Me31B silences translation of oocyte-localizing RNAs through the formation of cytoplasmic RNP complex during *Drosophila* oogenesis. *Development.***128**, 3233-3242.

Sano, H., A. Nakamura and S. Kobayashi (2001) Identification of a transcriptional regulatory region for germline-specific expression of *vasa* gene in *Drosophila* melanogaster. *Mech. Dev.* (in press)

Sano, H., M. Mukai and S. Kobayashi (2001) Maternal Nanos and Pumilio regulate zygotic *vasa* expression autonomously in the germline progenitors of *Drosophila* embryos. *Develop. Growth & Differ.* **43**, 545-552.

DEPARTMENT OF DEVELOPMENT, DIFFERENTIATION AND REGENERATION II

Professor: *TAKADA, Shinji*
Research Associate: *KOSHIDA, Sumito*

The research interest of this laboratory is to understand molecular mechanism of the vertebrate development. Particularly, roles of cell-to-cell signals, including members of Wnt and fibroblast growth factor (FGF) families, are characterized. 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. In addition, to reveal molecular networks in which these signals are involved, we are also trying genetical approach with the zebrafish.

I Roles of Wnt signals in somite development

The *Wnt* family of genes that encode cysteine rich secreted proteins consists of at least 15 members in the vertabrate. It has already been shown that some of them are expressed and play important roles during gasturulation. For instance, *Wnt-3a*, a member of the *Wnt* family genes, is expressed in the primitive streak ectoderm during gastrulation and in the tailbud in later development of the mouse (Fig. 1). For dissecion of the complex developmental events regulated by Wnt-3a signaling in these regions, it is important to identify genes regulated by this signal. It has already been demonstrated that *T* (*Brachyury*) is a direct target of Wnt-3a in the anterior primitive ectoderm, which is fated to give rise to the paraxial mesoderm, suggesting that Wnt-3a modulates a balance between mesodermal and neural cell fates via *T*.

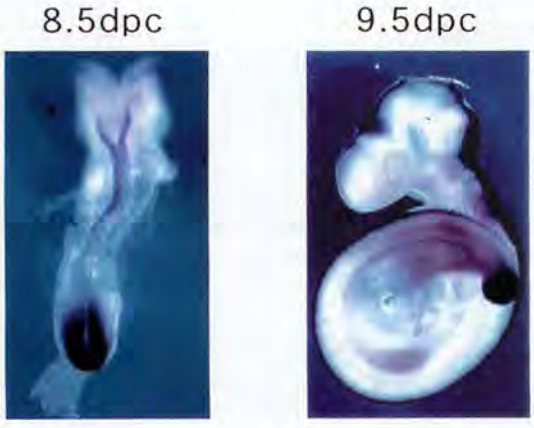


FIG.1 Expression of Wnt-3a at 8.5 and 9.5 days post coitus (dpc) in the mouse embryo. Wnt-3a is expressed in the primitive streak ectoderm (8.5 dpc) and the dorsal neural tub and the tail bud (9.5 dpc).

To gain more insight into the role of *Wnt-3a* during gastrulation, we searched for potential target genes of Wnt-3a. We found that Wnt signaling stimulated the *in vitro* expression of *cdx-1*, and that Wnt-3a was required for full activation of *cdx-1* expression in the primitive ectoderm and tailbud *in vivo*. Moreover, *Wnt-3a* mutants displayed a defect in their anteroposterior patterning similar to that in the *cdx-1* mutant mouse, in addition to another homeotic transformation that was not observed in *cdx-1* mutants (FIG. 2). These results suggest that *cdx-1* is one of the mediator genes of Wnt-3a signaling in the anteroposterior patterning of the vertebrae and that Wnt-3a is also involved in a Cdx-1 independent process in anteroposterior patterning

II A roles of Wnt signaling in neural development

In the dorsal half of the spinal cord, three subclasses of interneurons called D1, D2 and D3 from the dorsal side. These interneuron subclasses are thought to be derived from distinct neural progenitor domains. Recent studies indicate that the roof plate is a source of inductive signals that control the generation of D1 and D2 classes of dorsal interneurons.

Interestingly, Wnt-1 and Wnt-3a are expressed in largely overlapping regions within the central nervous system, predominantly in the roof plate from diencephalon to spinal cord. We show that mouse embryos lacking both Wnt1 and Wnt3a are indeed defective in determination of the dorsal interneurons. Generation of D1 and D2 classes of dorsal interneurons and their progenitors was markedly disrupted; this loss of the dorsal interneurons was compensated by a dorsal expansion D3 interneuron populations. Most importantly, expression of TGF β family members was not significantly affected in these mutant embryos. Moreover, we demonstrate that WNT3A can induce D1 and D2 class interneurons in the isolated intermediate region of the neural tube. Together, these observations clearly indicate that Wnt signaling has a critical role in the generation of dorsal interneurons specific D1 and D2 cell types.

III Screening of target genes of Wnt signaling by an gene trap approach

To gain more insight into roles of Wnt signaling during embryogenesis, we searched for potential target genes of this signaling by an induction gene trap screening in mouse ES cells. In at least three ES cell clones among 794 clones screened, expression of beta-geo reporter genes was dramatically changed in response to the conditioned medium of Wnt-3a expressing

cells. The expression analysis of the reporter genes in embryos generated from these ES cell clones revealed that the spatiotemporal expression patterns of these reporter genes were well correlated to those of several Wnt genes. These results suggested that an induction gene trap approach is effective for screening of target genes of Wnt signaling during embryogenesis.

IV Roles of Fgf signals in bone development

The Fgf family of cell signaling molecules is composed of at least 22 members in the mouse. Although Fgf signaling has been implicated in the bone development, study on null mutant mice have not yet fully shown the role of this family in the skeletal development.

As an attempt to identify a candidate *Fgf* gene essential for bone formation, we have examined the expression of mouse *Fgf18* gene using *in situ* hybridisation and demonstrated expression during calvarial and long bone development. Furthermore, to investigate the role of *Fgf18 in vivo*, we generated a null allele of the *Fgf18* locus by homologous recombination in ES cells. In *Fgf18*-deficient mouse embryos generated by gene targeting, progress of the suture closure is delayed. Furthermore, terminal differentiation of the osteoblast is specifically delayed in developing calvaria and long bones. Proliferation of calvarial osteogenic mesenchymal cells is transiently decreased. On the other hand, the number of proliferating and differentiated chondrocytes is increased in the limbs. *Fgf18* is thus essential for distinct aspects of cell proliferation and differentiation in bone formation. Taken together, Fgf18 plays essential roles in the osteogenesis and the chondrogenesis of the mammal and regulates differently cell proliferation and differentiation in these two processes..

V Screening of mutations affecting mesoderm development in zebrafish

To understand the molecular mechanism of mesoderm development in the tail bud, we have been screening mutations affecting mesoderm development in zebrafish.

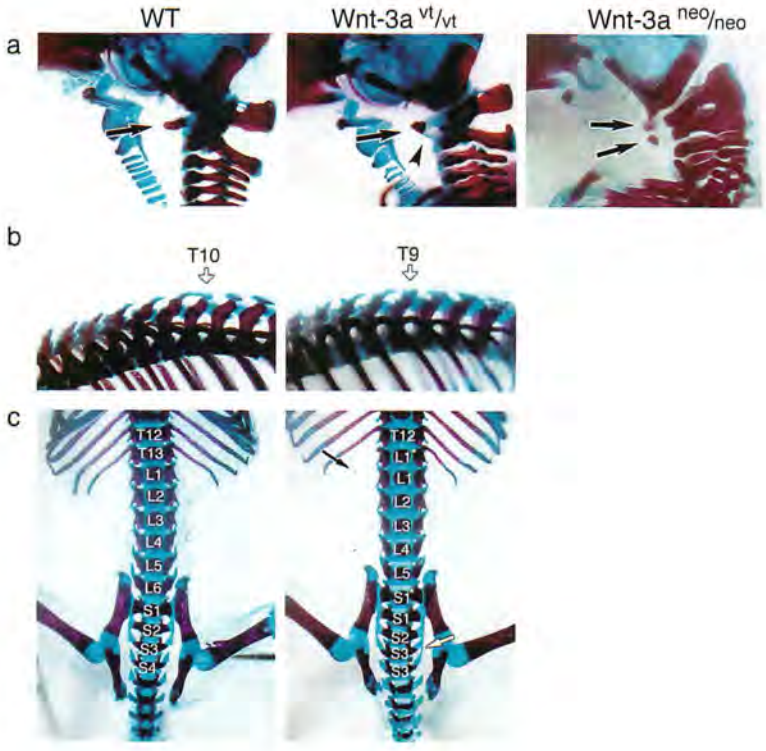


FIG. 2 Skeletal analysis of *Wnt-3a* mutants. a, In the cervical region, C2 to C1 transformation was observed in *Wnt-3a*$^{neo/neo}$ and *Wnt-3a*$^{vt/vt}$ mutants. b, Side view of the midthoracic region. Anterior is to the left. The transitional vertebra, which was normally observed in T10, was observed in T9 in the *Wnt-3a*$^{vt/vt}$ mutant. c, From the posterior thoracic to the anterior caudal region. In *Wnt-3a*$^{vt/vt}$, the T13 vertebra, which normally has ribs on both sides, did not have rib on the right side (partially transformed to L1) and the 6th lumbar vertebra fused together at the transverse processes to form sacral bone (L6 to S1 transformation). Black arrow indicates a rudimentary rib. White arrow indicates that the morphology of S4 was altered, its transverse processes being fused to those of S3 (partially transformed to S3).

Publication List:

Hino, S.-I., Kishida, S., Michiue, T., Fukui, A., Sakamoto, I., Takada, S., Asashima, M. & Kikuchi, A. (2001) Inhibition of the Wnt signaling pathway by Idax, a novel Dvl-binding protein. *Mol. Cell. Biol.*, **21**, 330-342.

Ikeya, M. & Takada, S. (2001) Wnt-3a is required for somite specification along anteroposterior axis of the mouse embryo and regulates cdx-1 expression. *Mech. Dev.* **103**, 27-33.

Jin, E.J., Erickson, C.A., Takada, S,. & Burrus, L.W. (2001) Wnt and bmp signaling govern lineage segregation of melanocytes in the avian embryo. *Dev. Biol.* **233**, 22-37.

Kawamura, Y., Kikuchi, A., Takada, R., Takada, S., Sudoh, S., Shibamoto, S., Yanagisawa K., & Komano, H. (2001) Inhibitory effect of *presenilin 1* mutation in the Wnt signaling pathway---*presenilin 1* mutations enhance the phosphorylation of β-catenin--- Eur. *J. Biochem.* **268** 3036-3041.

Koshida, S., Shinya, M., Nikaido, M., Ueno, N., Schulte-Merker, S., Kuroiwa, A., & Takeda, H. (2002) Inhibition of BMP activity by the FGF signal promotes posterior neural development in zebrafish *Dev. Biol.* (in press).

Mao, J., Wang, J., Liu, B., Pan, W., Farr, G.H., Flynn, C., Yuan, H., Takada, S., Kimelman, D., Li, L., & Wu, D. (2001) Low-density lipoprotein receptor-related protein-5 binds to axin and regulates the canonical Wnt signaling pathway. *Mol. Cell* **7**, 801-809.

Muroyama, Y., Fujihara, M., Ikeya, M,. Kondoh,. H., & Takada, S.(2002) Wnt signaling plays an essential role in neuronal specification of the dorsal spinal cord. *Genes Dev.* **16**, 548-55.

Nomi M., Oishi I., Kani S., Suzuki H., Natsuda T.,Yoda A., Kitamura M., Itoh K., Takeuchi S Tkeda K., Akira S., Ikeya M., Takada S., & Mnami Y. (2001) Loss of mRor1 enhances the heart and skeletal abnormalities in mRor2-deficient mice: kinase. *Mol Cell. Biol.* **21**, 8329-8335.

Ohbayashi, N., Shibayama, M., Kurotaki, Y., Imanishi, M., Fujimori, T., Itoh, N., & Takada, S. (2002) Fgf18 is required for normal cell proliferation and differentiation during osteogenesis and chondrogeneses. *Genes Dev.* (in press)

Shinya, M., Koshida, S., Sawada, A., Kuroiwa, A., & Takeda, H. (2001) Fgf signalling through MAPK cascade is required for development of the subpallial telencephalon in zebrafish embryos. *Development* **128**, 4153-4164.

Yamane, T., Kunisada, T., Tsukamoto, H., Yamazaki, H., Niwa, H., Takada, S. & Hayashi, S.-I. (2001) Wnt signaling regulates hematopoiesis through stromal cells. *J. Immunol.* **167**, 765-772.

DEPARTMENT OF BIOENVIRONMENTAL RESEARCH I

Professor: *IGUCHI, Taisen*
Associate Professor: *WATANABE, Hajime*
Research Associate: *KATSU, Yoshinao*
Technical Staff: *MIZUTANI, Takeshi*
Institute Research Fellow: *SONE, Kiyoaki*
Graduate Students: *KHONO, Satomi* [1)]
MIYAGAWA, Shinichi [1)]
Technical Assistants: *KAJIYAMA, Chieko*
SUZUKI, Atsuko
KOBAYASHI, Kaoru
TAKASU, Eri
Secretary: *IMAIZUMI, Taeko*
[1)] *Graduate School of Yokohama City University*

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 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 estrogen receptors 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.

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 the development 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 the estrogen receptor (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 EGF receptor levels, in addition to other hormone receptor levels.

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, PF 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.

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

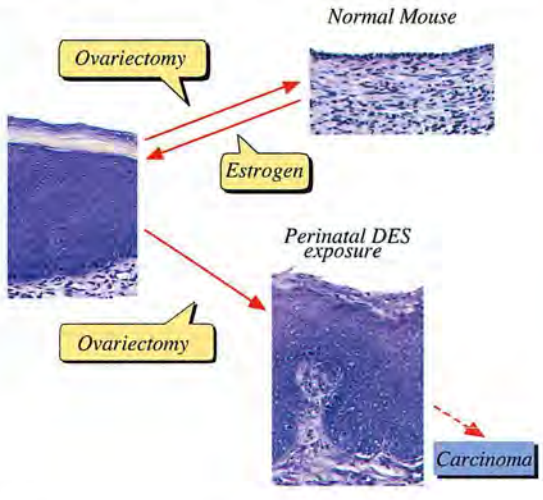


FIG. 1

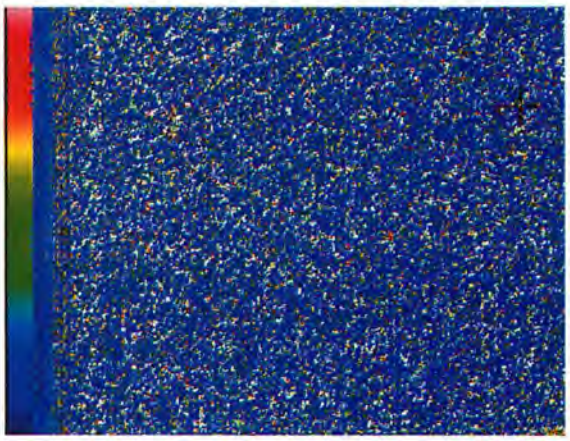


FIG. 2 Fluorescence image of an array

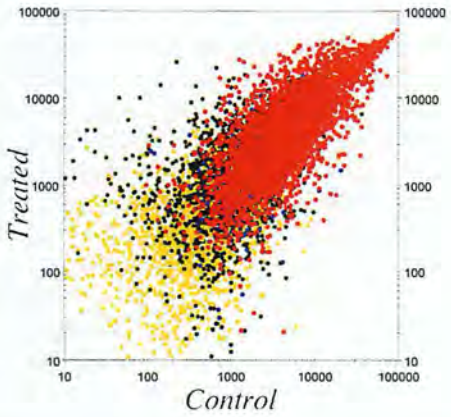


FIG.3 Scatter plot of average expression levels in control and chemical-treated uterus

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.

II. Effect of estrogen on amphibian and fishes.

During embryogenesis, exogenous estrogen exposure induces abnormal sex differentiation and the abnormal bone formation in African clawed frog, *Xenopus laevis* and the cyprinodont fish, mummichog (*Fundulus heteroclitus*). To analyze the function of estrogen, we have isolated cDNA clones of estrogen receptor α and β from *F. heteroclitus* and American alligators. The estrogen-responsive genes must play important roles. We tried to isolate the estrogen-responsive genes to understand the molecular physiology of estrogen action. Vitellogenin has been well characterized in avian, amphibian and fish as a precursor of egg yolk. As the vitellogenin gene is responsive to estrogen, we can examine the effect of endocrine disruptors in the environment using a vitellogenin-specific and sensitive enzyme-linked immunosorbent assay (ELISA). 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.

III. 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 using Surface Plasmon Resonance technology.

Publication List:

Buchanan, D.L., S. Ohsako, C. Tohyama, P.S. Cooke and T. Iguchi: 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) inhibition of estrogen-induced mouse uterine epithelial mitogenesis involves changes in cylin and transforming growth factor-beta expression. *Toxicol. Sci.* (in press)

Honma, S., A. Suzuki, D.L. Buchanan,Y. Katsu, H. Watanabe and T. Iguchi: Low dose effect of in utero exposure to bisphenol A and diethylstilbestrol onfemale mouse reproduction. *Reprod. Toxicol.* (in press)

Iguchi, T., H. Watanabe and Y. Katsu (2001) Developmental effects of estrogenic agents on mice, fish and frogs: a mini review. *Horm. Behav.* **40**: 248-251.

Miyagawa, S., D.L. Buchanan, T. Sato, Y. Ohta, Y. Nishina and T. Iguchi: Characterization of diethylstilbestrol-induced hypospadias in female mice. *Anat. Rec.* (in press)

Okada, A., T. Sato, Y. Ohta, D. L. Buchanan and T. Iguchi (2001) Effect of diethylstilbestrol on cell proliferation and expression of epidermal growth factor in the developing female rat reproductive tract. *J. Endocrinol.*, **170**:539-554.

Okada, A., Y. Ohta, D.L. Buchanan, T. Sato, S. Inoue, H. Hori, M. Muramatsu and T. Iguchi: Changes in ontogenetic expression of estrogen receptor α and not of estrogen receptor β in the female rat reproductive tract. *J. Mol. Endocrinol.* (in press)

Suzuki, A., A. Sugihara, K. Uchida, T. Sato, Y. Ohta, Y. Katsu, H. Watanabe and T. Iguchi: Developmental effects of perinatal exposure to bisphenol-A and diethylstilbestrol on reproductive organs in female mice. *Reprod. Toxicol.* (in press)

Yamamura, Y., M. Tamano, T. Iguchi and Y. Ohta (2001) Methallothionein expression and tumor growth in the transplantable pregnancy-independent mouse mammary tumor. *J. Vet. Med. Sci.*, **63**: 687-689.

Yamamura, Y., Y. Ohta, T. Iguchi and A. Matsuzawa (2001) Metallothionein expression and apoptosis in pregnancy-dependent and –independent mouse mammary tumors. *Anticancer Res.*, **21**: 1145-1150.

Watanabe, H., A. Suzuki, T. Mizutani, H. Handa and T. Iguchi: Large-scale gene expression analysis for evaluation of endocrine disruptors. *Proceedings of 1st International Forum for Toxicogenomics.* (in press)

Watanabe, H., D.L.Buchanan, H. Handa and T. Iguchi (2001) Global analysis of gene expression induced by environmental endocrine disruptors. Perspective in Comparative Endocrinology, Goos, H.J.Th., et al. (eds.), *Monduzzi Editore, pp.*147-151.

DEPARTMENT OF BIOENVIRONMENTAL RESEARCH II

Associate Professor: *TSUKAYA, Hirokazu, Ph.D.*
Assistant Professor: *KIM, Gyung-Tae, Ph.D.*
Technical Staff: *KONDO, Makiko*
Post Doctoral Fellow: *CHO, Kiuhyon, Ph.D.*
Graduate Student: *KOZUKA, Toshiaki*
Secretary: *KABEYA, Kazuko*

Diversity of plant form is mostly attributable to variation of leaf and floral organs, which are modified, leaves. The leaf is the fundamental unit of the shoot system, which is composed with leaf and stem. So the leaf is the key organ for a full understanding of plant morphogenesis. However, the genetic control of development of these shapes had remained unclear. Recently, studies of leaf morphogenesis has been in a turning point, after our successful application of the techniques of developmental and molecular genetics to it, using model plants, *Arabidopsis thaliana* (L.) Heynh (Dengler and Tsukaya, 2001). Based on it, a new perspective on understanding of mechanisms for leaf morphogenesis is proposed (Tsukaya, in press).

Focusing on mechanisms that govern polarized growth of leaves in a model plant, *Arabidopsis thaliana*, we found that the two genes act independently to each other on the processes of polar growth of leaves: the *AN* gene regulates width of leaves and the *ROT3* gene regulates length of leaves. The *AN* gene controls the width of leaf blades and the *ROT3* gene controls length. Cloning of the *AN* gene revealed that the gene is a member of gene family found from animal kingdom (Kim et al., submitted). The *ROT3* gene was cloned by us in 1998. Transgenic experiments proved that the *ROT3* gene regulates leaf-length without affect on leaf-width via biosynthesis of steroids (Kim et al., 1999). In relation to it, we recently revealed that a steroid hormone, brassinosteroid, controls both proliferation and expansion of leaf cells (Nalaya et al., 2002).

Apart from polar elongation, we identified the following genes involved in leaf expansion process. The *AS1* and *AS2* genes are needed for proportional growth of the leaf. Molecular and anatomical analysis of the *as2* mutant is now underway, in collaboration with a research team of Prof. Machida, Nagoya University (Endang et al., 2001). We also started analysis of *blp* mutation which strongly enhances the *as2* phenotype, in collaboration with a reaseach team of Prof. Nam, POSTECH, Korea.

On the other hand, we are trying to identify molecular mechanisms which distinguish developmental pathway of leaves from that of shoots. For such purposes, we introduced tropical plants having queer developmental program for leaf morphogenesis, namely, *Chisocheton*, *Guarea* and *Monophyllaea*, as materials for molecular studies.

In addition, we are interested in environmental adaptation of leaves, from view point of biodiversity (*e.g.*, Tsukaya and Tsuge, 2001; Tsukaya, in press). Leaf index, relative length of leaf to width, is also the most diverse factor of leaf shape, and is affected by environmental factors in some plants (Kuwabara et al., 2001). Are *AN* and *ROT3* genes are involved in regulation of adaptive change of leaf index in natural condition? Are these genes the responsible for evolution of rheophytes? So called "Evo/Devo" study of leaf morphogenesis is also one of our research project in NIBB.

Publication List:

Dengler, N. and Tsukaya, H. (2001). Leaf morphogenesis in dicotyledons: current issues. *Int. J. Plant Sci.* **162:** 459-464.

Kuwahara, A., Tsukaya, H., and Nagata, T. (2001) Identifca tion of factors that cause heterophylly in *Ludwigia arcuata* Walt. (Onagraceae) *Plant Biology* **3:** 98-105.

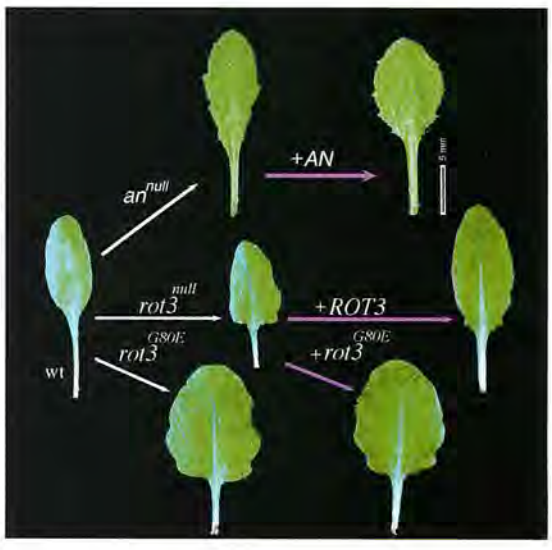
Nakaya, M., Tsukaya, H., Murakami, N., and Kato, M. (2002) Brassinosteroids control the proliferation of the leaf cells in *Arabidopsis thaliana. Plant Cell Physiol.* **43**: 239-244

Semiarti, E., Ueno, Y., Tsukaya, H., Iwakawa H., Machida C. and Machida, Y. (2001). The *ASYMMETRIC LEAVES2* gene of *Arabidopsis thaliana* regulates formation of symmetric lamina, establishment of venation and repression of meristem-related homeobox genes in leaves. *Development* **128:** 1771-1783.

Tsukaya, H. and Tsuge, T. (2001) Morphological adaptation of inflorescences in plants that bloom at low temperatures in early spring: the convergent evolution of "downy plants". *Plant Biology* **3:** 536-543.

Tsukaya, H. (2002). Interpretation of mutants in leaf morphology: genetic evidence for a compensatory system in leaf morphogenesis that provides a new link between Cell and Organismal theory. *Int. Rev. Cytol.* **217** (in press)

Tsukaya, H. (2002) Optical and anatomical characteristics of bracts from the Chinese "Glasshouse" plant, *Rheum alexandrae* Batalin (Polygonaceae), in Yunnan, China. *J. Plant Res.* **115**: 59-64



DEPARTMENT OF BIOENVIRONMENTAL RESEARCH III

Professor (Adjunct): *KANEHISA, Minoru*
(Kyoto University)

Though intermediary metabolism common to most organisms has been deeply investigated so far, variety of species specific pathways in secondary metabolism, which may work only in specific environmental conditions, are still unclear. The aim of this laboratory is to develop a database system for environmental biology, which integrates knowledge about organic compounds, chemical reactions between these compounds *in vivo*, enzymes (genes) involved in these reactions, and species whose genomes contain these genes. Through this database combining with data from transcriptome or proteome analyses in various environmental conditions, we intend to elucidate the principle of interactions between organisms and environmental chemical compounds to predict or design novel interactions.

Publication list:

Kanehisa, M. (2001) Prediction of higher order functional networks from genomic data. *Pharmacogenomics* **2**, 373-385.

Nakaya, A., Goto, S., and Kanehisa, M. (2001) Extraction of correlated gene clusters by multiple graph comparison. *Genome Informatics* **12**, 44-53.

CENTER FOR RADIOISOTOPE FACILITIES (CRF)

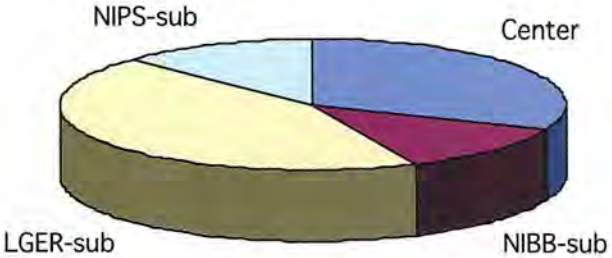
Head:YAMAMORI, Tetsuo
(Professor, concurrent post)

Associate Professor: *OGAWA, Kazuo*
Technical Staffs: *MATSUDA, Yoshimi*
(Radiation Protection Supervisor)
KATO, Yosuke
(Radiation Protection Supervisor)
MOROOKA, Naoki
(Radiation Protection Supervisor)
Supporting Staff: *ITO, Takayo*
IIDA, Yumi

I. Research supporting activity

Technical and supporting staffs of the CRF are serving the purchase of radioisotopes from JRA (Japan Radioisotope Association) and the transfer of radioisotope wastes to JRA. The physical maintenance of the controlled areas where radioisotopes are used is also one of our business.

The CRF consists of four controlled areas: Center, NIBB-sub, LGER (Laboratory of Gene Expression and Regulation)-sub, and NIPS (National Institute for Physiological Science)-sub. Users going in and out the controlled areas counted by the monitoring system are 7,559 in 2001. The items in each controlled area is presented in Figure 1.



	Actual counts
Center	2,359
NIBB-sub	956
LGER-sub	3,320
NIPS-sub	924
total	7,559

Figure 1. Percentage of users going in and out the controlled areas in 2001.

II. Academic activity

Academic activity by teaching staff is focused on the analysis of the structure and function of a dynein motor protein. Dyneins are a group of microtubule-activated ATPases that serve to convert chemical energy into mechanical energy and divided into axonemal and cytoplasmic dyneins. Figure 2 shows the localization of two isoforms of dynein in the outer arms of sperm axonemes (Ogawa et al., 1977) and the mitotic apparatus of cleaving egg (Mohri et al., 1976) visualized by anti-axonemal dynein (Fragement A) antibodies.

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

Outer arm dynein consists of two heavy chains with ATPase activity. The motor activity is closely related to this polypeptide. The first successful molecular cloning of this huge polypeptide (520 kDa) was performed in our laboratory in 1991. Since then cDNA clones for axonemal and cytoplasmic dyneins have been isolated in a variety of organisms. The sequences of heavy chains, without exception, contain four P-loop motives referred to as ATP-binding sites in the midregion of the molecules. Figure 3A and B draw the structure of heavy chain deduced from the amino acid sequence (Ogawa, 1992). Taking the recent works by Koonce et al. (1998) and Vallee et al. (1998) into consideration, this model might be seen as depicted in Figure 3C. In particular, Vallee et al. (1998) have described the importance of a hairpin structure formed between M and C domains which binds to microtubules and presented a novel mechanism for dynein force production different from that of myosin and kinesin.

Outer arm dynein contains three intermediate chains (IC1, IC2, and IC3) that range in molecular mass from 70 to 120 kDa. IC2 and IC3 were cloned by Ogawa et al. (1995) and contain the WD repeats in the carboxy-terminal halves of the molecules. By contrast, IC1 is not a member of the WD family. IC1 has a unique sequence such that the N-terminal part is homologous to the sequence of thioredoxin, the middle part consists of three repetitive sequences homologous to the sequence of NDP kinase, and the C-terminal part contains a high proportion of negatively charged glutamic acid residues (Ogawa et al., 1996). Thus, IC1 is a novel dynein intermediate chain distinct from IC2 and IC3 and may be a multifunctional protein.

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

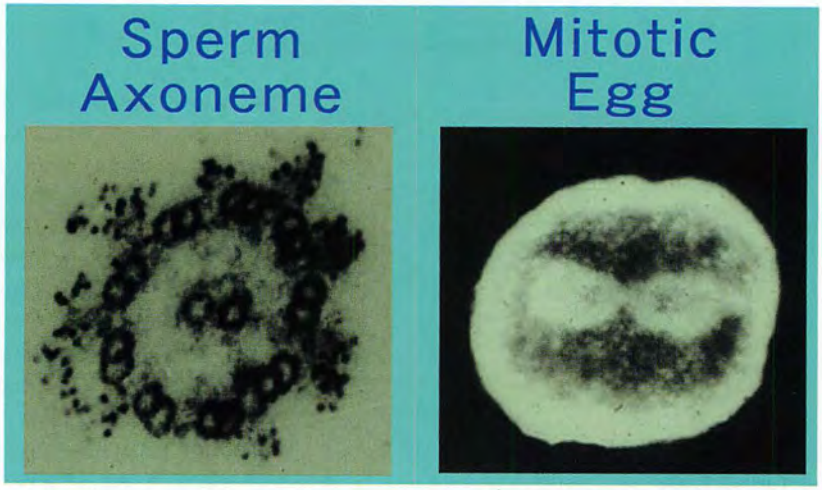


Figure 2. Localization of two dynein isotypes on outer arm of sperm axonemes and mitotic apparatus of cleaving egg.

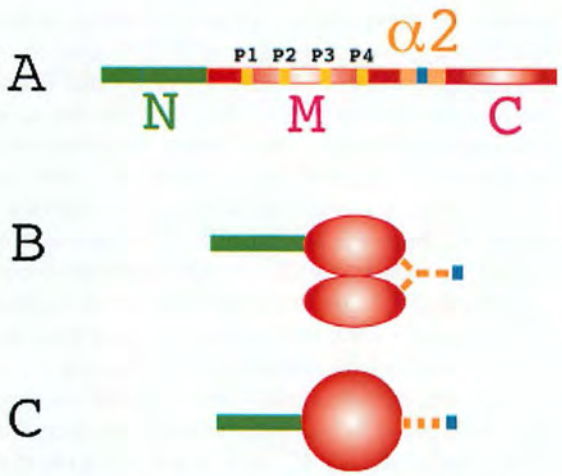
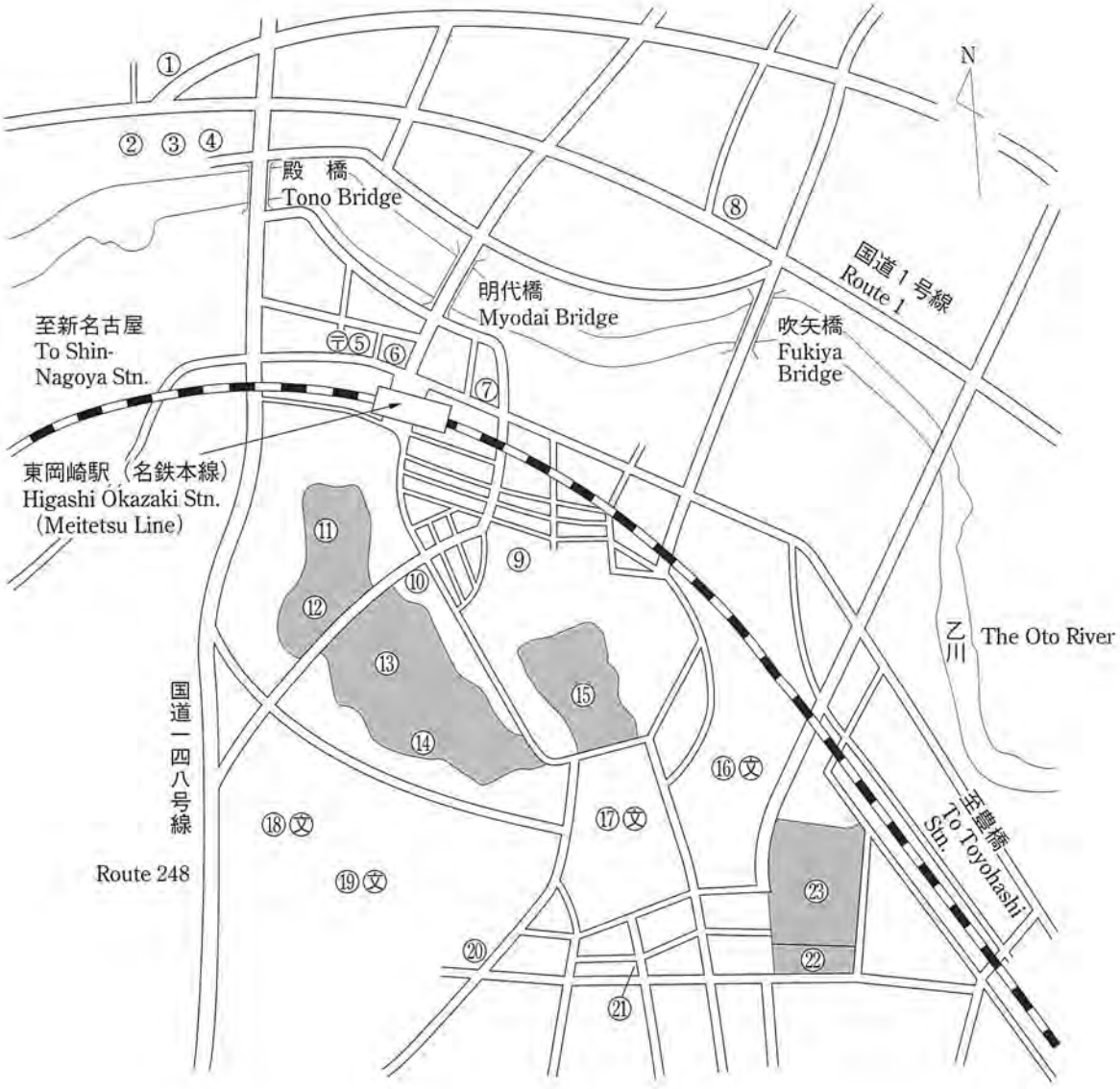


Figure 3. Structure of dynein heavy chain. A; Analysis of amino acid sequence of heavy chain reveals that it consists of three major domains referred to as N, M, and C from the N-terminus. B; M and C domains make larger domain (motor domain) by intramolecular association. C, According to Koonce et al. (1998) showthat recombinant motor domain would be spherical. Vallee et al. (1998) propose that a2 region corresponds to the B-link which is the stalk projected from the globular head structure of dynein, by demonstrating that the recombinant a2 actually binds to microtubules.



① 名鉄岡崎ホテル
Meitetsu Okazaki Hotel

② 岡崎城
Okazaki Castle

③ 岡崎ニューグランドホテル
Okazaki New Grand Hotel

④ 岡崎グランドホテル
Okazaki Grand Hotel

⑤ 郵便局
Post Office

⑥ 銀行
Bank

⑦ スーパーマーケット“ユニー”
Super Market “Uny”

⑧ 市役所
Okazaki City Office

⑨ 六所神社
Rokusho Shrine

⑩ 岡崎国立共同研究機構（看板）
Signboard of Okazaki National Research Institutes

⑪ 基礎生物学研究所
National Institute of Basic Biology

⑫ 生理学研究所
National Institute for Physiological Sciences

⑬ 管理局
Administration Bureau

⑭ 分子科学研究所
Institute for Molecular Science

⑮ 三島ロッジ・岡崎コンファレンスセンター
Mishima Lodge・Okazaki Conference Center

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Mishima Elementary School

⑰ 岡崎高校
Okazaki High School

⑱ 愛教大附中
Jr. High School affiliated to Aichi Univ. of Education

⑲ 竜海中
Ryukai Jr. High School

⑳ コンビニエンスストア
Convenience Store

㉑ 薬局
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㉒ 山手ロッジ
Yamate Lodge

㉓ 統合バイオサイエンスセンター
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