Department of Cell Biology and Anatomy

Outline and Research Objectives

When our institution was reformed from School of Medicine to Graduate School of Medicine, Department of Cell Biology and Anatomy has been built with the transition from the previous department of Anatomy. Therefore, directions of researches in this department are molecular cell biology based on structural cell biology.

At the same time this department has high responsibility for education of medical students, students of other faculties, and master course and Ph.D. course students (totally 518 hours per year). The teaching covers lectures and laboratory courses of gross anatomy, neuroanatomy, histology, cell biology and developmental biolgy.

Main subjects of researches are focused on 1) the mechanisms of intracellular transport, 2) the mechanism of cell morphogenesis, and 3) roles of cytoskeleton on development.

To solve these problems we combine structural biological approaches such as various kinds of light microscopy, immunocytochemistry, quick freeze deep etch electron microscopy, cryoelectron microscopy, X-ray crystallography with molecular biology, biochemistry, molecular biophysics, electrophysiology and molecular genetics.

Faculties and Students

Professor and Chair	Nobutaka Hirokawa, M. D., Ph.D. (from 1983~)
Associate Professor	Yoshimitsu Kanai, M. D., Ph. D.
	Takao Nakata, M. D., Ph. D.
Lecturer	Yasuko Noda, M. D., Ph. D.
	Sumio Terada, M. D., Ph. D.

Associate 8
Postdoctoral Fellow .....2 (Japan),1 (France),
.....1 (China)
Graduate Students .....7 (Japan), 2 (China)
Research Student .....3 (China), 1 (Korea)
Secretary & Technician 4

Past Research, Current Research and Major Accomplishments

I. Rapid Freeze Deep Etch Electron Microscopy and Identification of New Groups of Crossbridge Structures Among Microtubules, Intermediate Filaments, Actin and Membranous Organelles.

Hirokawa has developed a new microscopic method which rapidly freeze cells and tissues by making contact with a pure copper block cooled with liquid nitrogen (-190 C) or helium (-269 C) in Japan. He further developed this method in USA with Dr. John Heuser and visualised as yet unknown aspects of specialized membrane structures such as Gap Junction, Tight Junction, and Neuromuscular Junctions. Further he discovered a group of new filamentous crossbridges among microtubules, intermediate filaments, actin filalments and membranous organelles. Combining immunocytochemistry, biochemistry, and in vitro reconstitution he proved the chemical nature of these structures as 1) fodrin and myosin in brush borders of intestinal cells, a model of cellular cortex, 2) MAP1A, MAP1B, MAP2 and Tau in microtubule domains in neuronal axon and dendrites, 3) elongated C-terminus of Neurofilament M and H proteins in neurofilament domains in neuronal axons, 4) Synapsin I associated with actin and synaptic vesicles in presynaptic terminals.

II. Discovery of Kinesin Superfamily Proteins, KIFs and Elucidation of Mechanism of Intracellular Transport.

Hirokawa discovered various kinds of new filamentous structures between distinct kinds of membranous organelles and microtubules. He predicted these short crossbridges to be microtubule associated motor proteins carrying cargo vesicles along the microtubules.

The nerve axon is frequently very long (frequently ~1m long). Because of lack of protein synthesis machinery in the axon most of the proteins necessary in the axon and synaptic terminals ought to be transported down the axon as various kinds of membranous organelles and protein complexes after the synthesis in the cell body so that intracellular transport is fundamental for neuronal morphogenesis and functioning. At the same time because similar mechanism exists in every kinds of cells nerve cells serve as a good model system to elucidate this mechanism. Using molecular biology we discovered most of

kinesin superfamily motor proteins, KIFs which move along microtubule rails by hydrolysing ATP. Recently we identified all 45 kif genes in mammalian such as human and mouse. We have uncovered the structure, dynamics and functions of many members of KIFs usig molecular cell biological, biophysical, structural biological and molecular genetical approaches.

KIFs in Axonal Transport

KIF1A and KIF1Bβ transport synaptic vesicle precursors anterogradely from cell biology to synaptic terminals. They are fundamental for neuronal functioning and survivals. Further, we showed using molecular genetic approaches that KIF1Bβ is a responsible gene of human hereditary neuropathy Charcot-Marie-Tooth type 2A and proved that CMT2A is due to haploinsufficiency of functional KIF1Bβ and decrease of synaptic vesicle protein transport.

KIF1Bβ+/- mice serve as a model for CMT2A and we found a way of diagnosis and potential therapy of the symptom of this desease. KIF1B a and KIF5A, B, C redundantly convey mitochondria anterogradely. KIF3, composed of KIF3A and KIF3B heterdimer and associated protein KAP3, transports vesicles associated with a fodrin and important for neurites elongation through KAP3-α fodrin interaction. KIF2 and KIF4 are expressed specifically in juvenile neurons and functioning for neurite extension.

Receptor Transport in Dendrites

Our studies revealed the mechanism of transport of receptors in dendrites.

KIF17 transports NMDA type glutamate receptor containing vesicles in dendrites toward microtubule plus ends through interaction between KIF17 C-terminal tail-Mint 1 (mLin10)- CASK (mLin2)- Velis (mLin7) and NR2B subunit of NMDA receptor. Furthermore, we showed by transgenic mouse strategy that KIF17 plays a significant role on memory and learning. KIF5A, B, C convey AMPA type glutamate receptor containing vesicles towards microtubule plus end through the interaction between KIF5 heavy chain - GRIP1 (glutamate receptor interacting protein 1)-GluR 2 subunit of AMPA type glutamate receptors.

These studies elucidated also for the first time long standing questions how KIFs recognize and bind to cargoes by showing KIF tail - scaffolding protein complex or adaptor protein complex- membrane protein interaction is a typical way. This idea was supported by our other study revealing that KIF13A transports Mannose 6- phosphate Receptor containing vesicles from Golgi to plasma mambrane through the interaction between KIF13A tail - AP-1 adaptor protein complex and Mannose 6 phosphate receptors. Our study about the transport of AMPA type receptor by KIF5 also elucidated a mechanism how motor protein determines its direction, axon vs dendrites. GRIP1 steers KIF5 to dendrites via KIF5 heavy chain - GRIP1 interaction, while JSAP1 steers KIF5 to axon via KIF5 light chain- JSAP1 interaction.

KIF3 Determines Left-Right Asymmetery of Our Body

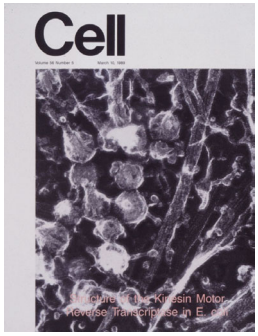
Our gene targeting study of KIF3 (KIF3A-/-, and KIF3B-/-) siginificantly contributued for the elucidation of mechanism of determination of left-right asymmetry of our body, a very important hot problem in developmental biology. Our study uncovered that KIF3 motor is essential for left-right determination of our body through intraciliary transportation of protein complexes for the ciliogenesis of motile primary cilia that generate leftward unidirectional flow of extraembryonic fluid, "nodal flow", which could produce a concentration gradient of putative secreted morphogen in the extraembryonic fluid along the left-right axis in the node of early embryo, very important region for the determination of our body plan.

Mechanism of Motility of Monomeric Motor, KIF1A

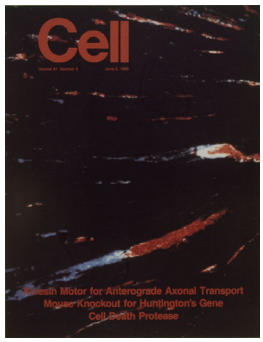
We have discovered monomeric motors KIF1 subfamily such as KIF1A, KIF1B α and β. Because motors previously identified are all dimers such as conventional kinesin (KIF5), dyneins and myosin the prevailing hypothesis for how the motor moves processively for certain distance along rails was the hand over hand model, which means a motor needs two legs to move as we walk on the rail. We, however, discovered monomeric motor such as KIF1A. Because the monomeric motor is the simplest motor it is a good model system to study the basic mechanism of motility of KIFs. We showed using molecular biophysics approach that a single KIF1A motor can move processively for more than 1μm on microtubules. Furthermore, our study revealed that the processive movement of KIF1A is based on the biased Brownian movement. This is the first clear experimental demonstration showing that motor protein can move by the biased Brownian movement. We further elucidated why single leg motor can move processively along microtubules without dissociation. We found polylysin loop (K-loop) in loop12 and showed that this K-loop is critical for processive movement through the interaction with C-terminal flexible end of tubulin (E-hook) especially at the weak binding state (ADP state) by biophysics, and cryoelectron microscopy.

We further analysed how the plus end bias can be emerged by combination of cryoelectron microscopy and X-ray crystallography. We found that counter clock wise 20 degree rotation of motor domain from ADP to ATP like state is critical for plus end biased

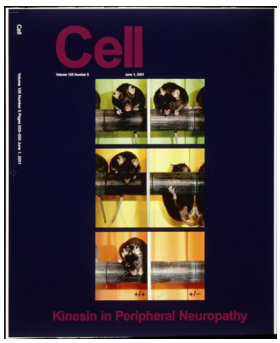
KIF review
(Hirokawa 1998)



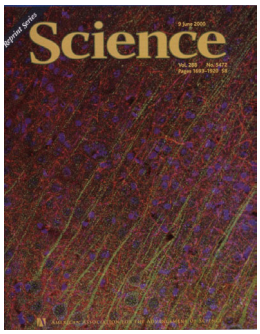
Kinesin structure
(Hirokawa et al. 1989)



KIF1Atransports synaptic vesicle precursor
(Okada et al. 1995)



KIF1B is responsible gene of hereditary neuropathy
(Zhao et al., 2001)

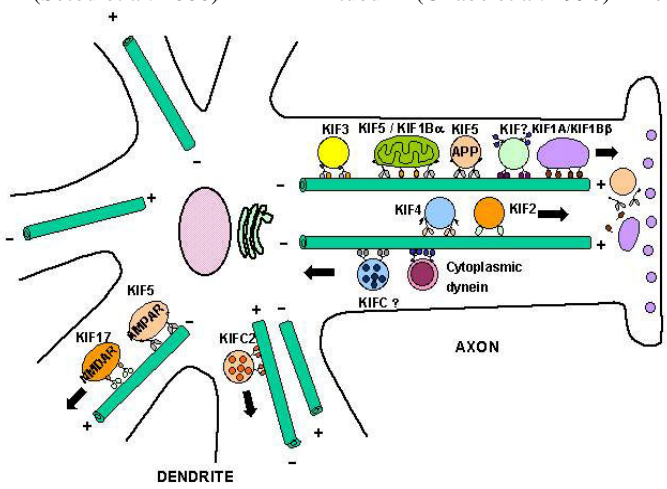


KIF17 transports NMDA receptors in dendrites
(Setou et al. 2000)

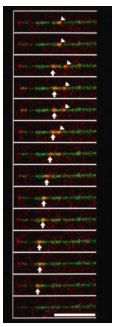
Photobleaching recovery study of fluorescent tubulin (Okabe et al. 1990)

Tau induced microtubule bundles and process elongation (Kanai et al. 1989)

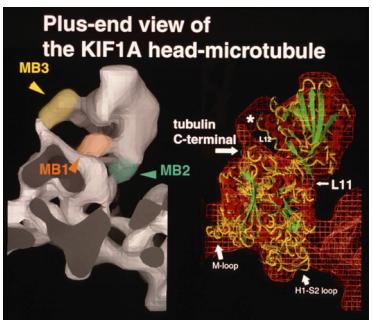
Tau/MAP1B double knockout mice
(Takei et al. 2000)



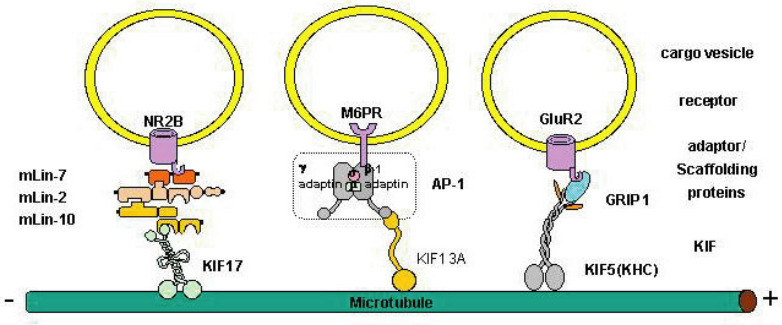
KIFs transport various kinds of cargoes in axon and dendrites



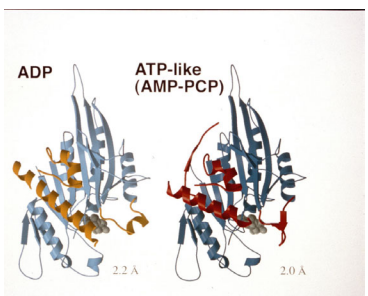
KIF1A monomer moves processively by biased Brownian movement
(Okada &Hirokawa Science 1999)



Cryo EM study of KIF1A motor domain-microtubule complex
(Kikkawa et al. Cell 2000)



KIFs recognize and bind functional membraneproteins, through KIF tail-scaffolding protein/adaptor protein complex interaction (Setou et al. Science 2000, Nakagawa et al. Cell 2001, Setou et al. Nature 2002)



X-ray crystallography and cryoEM study of monomeric KIF1A motor domain
(Kikkawa et al. Nature 2001)

movement of KIF1A monomeric motor.

Mechanism of Slow Axonal Transport

Cytoskeletal proteins such as tubulin, actin and neurofilament triplet proteins (NFH, NFM, NFL) and some cytosolic proteins were known to be transported down the axon slowly at 1~2mm/day. Using 1) microinjection of fluorescein labelled cytoskeletal proteins and subsequent analysis by fluorescent photobleach recovery, 2) microinjection of caged-fluorescein labelled cytoskeletal proteins and subsequent analysis by UV-photoactivation in cultured neurons we showed most of cytoskeletal proteins such as tubulin and actin are transported as small oligomers. Further, recently using microinjection of fluorescein labelled tubulin and cytosolic proteins into squid giant axons and subsequent analysis with fluorescence confocal laser scanning microscopy and fluorescence correlation spectroscopy combined with microinjection of anti KIF antibodies we demonstrated that tubulin is transported as small oligomers by conventional kinesin (KIF5) as a motor.

III. Microtubule Associated Proteins (MAPs) and Mechanism of Neuronal Morphogenesis.

Hirokawa identified various kinds of filamentous structures associated with microtubules by the quick freeze deep etch electron microscopy. The next questions were what are the chemical nature and what are the functions of these new structures. The microtubule associated proteins (MAPs) identified in brains were good candidates. Therefore, we have studied MAPs. We biochemically isolated major MAPs of mammalian brains such as MAP1A, MAP1B, MAP2 and tau, and studied molecular structure and localization. All these MAPs were filamentous flexible strutures from 185 to 50nm in length dependent on the difference of molecular weight. Immunocytochemistry and in vitro reconstitution comfirmed that these MAPs are components of filamentous structures associated with microtubules in neurons, To know the function of MAPs, we expressed tau and MAP2 in non neuronal cells such as fibroblasts and Sf9 cells by cDNA transfection. These studies showed that tau and MAP2 induce microtubule polymerization and bundling and process extention. They also revealed that C-terminal domain are critical for microtubule polymerization and N-terminal projection domain determines the different spacings between adjacent microtubules in microtubule domains in axon vs dendrites. These data suggest the important role of tau and MAP2 in formation of axon and dendrites. In order to elucidate function of MAPs in vivo we generated single knockout mice of tau, MAP1B, and MAP2 and analysed them. Generally, the phenotypes of single knockout mice were subtle. However, tau/MAP1B and MAP2/MAP1B double knockout mice showed predominant disturbance of axonal elongation and dendritic elongation respectively. Cellular bases of these phenotypes are suppression of microtubule stability and bundle formation in growth cones of axon and dendrites leading to the inhibition of proper neurite extention and neuronal cell migration. Thus, our studies revealed Tau/MAP1B and MAP2/MAP1B synergistically play fundamental roles in axonal and dendritic formation respectively.

Future Prospects

The further studies in near future will include following subjects as the continuation of on going researches.

I. Mechanism of Intracellular Transport

1) The functions of new KIFs especially in neurons, epithelial cells and fibroblasts to elucidate yet unknown mechanism of intracellular transport of various kinds of membranous organelles, protein complexes and mRNA.
2) Understanding how each KIFs recognize and bind their specific cargoes.
3) The studies how the binding and unbinding of KIFs with cargoes are regulated.
4) The mechanism how neurons determine the direction of transport by KIFs, toward axon vs dendrites.
5) The study of relationship between KIFs and neuronal functions such as memory and learning by molecular genetic approach.
6) The relationship between KIFs and diseases using molecular genetical approaches.
7) The detailed mechanism of motility of KIF1A motor using structural biology and molecular biophysics.

II. Mechanism of Neuronal Morphophogenesis and MAPs

Using molecular genetical approaches we will unravel the function of MAP1A, one member of major MAPs whose function has been unknown.

Research Grants

1. Center of Excellence (COE) grant from the Ministry of Education, Culture, Science and Sports, "Molecular Cell Biological and Molecular Genetical Study of the Cytoskeleton: Mechanism of Intracellular Transport, Signal Transmission and Cellular Morphogenesis." From April 1996 to March 2001. 1,885,000,000 yen
2. Grant in Aid for Basic Research, "Molecular Cell Biology of New Molecular Motors, KIFs," from the Ministry of Education, Culture, Science and Sports. From April 1996 to Mach 1999. 34,500,000 yen
3. Center of Excellence (COE) grant from the Ministry of Education, Culture, Science, Sports and Technology, "Mechanism of Intracellular Transport

:Molecular Cel Biology, Structure Biology and Molecular Genetics."From April 2001 to March 2006. 2,020,000,000 yen

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Department of Molecular Biology

Outline and Research Objectives

This department was established in 1893 as the first Biochemistry Department in Japan. Since then, this department has been devoted to biochemical studies on vitamin, oxidative phosphorylation, lipid, carbohydrate and nucleic acid metabolisms, making remarkable contributions to advancements in medical biochemistry. After H. Okayama succeeded Chair in 1992, research has been focused on cancer, the cell cycle and differentiation in order to understand the molecular mechanism of malignant transformation and growth control.

Faculties and Students

Professor and Chair Hiroto Okayama, MD & PhD (1992~)
Associate 3
Postdoctoral Fellow 1
Graduate Student.....5
Research Student 5
Secretary 2

Past Research and Major Accomplishments

Development of high-efficiency full-length cDNA cloning method and eukaryotic expression cDNA cloning system

Most of our past and current research stemmed from the development of the full-length cDNA cloning method and the cDNA expression cloning vector system that was done in the early 1980s by Hiroto Okayama and Paul Berg (Fig. 1)(1,2).

The full-length cDNA cloning method and the cDNA expression libraries constructed thereby have been used to clone numerous functional genes in laboratories over the world, the earliest one of which is the human Lesch-Nyhan syndrome causative gene (3). The cDNA expression cloning vector system was originally designed to clone genes on the basis of the functions they express in a wide range of host cells, from fission yeast up to mammalian cells. This was enabled by development of highly efficient methods for introducing cDNA libraries into mammalian cells and fission yeast and construction of vectors specialized for library transduction into fission yeast (Fig. 1) (8, 10, 11). One of the most successful applications of this expression cloning system was isolation of human orthologues of several key cell cycle regulators initially found in fission yeast. Paul Nurse cloned the human *cdc2* cDNA from one of our expression libraries on the basis of trans-complementation of a fission yeast temperature-sensitive mutant of *cdc2*$^+$ essential for mitosis, and we cloned human orthologues of *wee1*$^+$ and *cdc25*$^+$ genes (13, 14), which critically regulate Cdc2 kinase during this transition, as described below.

Cell cycle control

Cdc2 kinase is a protein kinase required for the onset of mitosis, initially found in fission yeast, but present in all eukaryotes. This kinase, associated with cyclins, is regulated by inhibitory tyrosine phosphorylation and activating dephosphorylation by Wee1 kinase and Cdc25 phosphatase, constituting a core element of DNA damage-responsive cell cycle checkpoint control for the G_2-M transition in fission yeast.

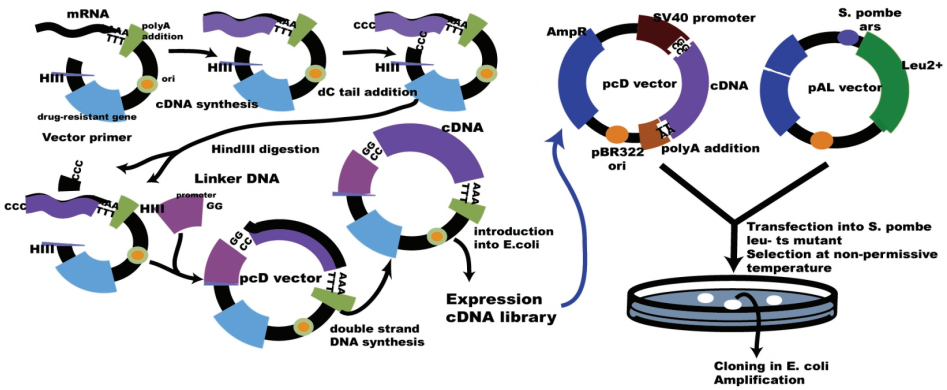

Fig.1. Methods for construction of full-length expression cDNA librareis and trans-copmplementation cloning with fission yeast as host

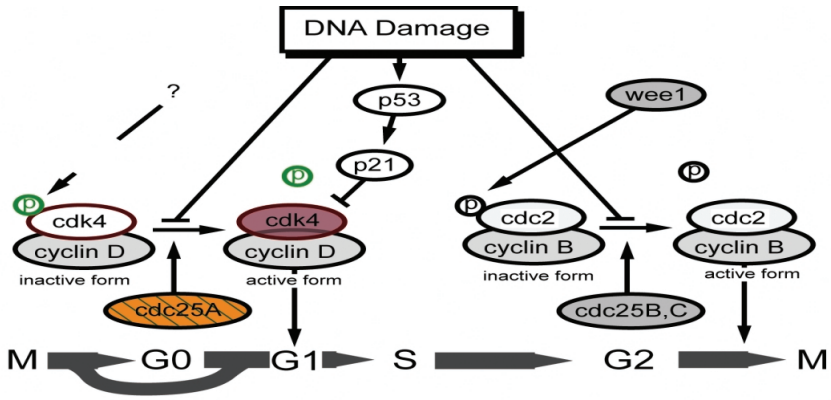

Fig. 2. Similarity in DNA damage-invoked G1/G2 checkpoint control

The identification of human orthologues (cdc2, wee1 and cdc25B) of these fission yeast cell cycle regulators demonstrated the evolutionary conservation of a cell cycle control system up to mammals (13, 14, 30). Using the same expression cloning system, we also identified an additional mammalian homologue (cdc25A) of *cdc25*$^{+}$ gene, which was found to play an unexpected role (23).

This gene is expressed in early G_1 and required for the cell cycle start. Search for targets for this novel phosphatase led us to discover that Cdk4, a cyclin-dependent kinase essential for the cell cycle start, is regulated by tyrosine phosphorylation and that its regulation constitutes a key part of DNA damage-induced G_1 checkpoint arrest during cell cycle entry from quiescence (23, 28, 37), revealing the presence of mirror image mechanisms for controlling DNA damage-responsive G_1 and G_2 checkpoint arrest in mammals (Fig. 2).

In addition to the studies on mammalian cell cycle control, we identified genes for 11 fission yeast novel cell cycle factors and cyclin G a novel mammalian cyclin (22), which control the G_1-S transition, DNA-replication checkpoint or the formation and maintenance of sister chromatid cohesion (20, 24-27, 29, 33, 35, 40, 42-44, 48). Recent studies on two newly identified factors Eso1 and Pds5 involved in the formation of sister chromatid cohesion led us to discover that sister chromatid cohesion is formed and maintained by a highly unexpected mechanism, which functions as a molecular zipper and thereby promotes the formation of cohesions only between sister chromatids (48). This finding provided a new clue to the understanding of the mechanism for the genetic instability associated with malignant transformation.

Differentiation control

One of the aspects of the cell we have been interested in understanding concerning carcinogenesis is differentiation control. To this end, we also used fission yeast as a model organism and discovered five novel genes controlling the commitment to differentiation. They encode Esc1 a MyoD-like helix-loop-helix protein, Nrd1 an RNA-binding protein, Phh1 a stress MAP kinase similar to p38, Rcd1 an evolutionally conserved protein and Pas1 and Cyc17 novel cyclins (21. 26, 31, 33, 36, 43, 44). These proteins were found to be a modifier of cyclin AMP signaling, a determinant of the threshold to nutrient starvation for the commitment to differentiation, a stress signal transducer as an absolute requirement for the differentiation commitment, a key mediator of nitrogen starvation signal and factors that ensure mutual exclusiveness between the cell cycle start and the commitment to differentiation. In addition, using trans-complementation cloning, we isolated a human functional counterpart of the RNA-binding protein with predicted functions, which were shown in hematopoietic cells (38).

Furthermore, we recently found that the mammalian counterpart of Rcd1 is a novel transcriptional cofactor mediating retinoic acid-induced cell differentiation and is deeply involved in mouse lung development (50). These findings not only reveal remarkable conservation of the differentiation commitment system as well as the key elements that control the commitment step from yeast up to mammals (Fig. 3), but also greatly help understand the highly complex mechanism controlling the differentiation commitment in mammals.

Anchorage-independent cell cycle start: a key mechanism for malignant transformation

One of the fundamental phenotypes that distinguish malignant from benign cells is the metastatic capability, which is underlain by the acquirement of the ability to perform S phase entry in the absence of anchorage. In the late 1980s, we began studies to understand the nature and mechanism of the acquirement of this unique property upon malignant transformation, by using growth factor-transformable NRK cells as a model system. The generally held concept was that anchorage-independent S phase entry is a mere consequence of excessive activation of growth signaling or defects in a growth arrest system, which

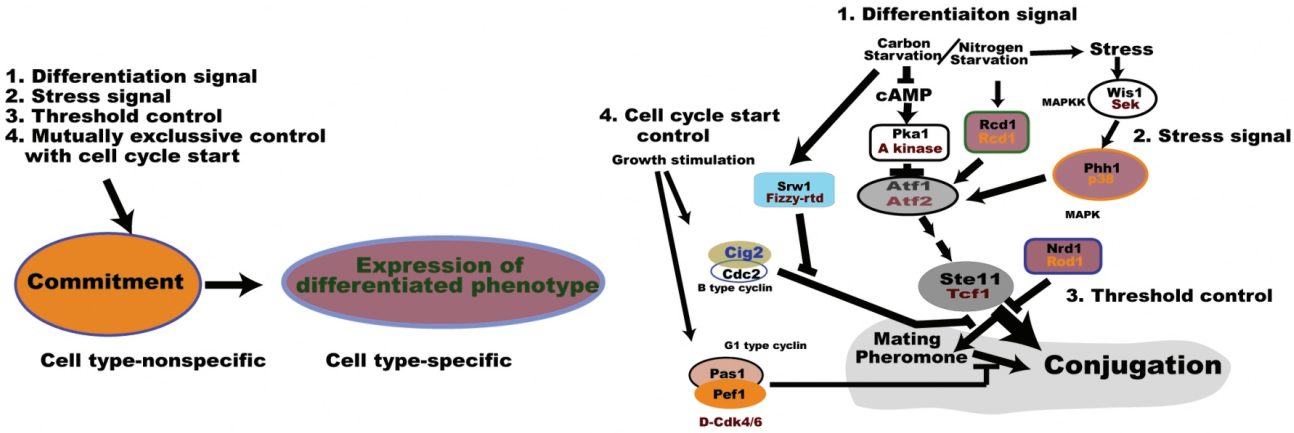

Fig. 3. Elements and evolutionary conservation of eukaryotic differentiation commitment control

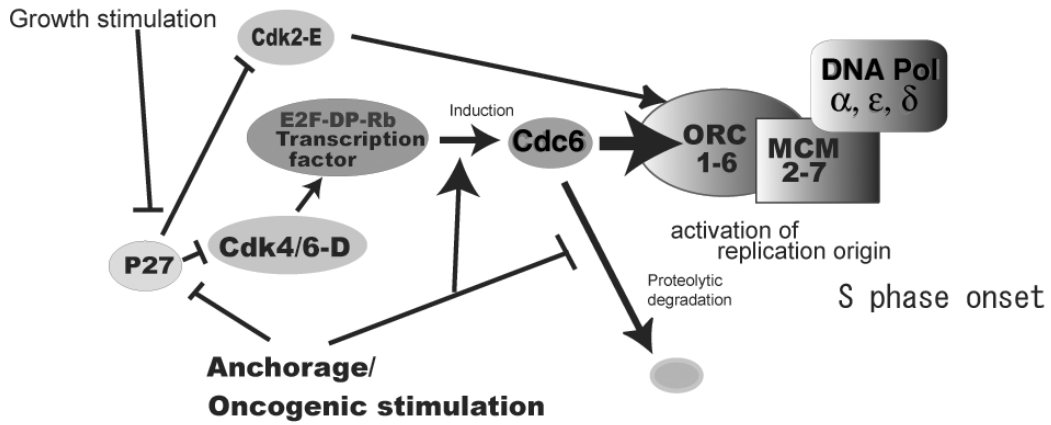

Fig.4. Cell cycle start control by anchorage

are caused by activation of oncogenes and inactivation of recessive oncogenes. For the past 10 years, we studied genetically and cytologically the properties of oncogenically stimulated NRK cells and came to the several remarkable conclusions: 1) In NRK cells, epidermal growth factor and platelet-derived growth factor signals merge into a common pathway composed of CrkII, Ras and Raf-1, through which many oncogenes induce transformation; 2) Activation of this pathway induces anchorage-independent S phase entry in a synchronized fashion, but is dispensable for cell proliferation in the presence of anchorage; 3) Oncogenic stimulation recruits Cdk6 to participate in an essential step of anchorage-independent S phase onset; 4) Activation of G_1 cyclin-dependent kinases (Cdk4/6, Cdk2) is insufficient to induce anchorage-independent S phase onset (15, 16, 32, 39). Recently, we found that Cdc6, a key protein for the activation of replication origins, requires anchorage or oncogenic stimulation for its expression, which is regulated at the levels of transcription and proteolysis via a calpain-like protease, and moreover, that G_1 cyclin-dependent kinases and Cdc6 constitute key targets for controlling the G_1-S transition by anchorage and oncogenic signals (Fig. 4)(49).

We believe that this latest finding would provide a major breakthrough toward the understanding of the mechanisms for anchorage loss-invoked restriction of S phase onset and oncogenically induced anchorage-independent S phase onset, the latter being responsible for the metastatic capability of malignant cells. In addition, all these findings taken together raise the possibility that oncogenic transformation is not merely resulted from a failure to arrest due to excessive growth stimulation or defective cell cycle arrest systems, but from constitutive activation of a dormant mechanism that is present in normal cells and enables the cell cycle start in the absence of anchorage (46).

Current Research

We are currently devoting most of our efforts to the understanding of how Cdc6 expression is regulated by anchorage and oncogenic signals.

Requirement for PI3 kinase in Cdc6 expression

It has been known that phosphoinositol-3 (PI3) kinase is required for the cell cycle start and one of its targets is p27 cyclin-dependent kinase inhibitor. We recently found that entirely independent from p27 downregulation, Cdc6 requires PI3 kinase for its expression during anchorage-dependent, but not

anchorage-independent, S phase entry. Treatment of cells with a specific PI3 kinase inhibitor shuts off Cdc6 expression despite E2F activation, but interestingly this shutoff is overridden by oncogenic stimulation. Further analysis is underway to identify how PI3 kinase and anchorage signal cooperate to express Cdc6.

Identification of the protease responsible for the Cdc6 degradation and its regulation

The most crucial point in understanding the regulatory mechanism of Cdc6 expression by anchorage and oncogenic stimulation is identification of the responsible protease and its regulatory mechanism (Fig.4). To this end, a human cdc6 cDNA was isolated, tagged with a histidine tag and transcribed in vitro and expressed in reticulocyte lysates, and the expressed His-tagged Cdc6 protein was purified by affinity chromatography in order to establish an in vitro assay system. Analysis of the nature of the putative protease suggests that the cells arrested in G_1 by anchorage loss contain the proteolytic activity that seems to be responsible for Cdc6 degradation. Analysis of the nature of this protease and its regulation is currently in progress.

Cdk6-cyclin D3 as a highly potent sensitizer of cells to chemical and physical transformation

We recently found that among D-type cyclin (D1, D2, D3) and partner kinase (Cdk4, Cdk6) combinations, the Cdk6-D3 complex is unique and can evades inhibition by p27 and p21, consequently enabling this complex to control proliferation competence under growth suppressive conditions (47). This finding led us to investigate the possible effects of the elevated expression of this complex on cell's susceptibility to malignant transformation, and we have found that 2-5 fold overexpression of Cdk6-D3 elevates 10^3-10^6 folds the susceptibility of rodent fibroblasts to UV irradiation- or 3-methylcholanthrene-induced malignant transformation. Analysis is in progress to understand this sensitization mechanism.

Future Prospects

There are three key questions regarding malignant transformation: 1) What is the mechanism by which transformed cells acquire the ability to perform anchorage-independent S phase onset as a basis for metastatic capability?; 2) What causes the genetic instability of cancer, which is believed to be a driving force for malignant progression and what are the key cell cycle factors activated or inactivated during this process?; 3) What is the mechanism for dedifferentiation of malignant cells? We believe that the series of our research will certainly offer critical clues to solve these questions. We particularly believe that the finding that Cdc6, a key factor for the activation of replication origins, absolutely requires anchorage or oncogenic stimulation for its expression is a major breakthrough to understanding the central mechanism of oncogenically induced anchorage-independent S phase onset.

Research Grants

1. H. Okayama, Grant-in-Aid for Scientific Research (A), JSPS, 1992-present. ¥10,000,000 a year.
2. H. Okayama, Grant-in-Aid for Scientific Research on Priority Areas "Advanced Cancer Research", MEXT, 1992-1999. ¥32,000,000 a year.
3. H. Okayama, Grant-in-Aid for Scientific Research on Priority Areas "Cell Cycle Control", MEXT, 2000-present. ¥60,000,000 a year.

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Department of Cellular Signaling

Outline and Research Objectives

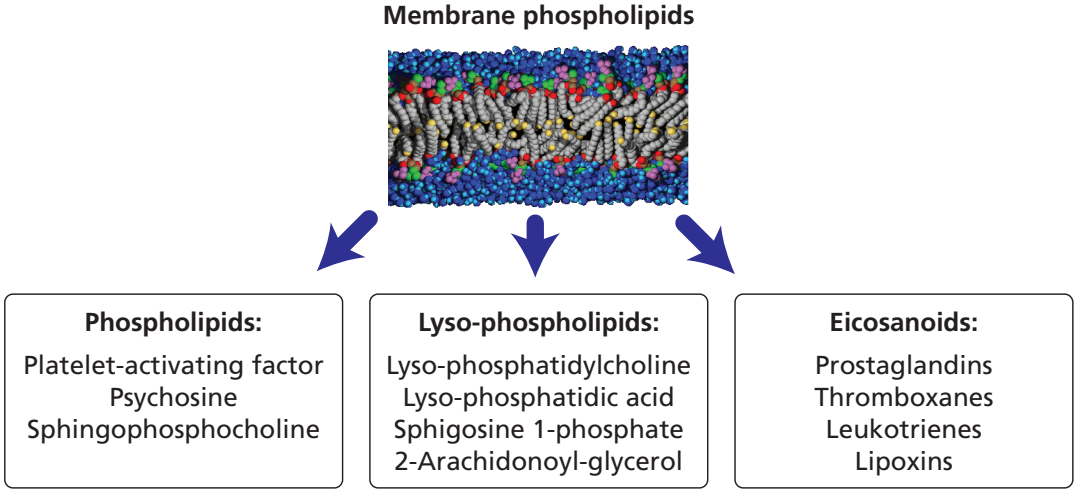
When cells are stimulated, by the serial actions of various enzymes, biologically active lipid molecules are produced and released. They include prostaglandins, leukotrienes, platelet-activating factor (PAF), lysophosphatidic acid (LPA) etc., collectively termed lipid mediators (Fig 1). In concert with various neurotransmitters and hormones, these lipid mediators are playing important roles in self-defenses and maintenance of homeostasis. Unlike the biogenic amines or peptide mediators, lipid mediators are not stored in granules, but they are biosynthesized from precursor lipids when necessary. To elucidate the functions of lipid mediators, we isolated enzymes involved in the biosyntheses and metabolism of lipid mediators, isolated G-protein-coupled receptors, and identification of intracellular signaling. We are especially interested in identifying the specific roles of lipid mediators in inflammatory responses as well as neuronal functions. We generated several transgenic or knockout mice and analysed their roles in vivo. By mass spectrometric analyses, we also measured dynamic changes in membrane lipid compositions, the significance of these changes being in general signaling pathway. In the postsequece of whole human genome, the intensive studies of protein regulation as well as behavior of small lipid metabolites are of the highest importance.

Faculties and Students

Professor	Takao Shimizu, M.D., Ph.D. (1991-)
Associate Professor	Takehiko Yokomizo, M.D., Ph.D.
Associate	3
Postdoctoral Fellows	4
Graduate Students	12
Research Students	7
Secretary	2

Past Research and Major Accomplishments

We have purified and cDNA cloned various enzymes involved in the biosyntheses and metabolism of lipid mediators. Leukotriene A4 hydrolase, one of the key enzyme in the biosynthesis of chemotactic leukotrienes, was cloned and characterized in our laboratory. This achievement was the first successful example of molecular cloning of the enzymes in the field of lipid mediators. We also obtained PAF receptor, as the first successful example of receptor cloning of lipid mediators, followed by cloning receptors of various lipid mediators. We determined the regulation of these enzymes by Ca-dependent intracellular translocation, phosphorylation by various kinases, and other posttranslational modifications. We also

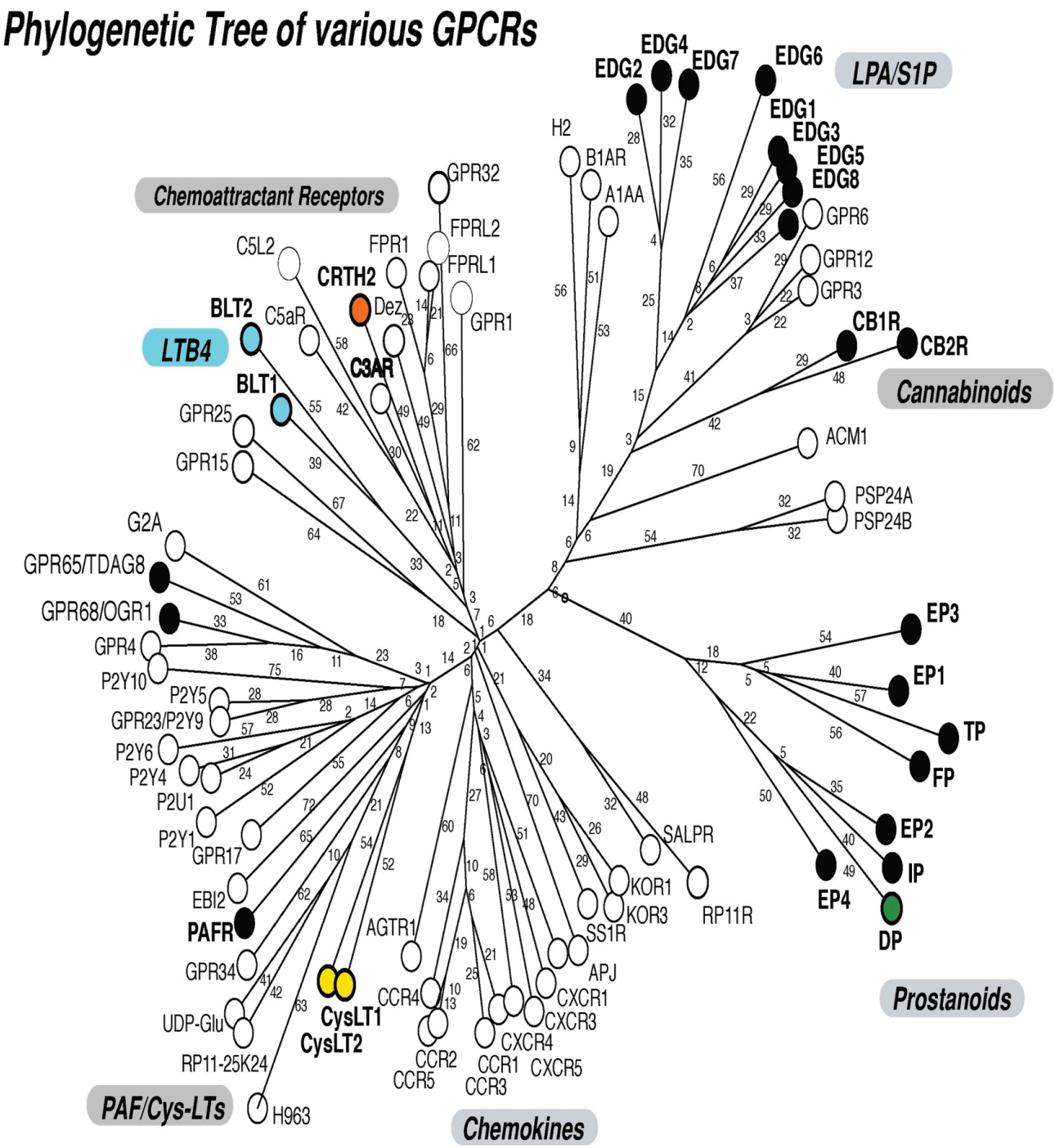


generated PAF receptor-overexpressing mice, PAF receptor-deficient mice, leukotriene receptor-deficient mice, and the mice deficient in cytosolic phospholipase A2 (cPLA2), which are involved in the syntheses of eicosanoids and PAF. By analyzing the phenotypes of these mice, we reported that the mediators are involved in the pathogenesis of various inflammatory and immune disorders, as well as normal physiology including reproduction and synaptic plasticity. Independently, one of the Associate is working on axon guidance molecules, by establishing knockout mice of guidance molecules (Sema 3A), and analyses of their multiple phenotypes.

Current Research

Followings are ongoing projects in our laboratory.

1. Elucidation of enzymes in the biosyntheses of lipid mediators, their regulation and mechanism of intracellular translocation by stimuli.
2. Deorphaning projects of putative lipid mediator GPCRs, which include indentificaion of novel lipid mediators (Fig. 2).
3. Elucidation of GPCR-sorting mechanism in polarized cells (kidney epithelial cells, neuron etc.)
4. Phenotype analyses of various genetic engineered mice. Understanding of the molecular mechanism of individual phenotypes in depth.



5. Establishment of novel gene-targeted mice (enzymes and receptors), and their congenic line (B6, Balb/c, DBA etc.)
6. Identification of Sema3A knockout mice, in nervous systems, olfactory systems, bone formation.

Future Prospects

We will pursue following different projects, lead by each faculty member.

1. Identification of the roles of lipid mediators in the central nervous system. For this purpose, we will analyze the localization and subcellular localization of enzymes involved in the biosyntheses of lipid mediators. It is the most important to determine, how and which direction (either axon or dendrite), these enzyme cause translocation in a stimulus-dependent manner. The mice deficient of enzymes or receptors of lipid mediators are useful to identify the roles of mediators in the central nervous systems. Some link has been reported between Sema3A axon guidance molecules and lipid mediators. By the use of double knockout mice (Sema3A and lipid mediators), more confirmative and direct evidence will be obtained.
2. We have so far obtained various phenotypes of genetic engineered mice, but molecular mechanisms underlying these phenotypes remain mostly unknown. The link between lipid mediators and hormones, neurotransmitters, and cytokines will be analyzed to obtain a whole view.
3. By mass spectrometric analyses, we like to find out the temporal and positional dynamic changes of membrane compositions including Raft and caveolae. Also, mass spectrometry attached with HPLC will aid in the identification of novel natural lipid mediators (deorphaning project). For these purposes, we recently built a new research group called Department of Lipid Metabolome, by donation. We have collected several excellent lipid biochemists, and mass spectrometer specialists. Metabolome (metabolomics) is a new research strategy after genome and proteome research. The study is to analyze in detail small-sized molecules (metabolites), and in combination with proteomics, we will gain a more broad view how cells adapt to a new environment, change membrane fluidity, and cause metabolic changes, which finally yield to gene expression.

Research Grants

1. 1998-2003 CREST of Japan Science and Technology Corporation
2. 2001-2004 PREST of Japan Science and Technology Corporation
3. 1999-2002 Grant from the Ministry of Education, Science, Culture, Sports, and Technology of Japan (A, B, and C)
4. 1997 Priority Area A, from the Ministry of Education, Science, Culture, Sports, and Technology of Japan.
5. 1996-2000 Ministry of Welfare, Health and Labor.

Select Publications (50 among 128 publications between 1991 and 2002)

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Department of Physiological Chemistry and Metabolism

Outline and Research Objectives

The Department of Physiological Chemistry and Nutrition, the predecessor of the present department, was founded in 1952. The successive professors were as follows;

The first professor: Haruhisa Yoshikawa (1952~1969)
The second professor: Osamu Hayaishi (1970~1974)
The third professor: Yoshitake Mano (1974~1982)
The fourth professor: Yousuke Seyama (1983~2001)

During the past 50 years, these professors lead high-quality researches in the field of biochemistry, metabolism and nutrition and greatly contributed to scientific progresses in Japan. Upon the restructuring of the university system in 1997, the department was renamed 'Department of Physiological Chemistry and Metabolism' as one unit of the Specialty of Molecular Cell Biology. In 2002, Dr. Hiroki Kurihara was designated as a successor of Professor Yousuke Seyama, and started new researches on developmental biology and technology as well as regenerative medicine.

Faculties and Students

Professor and Chair Hiroki Kurihara, M.D., Ph.D. (2002~)
Associate.....3
Postdoctoral Fellow 1
Graduate Student.....2
Secretary 1

Past Research and Major Accomplishments

1. Vascular Biology

(1) Biology of Vasoactive Peptides

i) Endothelin

a. Discovery and Basic Characterization of Endothelin

Our researches started with contribution to the discovery of endothelin (ET-1), an endothelium-derived vasoactive peptide, with Drs. Masashi Yanagisawa, Tomoh Masaki and colleagues in 1987. Then, we studied the role of ET-1 in vascular physiology and pathophysiology and revealed its vasoconstrictive effect on in vivo canine coronary arteries and induction of the ET-1 gene by some cytokines and flow-induced mechanical stress in vascular endothelial cells.

b. Endothelin-1 Knockout Mice

In 1991, we introduced the technique of gene targeting in mice to our lab to further analyze the biological implication of ET-1. As a result, we found two novel and important roles of ET-1. First, ET-1 proved to lower blood pressure when it acts in the central nervous system although it mainly acts as a pressor in the periphery. ET-1 also modulates stress responses in the central nervous system by regulating catecholamine metabolism. Second, ET-1 proved to be essential for embryogenesis. The phenotype of mice lacking ET-1 involved craniofacial and cardiac neural crest-derived structures including the branchial arches and great vessels. Indeed, ET-1 was the first vasoactive substance bound to G protein-coupled receptors that proved to be involved in embryonic development (See 2-1)).

c. Endothelin-1 Overexpressing Mice

We found that the expression of ET-1 and its processing enzyme ECE-1 is increased in the lesion of human atherosclerosis and experimental vascular injury. The effect of overexpressed ET-1 was further analyzed in transgenic mice and proved to cause mesangial hyperplasia and glomerulosclerosis in the kidney. This result was indicative of the stimulating effect of ET-1 on cellular proliferation and remodeling in vivo.

ii) Adrenomedullin

a. Adrenomedullin Overexpressing Mice

Adrenomedullin (AM), a vasodilating peptide, can be regarded as a counterpart of ET-1 in vascular tone regulation. We investigated the in vivo effect of AM by producing transgenic mice overexpressing AM and found NO-dependent hypotension and resistance to endotoxin shock (decrease in lethality) in transgenic mice. These results suggested that AM may lower blood pressure by stim-

ulating NO production and that AM may protect tissues and organs from shock-induced damage.

b. Adrenomedullin Knockout Mice

The biological importance of AM was further investigated by gene targeting. We demonstrated that AM is indispensable for the vascular morphogenesis during embryonic development and for postnatal regulation of blood pressure by stimulating NO production, confirming the hypothesis derived from AM overexpression mice.

iii) CGRP

CGRP is structural related to AM and shares the common receptor CRLR. To investigate systematically the differential role of the AM/CGRP family members, we established alphaCGRP knockout mice. The resultant phenotype revealed that alphaCGRP contributes to the regulation of cardiovascular function through inhibitory modulation of sympathetic nervous activity and that AM and CGRP have distinct physiological roles.

(2) Transgenic techniques

During gene manipulation studies in mice, we found that the ET-1 gene promoter is useful for vessel-selective gene expression. This promoter was used not only to overexpress ET-1 and AM but also to make endothelial NO synthase and 15-lipoxygenase overexpressing mice by our collaborators with fruitful results. Recently, we realized targeting gene expression in vascular smooth muscle cells by using the ET-A receptor (ETAR) gene promoter.

2. Developmental Biology

(1) Neural Crest Development

Craniofacial and cardiovascular defects in ET-1 knockout embryos gave a clue to the elucidation of mechanisms for branchial arch formation contributed by neural crest cells. By subsequent analysis, we revealed that ET-1 regulates several downstream genes such as HAND1/2 and Goosecoid transcription factors to contribute to cranial/cardiac neural crest development. Recently, we established ETAR promoter::GFP and ETAR promoter::TVA (avian retrovirus receptor) to visualize and isolate ETAR+ neural crest cells and to transfer genes specifically to them via retroviral vectors. These systems serve as very useful tools for the analysis of neural crest-derived branchial arch development.

(2) Vascular Development

ET-1 knockout mice also demonstrated defects in the great vessels and cardiac outflow tract, to which cardiac neural crest cells largely contribute. From this result, we found that ET-1 is important in vascular smooth muscle cell development originated from neural crest cells and that the ET-1to HAND2 signaling pathway seems to be critical. The outcome from AM knockout mice showed that AM is important in the formation of basement membrane during angiogenesis to stabilize vascular network.

3. Collaborative Works

(1) Biology of Metalloproteinase

In collaboration with Dr. Kouji Matsushima (Dept. of Molecular Preventive Medicine), we exploited the biological implication of ADAMTS-1, a member of the ADAM-type metalloproteinase family, by gene targeting. We found that ADAMTS-1 is important for normal growth, fertility and organ developemnt (the kidneys, adrenal glands, adipose tissues etc.).

(2) Biology of Antimicrobial Peptides

In collaboration with Drs. Yasuyoshi Ouchi and Takahide Nagase (Dept. of Geriatrics), we are studying about defensins, endogenous antimicrobial peptides. We have identified several novel types of human and mouse beta-defensins which are expressed skeletal muscle and epididymis.

Current Research

1. Vascular Biology

We have converged our previous achievement of vascular biology on the aspect of developmental biology including vascular development as described below.

2. Developmental Biology

(1) Neural Crest Development

Extending our achievement of ET-1 knockout mice, we are studying the mechanism how the intercellular and intracellular signaling system related ET-1 is involved in neural crest development and branchial arch formation because it may give a clue to the insight how signaling interactions can change cellular behavior to lead to morphogenesis. For this purpose, we have established GFP and TVA transgenic mice as described and are now establishing some other mice which will be useful for the analysis of branchial arch formation. We are also performing a systematic screening for differential gene expression by DNA microarray followed by in situ hybridization. By this method, several important genes are to be obtained.

We are also studying about neural crest differentiation using murine neural crest cell culture and Sendai virus vectors developed by Drs. Yoshiyuki Nagai and Atsushi Kato. In our preliminary result, we realized nearly 100 % gene transfer into neural crest cells and are analyzing the effect of some genes on neural crest

differentiation. Using some markers, we are also trying to isolate neural crest stem cells.

We are further looking for the mechanism how some transcription factors important for neural crest development can exert their effects. For this purpose, we are performing yeast two-hybrid screening using Pax3 and HAND2 as baits. We have obtained several interesting clones and are analyzing their molecular characteristics.

(2) Vascular Development

Above-mentioned studies concerning neural crest differentiation include the issue of vascular smooth muscle cell differentiation and we are trying to identify the target genes of the ET-1-HAND2 pathway that is important for smooth muscle differentiation. Two-hybrid studies also involve this theme. Abnormalities in vascular formation in AM knockout mice are also being investigated further concerning the mechanism.

In addition, we have started studies on vascular endothelial cell differentiation using the embryonic stem cell system and are looking for possible interaction between neural crest-derived smooth muscle precursors and endothelial precursors. It may clarify a novel cellular interaction mechanism which can stimulate vascular formation.

3. Collaborative Works

In collaboration with Drs. Yasuyoshi Ouchi and Takahide Nagase (Dept. of Geriatrics), we are starting further study on defensins using gene manipulation in mice.

Future Prospects

(1) Basic research

Our future study will focus mainly on neural crest and vascular development. Especially, major questions are (1) how do intracellular mechanisms modulate stem cell behavior including fate determination and differentiation, and (2) how are cell behaviors integrated into morphogenesis and organogenesis. By studying neural crest development as a model, we expect that we can approach these general issues. For our coming research, we are introducing some new techniques such as nuclear transfer.

(2) Applied research

Our current researches on vascular development using embryonic stem cells are close to applied researches in the field of regenerative medicine. On this background together with nuclear transfer technique, we are planning translational researches to generate vessels and other tissues and organs related neural crest cells.

Research Grants

1. JSPS (Japan Society for the Promotion of Science) Research for the Future Program (2000.4~2004.3) total 240,000,000 yen.
2. Grants-in-Aid for Scientific Research from JSPS, Scientific Research (B)
   (2002.4~2004.3) total 14,900,000 yen
3. Grants-in-Aid for Scientific Research from JSPS, Exploratory Research (C)
   (2002.4~2004.3) total 9,900,000 yen
4. Grants-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan, Priority Areas Research (A), Allotted.
   (2000.4~2001.3) total 1,800,000 yen.
5. the Research Grant for Cardiovascular Diseases (14C-1) from the Ministry of Health and Welfare
   (2002.4~2005.3) total 6,300,000 yen.

Select Publications

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