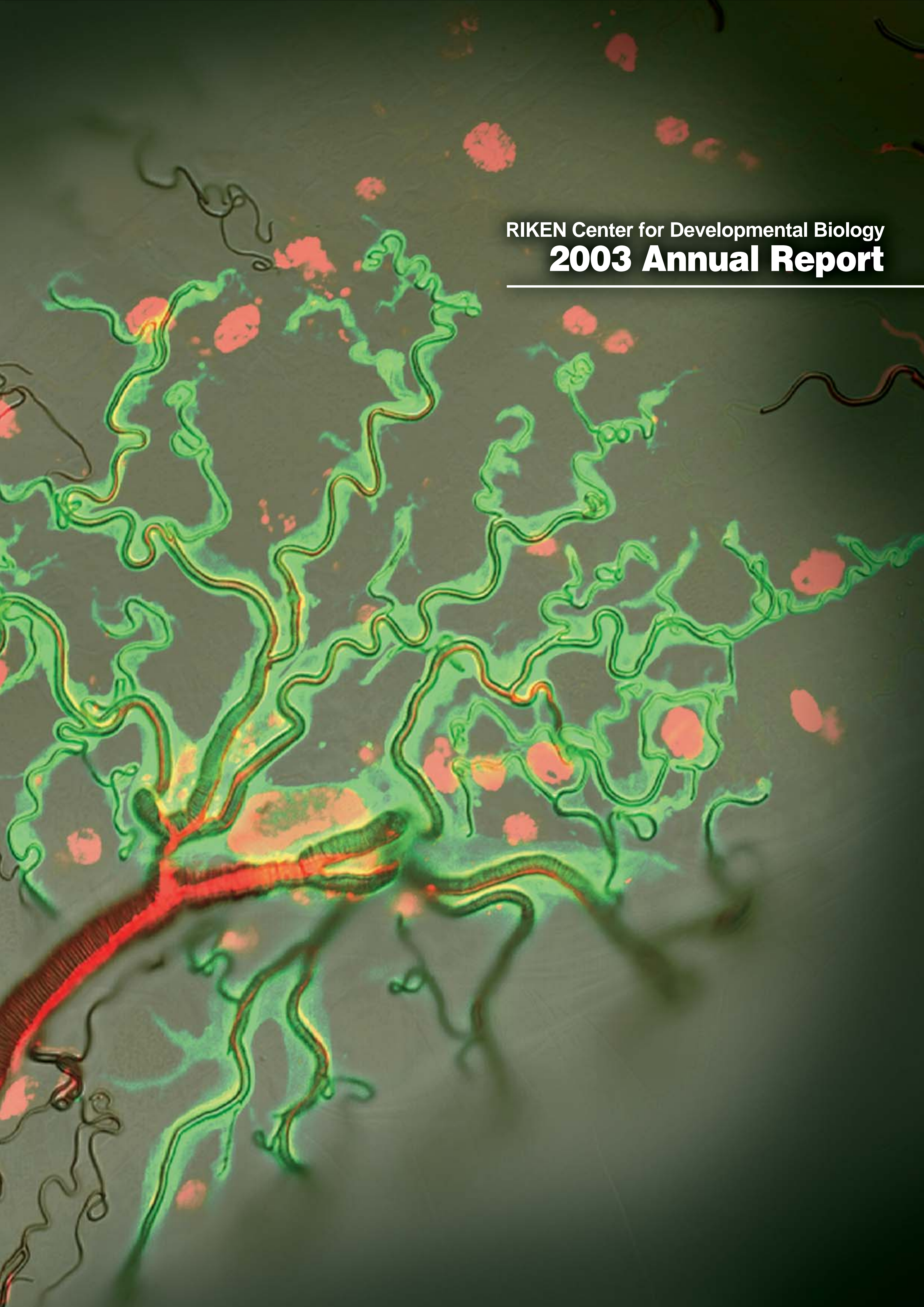

RIKEN Center for Developmental Biology

2003 Annual Report

RIKEN
Center for Developmental Biology

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Middle ear of the chick. PHOTO: Raj Ladher

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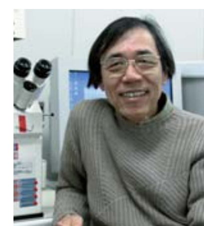
Center for Developmental Biology



Message from the Director

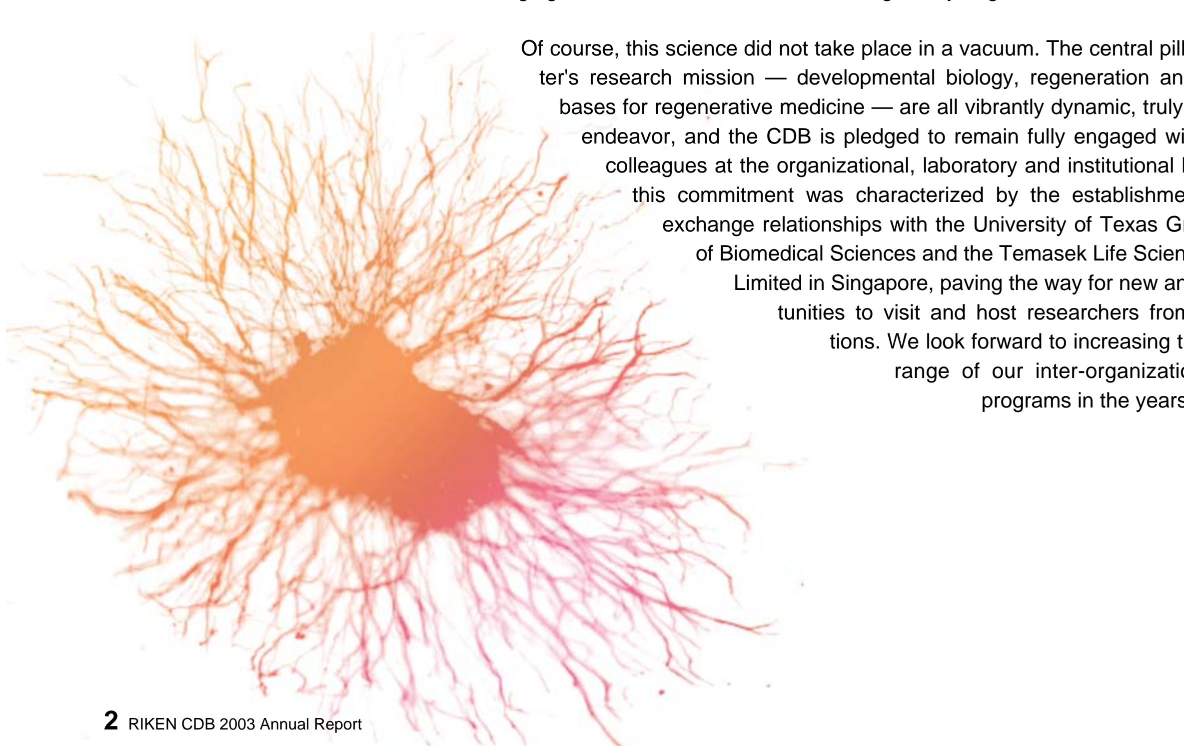
Masatoshi Takeichi
Director, CDB

The days leading up to the year's end tend to be chaotic, but they also provide one with a valuable stimulus to look back on milestones passed and ahead to new objectives. As 2003 comes to a close, it's good to be able to take stock of the achievements made by researchers at the RIKEN Center for Developmental Biology, the challenges we face in the years ahead, and the state of our rapidly evolving science. This year has seen the emergence of news and civic issues that have placed the fields of developmental biology, regeneration and regenerative medicine frequently in the spotlight and sometimes at the center of controversy on the world stage, a position accompanied by all the advantages and drawbacks that public prominence entails.

For us at the CDB, the year began in March with the unqualified (and uncontroversial) success of our first annual symposium on The Origin and Formation of Multicellular Systems, a three-day meeting of more than 200 distinguished speakers, poster presenters and attendees who gathered to discuss metazoan biology, from its single-celled forebears to its greatest evolutionary achievement in the formation of neural networks comprising billions of cells. We look forward to hosting more such meetings on a yearly basis, beginning with our next symposium, on Developmental Remodeling, in March of 2004, which will be held on the CDB campus in an auditorium designed to increase the Center's capacity for hosting scientific meetings and expand its ability to provide a venue for international exchange across fields.

Research advances from CDB labs have also provided a string of highlights this year. As you will see from the pages of this report, scientists at the CDB have made strides forward on many fronts, from uncovering new molecular mechanisms at work in the asymmetric division, signaling and guided migration of cells, to developing new technologies that may one day enable the targeted differentiation of embryonic stem cells toward clinically relevant ends. Studies in evolutionary development have also provided broadened contexts and perspectives, finding possible roots for the central nervous system in the genome of a tiny flatworm and challenging conventional wisdom about the regulatory origins of the vertebrate hindbrain.

Of course, this science did not take place in a vacuum. The central pillars of the Center's research mission — developmental biology, regeneration and the scientific bases for regenerative medicine — are all vibrantly dynamic, truly global fields of endeavor, and the CDB is pledged to remain fully engaged with international colleagues at the organizational, laboratory and institutional levels. In 2003, this commitment was characterized by the establishment of scientific exchange relationships with the University of Texas Graduate School of Biomedical Sciences and the Temasek Life Sciences Laboratory Limited in Singapore, paving the way for new and better opportunities to visit and host researchers from these institutions. We look forward to increasing the number and range of our inter-organizational exchange programs in the years ahead.



Domestically, Japan saw increased attention to a number of related issues and research fields. The national government unveiled plans for the Leading Projects, initiatives set up to fund mid-term research into a number of key areas identified as having the potential to improve public health, welfare and economic well-being. As the largest center for the study of developmental biology in Japan, the CDB received funding to carry out Leading Project research into potential applications for stem cells in regenerative medicine. Embryonic stem cells also featured in the national news this year, which saw the first establishment of a new line of human ES cells by a Japanese laboratory in Kyoto University. ES cells represent a biological resource of potentially revolutionary importance, but whose study demands careful consideration and ethical restraint. The use of human ES cells is for biomedical research is permitted in Japan, and in 2003 a number of CDB labs applied for permission to use the new Kyoto cell line. Approval of these research plans will allow scientists here to explore the secrets of cell differentiation and pluripotency, and perhaps one day to contribute to the development of cell-replacement therapies for intractable illnesses such as diabetes and Parkinson's disease.

As a basic research institute, the CDB also appreciates the fundamental importance of maintaining ties with the world of academia, and we have pursued active affiliations with some of Japan's top graduate school programs in the biosciences and regenerative medicine, allowing students from affiliated programs to conduct their academic research using the Center's facilities. This year, we were proud to formalize relationships with the Kyoto University Graduate School of Medicine, the Nara Advanced Institute of Science and Technology Graduate School of Biological Sciences, and the Kwansei Gakuin University Bioscience Department, bringing the total number of such affiliations to six.

Public communications and outreach also featured prominently on the Center's calendar this year. In May, the CDB opened its doors to the public for its first Open House, which was attended by more than 1,000 visitors who took advantage of the opportunity to take in talks on stem cells and programmed cell death, join in mock experiments, and sneak a peek at a day in the life of a developmental biology lab. The Center also joined in the public educational activities at the Kobe BioWeek event in August, a biotechnology convention that saw more than 8,000 attendees, including many who visited the CDB in a virtual sense via two-way video lectures and realistic software simulations of laboratory equipment.

In retrospect, the year has been a full one, and it has been gratifying to participate in the Center's activities both as a scientist and an administrator. So it is a great pleasure for me to introduce our 2003 Annual Report, which illustrates the range and depth of research at the CDB and serves both as a spark for happy memories and as a source of inspiration as we enter a new year, with its promise of new discoveries and change.

CDB RIKEN Kobe since 2000

2003 HIGHLIGHTS

March 24 -26

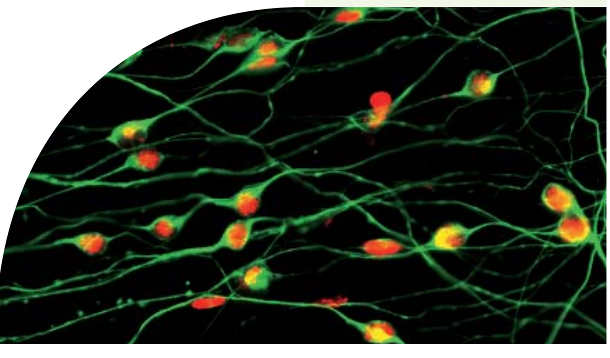
First CDB Symposium: Origin and Formation of Multicellular Systems

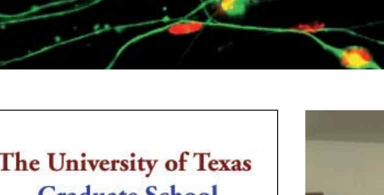
The CDB held its first annual symposium at the nearby Portopia Hotel. The meeting drew more than 200 speakers and attendees from around the world, with lectures, poster presentations and lively discussion on the origins of metazoan biology, the fundamental processes of development and regeneration and the formation of higher-order structures, such as neural networks.

April 1

New Graduate School Affiliations

The CDB formalized its affiliations with the Kyoto University Graduate School of Medicine and the NAIST Graduate School of Biological Sciences, allowing doctoral students from those schools to conduct their thesis research in CDB laboratories under the guidance of lab heads designated as visiting professors.





May 2

Scientific exchange agreement with University of Texas Graduate School of Biomedical Sciences

As part of its ongoing efforts to develop organizational ties with the international developmental biology research community, the CDB formalized an agreement for active scientific exchange with the University of Texas Graduate School of Biomedical Sciences at Houston. The agreement facilitates the hosting and exchange of research staff and the joint sponsorship of scientific meetings with one of the foremost academic centers for developmental biology research in the United States.

May 13

Publication of "Generation of neural crest-derived peripheral neurons and floor plate cells from mouse and primate embryonic stem cells"

In a study published in the May 13 issue of the *Proceedings of the National Academy of Sciences (PNAS)*, the Sasai research group demonstrated the application of stromal cell derived inducing activity (SDIA) in the high-efficiency induction of peripheral neurons from embryonic stem cells.

May 31

CDB Open House

The CDB opened its doors to the public for a one-day event featuring walk-through experiments, lab visits, talks on *C. elegans* and ES cell biology, quizzes, games and video and poster presentations.

June 24

Publication of "Origin and evolutionary process of the CNS elucidated by comparative genomics analysis of planarian ESTs"

On June 24, *PNAS* published this study by the Laboratory for Evolutionary Regeneration Biology providing evidence of genetic roots for the central nervous system in a primitive bilaterian, the flatworm *Dugesia japonica*.

July 2

Scientific exchange agreement with Temasek Life Sciences Laboratory Limited

The CDB established formal ties with the Temasek Life Sciences Laboratory in Singapore, a non-profit organization with affiliations to the National University of Singapore and Nanyang Technological University established to conduct molecular biology and genetics research.

August 19

Publication of "Traveling stripes on the skin of a mutant mouse"

The August 19 issue of *PNAS* featured a cover image taken from a research article from Shigeru Kondo's lab describing the mathematical properties of a strikingly patterned mutant mouse strain.

N Suzuki, M Hirata and S Kondo. Traveling Stripes on the Skin of a Mutant Mouse. Proc Natl Acad Sci U S A 100:9680-5 (2003). Copyright (2003) National Academy of Sciences, U.S.A.

August 27 - 9

Kobe BioWeek

The city of Kobe played host to more than 8,000 visitors participating in Kobe BioWeek, a series of meetings and events focusing on the growth and future directions of the biotechnology sector in the region and around Japan. The CDB joined in the public education activities, with two-way videoconference lectures and Q&A sessions, hands-on simulations of lab equipment and a virtual lab tour program using web cameras that allowed the public to watch CDB labs at work in real time.

October 1

RIKEN Becomes Independent Administrative Institution

As part of a restructuring of Japanese public corporations, RIKEN was officially re-designated as an independent administrative institution, a move intended to simultaneously increase the Institute's autonomy and accountability. As part of this transition, the Nobel Prize-winning chemist Ryoji Noyori was named the new president, ushering in a new era for RIKEN, which was first established in 1917.

October 20 -1

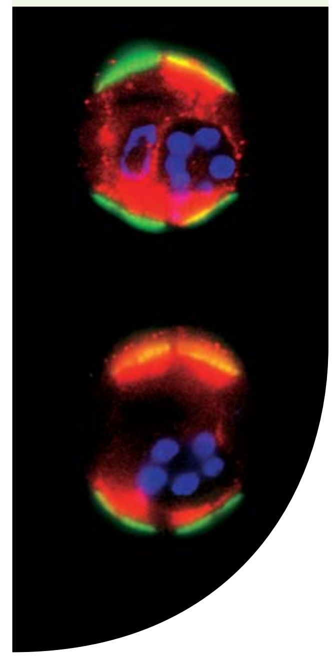
2nd CDB Retreat

The CDB held its second annual retreat on the island of Awaji, located a short distance from Kobe in Japan's Inland Sea. The retreat gave CDB research staff the chance to relax, mingle and exchange research findings and opinions in two days of oral presentation and poster sessions.

December 21

Publication of "An NDPase links ADAM protease glycosylation with organ morphogenesis in *C. elegans*"

In work published in the online edition of *Nature Cell Biology*, the Laboratory for Cell Migration, under team leader Kiyoji Nishiwaki, reported the role of a protein modification known as glycosylation in guiding the migration of gonadal cells in the nematode, *C. elegans*.



Core Program

The Core Program aims to promote highly innovative developmental and regeneration studies in a strategic and multi-disciplinary manner. This program constitutes the core research framework to achieve the aims of the Millennium Project, and focuses on the three main themes of the CDB:

- Mechanisms of Development
- Mechanisms of Regeneration
- Scientific Bases of Regenerative Medicine

The Core Program consists of seven research groups, each led by an eminent scientist. In addition to the group director, each group includes a number of research fellows and technical staff and in some cases a senior research fellow.

Zebrafish embryo PHOTO: Masahiko Hibi

Core Program

evolutionary regeneration biology

Kiyokazu Agata
Ph. D.

Kiyokazu Agata received his doctorate from Kyoto University in 1985 for his work on molecular cloning and gene expression of crystallin genes in chicken. From 1983 to 1991, he worked at the National Institute for Basic Biology, where he studied the molecular characteristics of dedifferentiated cells in transdifferentiation. He took an associate professorship at the Himeji Institute of Technology in 1991, and started his study of planarian regeneration with Kenji Watanabe. He remained at the Institute until 2000, when he left to assume a professorship at Okayama University. He joined the RIKEN Center for Developmental Biology as a group director in 2000.

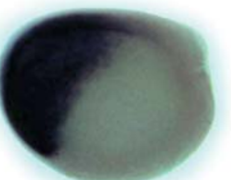
Its position on an important but scientifically underexplored branch of the evolutionary tree and its remarkable biology combine to make the planarian flatworm a fascinating and invaluable model for basic research in fields from evolutionary development to regenerative medicine. Kiyokazu Agata has adopted the freshwater planarian, *Dugesia japonica*, as a model species in his study of the origins and properties of stem cells, which are prevalent and experimentally accessible in these worms and which he believes hold the key to understanding the development, organization and maintenance of the cellular diversity that characterizes metazoan life.

Isolating planarian stem cells

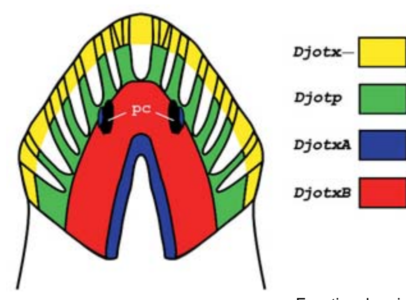
Agata's laboratory has developed a method for identifying subsets of planarian stem cells, also called neoblasts. These somatic stem cells are the only mitotically active cells in the planarian body, making them susceptible to X-ray irradiation. Examination of cell populations shown to be vulnerable to elimination by X-rays, revealed that planarian neoblasts, like stem cells in other species, seem to exist in proliferating and resting states. Real-time PCR analysis indicates that each of these sub-populations expresses discrete sets of genes. One fraction, X1, expresses genes specific to actively cycling cells, while a second fraction, X2, lacks this expression profile and is thought to comprise stem cells in a state of quiescence. Interestingly, many X1 cells also express signal receptor molecules which are switched off in the X2 fraction, while the corresponding ligands are expressed in a variety of differentiated cells resistant to X-ray irradiation.

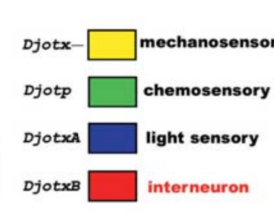
Brain regeneration by stages

A planarian can re-grow a fully functional brain within five days following the amputation of its entire head, an extraordinary feat of self-healing that involves recapitulating the development of the worm's entire nervous system. Researchers in the Agata group studied this process and identified patterns of gene expression that indicate the regeneration of the brain comprises five distinct stages. In the first stage, at about eight hours after wound closure, a *noggin*-like gene (*Djnlg*) is activated in the stump prior to the formation of a blastema, a mass of proliferating undifferentiated cells that serves as the frontline of regeneration. Soon thereafter, the brain-specifying gene *nou-darake* is switched on in the anterior fringe of the blastema, allowing the brain rudiment to be formed. These first two steps occur within 24 hours of decapitation, and set the stage for the third phase in which brain patterning is established by the expression of a set of three *otd/Otx*-related genes (relatives of which also function to pattern brain development in many other taxa, including vertebrates) followed by that of the planarian homolog of *Wnt* at about 48 hours into the regenerative pro-



Evolutionarily conserved expression patterns of planarian (right) and *Xenopus nou-darake*/FGFRL1 (left)





Functional regionalization of the planarian brain

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cess. In the fourth step, which occurs by day four of regeneration, a homolog of the axon-guidance gene *netrin* is triggered. At this stage the discrete components of the regenerating brain begin to form connections and higher-order organizations; chiasmata cross-linking eyes and brain develop, and connections are set up between the brain and the ventral nerve cords, which serve as a rudimentary peripheral nervous system. Thus, the basic components and connections of the planarian neural network are in place by the fourth day, but the functional recovery of the brain is not completed until a final stage in which two newly-identified genes are expressed is entered.

A parallel study revealed that these two genes, *1020HH* and *eye53*, are necessary for planarians to regain their normal light avoidance behavior, known as negative phototaxis. Knockdown of these genes by RNAi had no discernible effects on brain or eye morphology, but the worms failed to respond to light stimuli in the normal manner, even after five days of regeneration, when the structure of the brain has been fully reinstated.

Evolution of the central nervous system

The planarian is one of the lowest forms of animal known to possess a central nervous system, making it an apt model for the study of the evolution of this most complex and elaborately organized biological system. Using clones of over 3,000 expressed sequence tags (ESTs) isolated from the planarian head region, the team was able to identify 116 clones with significant similarities to genes closely linked to the nervous system in other species, including genes involved in neurotransmission, the neural network, brain morphogenesis and neural differentiation. These results point toward a shared evolutionary origin for many nervous system genes, indicating that the nervous system is likely to have arisen in a single common ancestor of bilaterian animals. Intriguingly, nearly 30% of the genes identified also have homologous sequences in yeast and the plant species, *Arabidopsis thaliensis*, both of which entirely lack neural development, which suggests that a significant number of genes that predate the advent of the nervous system have been co-opted to perform functions specific to neurobiology.

A planarian can re-grow a fully functional brain within five days following the amputation of its head

The Agata lab is also now participating in an international collaboration established to sequence and annotate a set of more than 10,000 planarian ESTs, which promises to provide an invaluable resource for the study of evolutionary biology, comparative genomics, and the genetics underlying the unique characteristics of these organisms. The availability of an annotated database of planarian cDNAs may provide keys to the understanding of stem cell biology, tissue plasticity and maintenance and other fundamentally important processes.

Expression of various neural-related genes

Publications

Hayasi S, Itho M, Agata K and Taira M. Conserved Expression Patterns of FGF Receptor-like 1/nou-darake in Xenopus and Planarian. *Dev Dyn* (2004).

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Core Program

vertebrate body plan

Shinichi Aizawa
Ph. D.

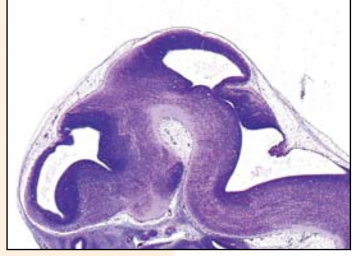
Shinichi Aizawa received his Ph. D. in Zoology from the Tokyo Kyoiku University, Department of Zoology in 1973. He spent the period from 1974 to 1979 as an investigator at the Tokyo Metropolitan Institute of Gerontology, then two years as a research fellow in the Laboratory of Genetic Pathology at the University of Washington (US). He returned to the Tokyo Metropolitan Institute of Gerontology in 1982, where he remained until 1986 when he moved to the RIKEN Tsukuba Life Science Center as a senior research associate. He was appointed professor in the Kumamoto University School of Medicine Department of Morphogenesis in 1994, and served in that position until 2002. Since 2000 he has served as CDB deputy director and group director of the Vertebrate Body Plan, as well as team leader of the Laboratory for Animal Resources and genetic Engineering. He also serves as a Managing Editor for the journal, *Mechanisms of Development.*

Despite their many differences, taxa as diverse as fish, amphibians, reptiles, birds and mammals share a common body plan comprising three regions: the trunk, the hindbrain/pharyngeal region, and the rostral head. Shin Aizawa is interested in the molecular bases for and phylogenetic origins of this regionalization, concentrating primarily on the genetic activity and molecular attributes of head development.

All animals develop from the head. The formation of inductive head organizer precedes that of the trunk organizer, as was first demonstrated by Spemann and Mangold in their studies of amphibian embryogenesis. Gene knockout studies in mice, have demonstrated that the rhombomere r1/2 is the ground state in the mammalian body plan. The head organizer induces the rostral head anteriorly to this rhombomere, while the trunk organizer guides trunk development caudally to the same r1/2 landmark. Studying mutations in genes responsible for body patterning in this region, the Aizawa research group hopes to reveal the genetic cascades for the constitution of the anterior head as conserved across vertebrate phyla. The group's research focuses on identifying and studying the functions of factors acting upstream and downstream of *Otx2*, a master gene in vertebrate head development, and the molecular bases for the processes of anterior-posterior axis formation, head induction, brain regionalization and cortical development.

Roles of *Otx2* in head development

Otx2 plays central roles in each step of head development in vertebrate, but the regulatory mechanisms by which this gene's activity is mediated have remained largely unknown. The development of the head traces back to before gastrulation, and is inseparably linked to the formation of the anterior-posterior axis. In mice, this process begins prior to gastrulation when the cells of the distal ventral endoderm migrate to the region that will become the animal's anterior, forming the anterior visceral endoderm (AVE), which suppresses posteriorizing signals in the adjacent epiblast. In previous work, members of the Aizawa lab demonstrated that *Otx2* plays an essential role in triggering this anterior migration. Following gastrulation, organizing centers in the epiblast induce the formation of the anterior neuroectoderm, which subsequently regionalizes into multiple primitive structures destined to form the areas of the brain. In this process, the isthmus and anterior neural ridge act as local organizing centers for mid- and forebrain development.



Fore- and midbrain structure at E12.5 in wildtype (left) and loss of archicortex and diencephalon in Emx2/Otx2 double mutants (right)

Otx2 functions as a master gene in each phase of head ontogeny. Aizawa and colleagues have been working to identify and characterize regulatory factors that control the gene's expression in

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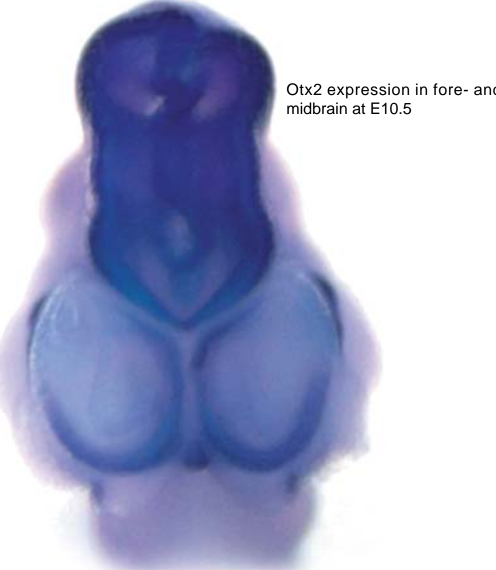
specific sites and stages of development. In the past, the lab found enhancers responsible for promoting *Otx2* expression in the visceral endoderm, definitive anterior mesendoderm and the cephalic neural crest cells. These cis-regulatory elements were all located relatively near the transcriptional start site for the gene. More recently, the group has identified and analyzed enhancers that guide *Otx2* expression in epiblast, anterior neuroectoderm and fore- and midbrain, which they have named the EP, AN and FM enhancers, respectively. All of these elements are located more remotely from the coding region (more than 80 kb upstream) than are the previously identified *Otx2* enhancers.

The development of the head is inseparably linked to the formation of the anterior-posterior axis

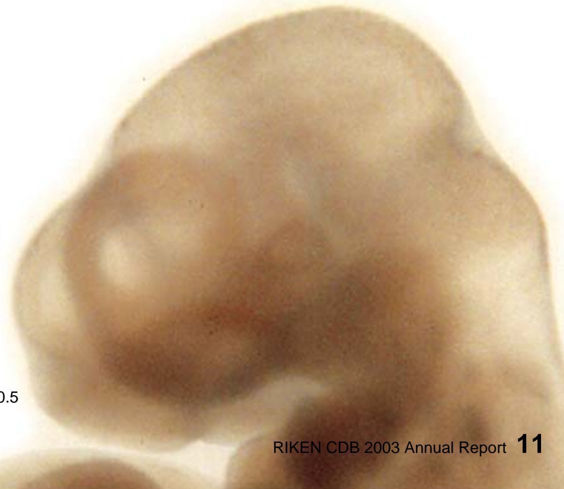
The activity of the AN enhancer is independent of that of the EP enhancer, and plays an essential role in maintaining the anterior neuroectoderm through *Otx2* expression, once that region has been induced. The Aizawa group's studies have also indicated a phylogenetic relationship between these elements, in which FM is the most deeply conserved in the gnathostome (jawed vertebrate) lineage, while the epiblast enhancer appears to have been acquired later, perhaps after the ascent of amphibians, and to include the anterior neuroectoderm enhancer as an essential component. Using these results as a springboard, Aizawa and colleagues next plan to investigate the significance of this phylogenetic specialization of enhancers in terms of the evolution of the vertebrate head, and to continue the search for upstream factors at work in the regulation of *Otx2*.

Emx genes in early cortical development

In the mouse, the development of the cerebrum is immediately preceded by the closure of the anterior neural plate at around E8.5 in the presumptive forebrain-midbrain junction. In the earliest stages of corticogenesis, the structures of the archipallium, the non-neuronal components of the choroid plexus, the hippocampal complex and the fimbria, are generated. It has been suggested that the last of these structures, the fimbria, which are located at the border of the cerebral cortex and the choroid plexus, function as a local signaling center in cortical development. One model of archipallial patterning involves the expression of ligands, receptors, transcriptional factors and inhibitor molecules to form morphogenetic gradients that direct the differentiation of areas on either side of the cortical hem, but the actions of specific players in this model remain to be worked out. The Aizawa group's studies of *Emx1* and *Emx2*, mouse homologs of the *Drosophila* head gap gene *ems*, have shown that these two genes cooperate in two phases of cortical development. Previous work demonstrated that *Emx1* and *Emx2* work together to generate Cajal-Retzius cells and subplate neurons. Recent work now indicates that the two genes also play a combinatory role in establishing the archipallium as distinct from the roof plate, immediately following the closure of the neural tube.



Otx2 expression in fore- and midbrain at E10.5



Forebrain/midbrain at E10.5

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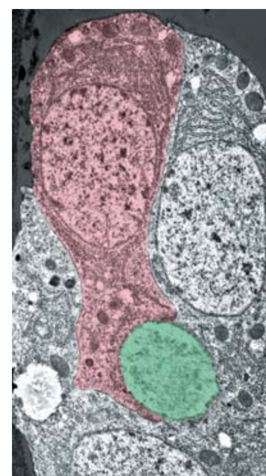
Core Program

morphogenetic signaling

Shigeo Hayashi
Ph. D.

Shigeo Hayashi received his B. Sc. in Biology from Kyoto University in 1982, and his Ph. D. in Biophysics from the same institution in 1987 for his work on lens-specific regulation of the chicken delta crystallin gene. Inspired by the discovery of the homeobox, he changed his research focus to the developmental genetics of *Drosophila* and spent three years as a postdoctoral research associate in Matthew Scott's lab at the University of Colorado before returning to Japan to work in the National Institute of Genetics. He became an associate professor at the same Institute in 1994, and professor in 1999. Also in 1999, he received the incentive prize of the Genetics Society of Japan for Genetic Studies on *Drosophila* Development. He was named group director of the Morphogenetic Signaling research group at the RIKEN CDB in May 2000.



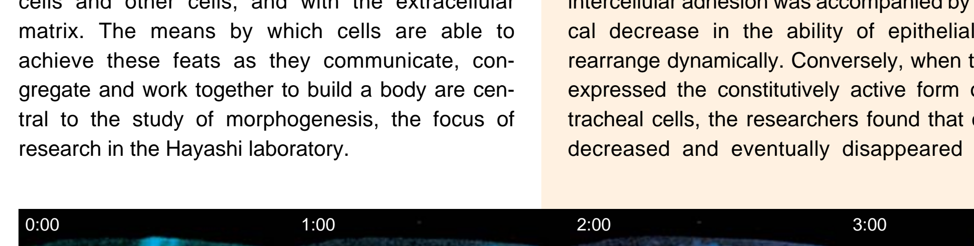
Transmission electron micrograph of dorsal trunk of the trachea, which consists of multiple epithelial cells joined tightly by adherens junctions. One of the tracheal cells is colored red and the tracheal lumen is colored green.

Epithelial tissues are constructed from sheets of cells with distinct apical-basal (top-bottom) polarities, which form junctions with each other and interact with the extracellular matrix, the macromolecular environment in the spaces between cells. A range of vital developmental processes, including the abilities to build complex structures through selective cell adhesion, to move through the body, and to form epithelial tissues rely on interactions between cells and other cells, and with the extracellular matrix. The means by which cells are able to achieve these feats as they communicate, congregate and work together to build a body are central to the study of morphogenesis, the focus of research in the Hayashi laboratory.

The regulation of epithelial adhesion

The ability of epithelial cells to attach to and disengage from their neighbors is closely linked to the activity of cadherin family molecules, which span the cell membrane to regulate this dynamic cell-cell adhesion. A number of studies have also indicated roles for the small GTPase Rac as another key regulator of cell adhesion, but these roles have yet to be confirmed in the context of embryonic development. In work published in 2003, members of the Hayashi research group reported the results of their investigations into Rac's role in epithelial cell rearrangement in the development of the *Drosophila* tracheal system, a tubular network formed from epithelium.

The group found that reductions in Rac activity led to increases in the amount and range of cadherin cell adhesion molecules in both embryonic epidermis and tracheal epithelia, which they attribute to a post-transcriptional mechanism and changes in epithelial structural organization. This strengthening of intercellular adhesion was accompanied by a reciprocal decrease in the ability of epithelial cells to rearrange dynamically. Conversely, when they overexpressed the constitutively active form of Rac in tracheal cells, the researchers found that cadherins decreased and eventually disappeared from cell



junction regions, resulting in the loss of tracheal cell adhesion and the detachment of these cells from the epithelium. These complementary findings strongly suggest that a strictly maintained balance of Rac activity is essential in determining the proper junctional assembly and disassembly of cadherins in the *Drosophila* tracheal epithelium, a role it is thought to fulfill by regulating cell adhesion and motility to ensure the appropriate remodeling of epithelial sheets into tubules.

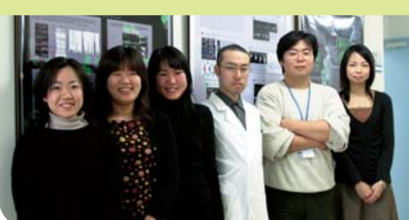
Hayashi seeks to develop an improved understanding of the genetic control of epithelial morphogenesis

A number of other signaling molecules that function in the tracheal system are known to cause gain-of-function phenotypes when overexpressed. In work related to the Rac study, the Hayashi lab has conducted a gain-of-function screen for genes that disrupt the tracheal epithelium on overexpression. The lab has identified several candidate genes whose hyperactivation leads to disruptions in epithelial integrity, including genes encoding regulators of actin dynamics, cell adhesion and cell motility. The functions of the individual candidate genes are now being analyzed, with the goal of developing an improved understanding of the genetic control of epithelial morphogenesis.

Leg and wing specification

The insect wing is an evolutionary innovation that is thought to have arisen as a derivative of the leg during an early stage in the evolution of the insect body plan. The close relationship between the wing and the leg can be observed in the embryogenesis of *Drosophila*, which is a highly derived winged insect. It has been shown that wing and leg both derive from a common precursive structure called the limb primordium. This structure gives rise to both the limb and wing imaginal discs, pouches of undifferentiated cells in the larva that form the leg and wing in the adult.

The Hayashi lab has investigated the role of *Wingless* (*Wg*) signaling in the wing and leg primordia. The Wg protein, which mediates a wide range of inductive interactions in *Drosophila*, is known to be required for the induction of imaginal discs during embryonic development. In the leg imaginal disc, Wg serves as an axis determinant, triggering dorsal-ventral pattern formation by specifying the ventral region of the leg, and by activating the *Distal-less* (*Dll*) gene in the distal region. However, *Wingless's* roles in leg development have not been fully explored.



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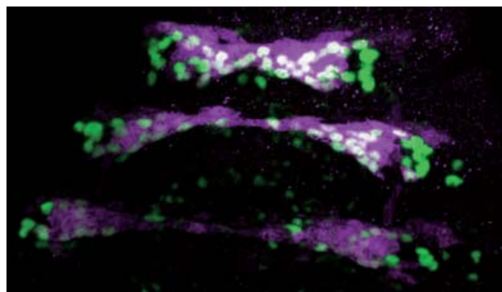
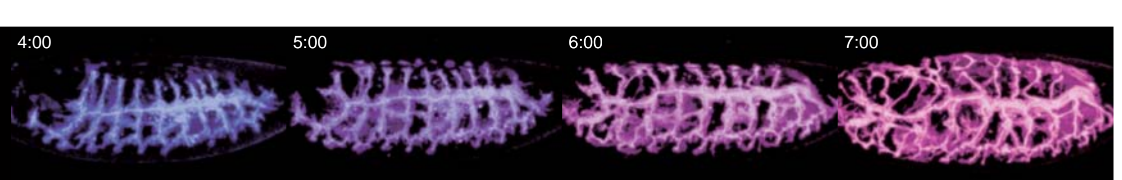
In a study published this year, the Hayashi group determined that Wg signaling is required for proximal-distal patterning during embryonic leg development, a role it performs by helping to recruit cell types specific to the proximal and distal



Ventral view of the thoracic region of a *Drosophila* embryo. Stripes of Wingless (purple) expression overlap the leg primordia (green) and specify the proximal leg identity.

domains. This finding counters previous work that suggested that Wg plays no role in late embryonic leg disc development, and led Hayashi to propose a new model for leg specification in which dorsal-proximal, ventral-proximal and distal cells are distinguished by differences in the expression domains of specific genes, including *Wg*. The group also found that ectopic Wg signaling inhibits wing disc development; ectopic activation of the Wg signal in the wing disc causes a reduction in the number of wing cells that develop. These results indicate that Wg determines leg cell specification while inhibiting that of the wing, thereby playing the role of a crucial binary switch in the establishment of wing and leg identities.



Time course of tracheal development. Time lapse pictures of an embryo expressing GFP in the tracheal system.

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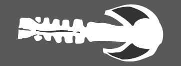


Core Program

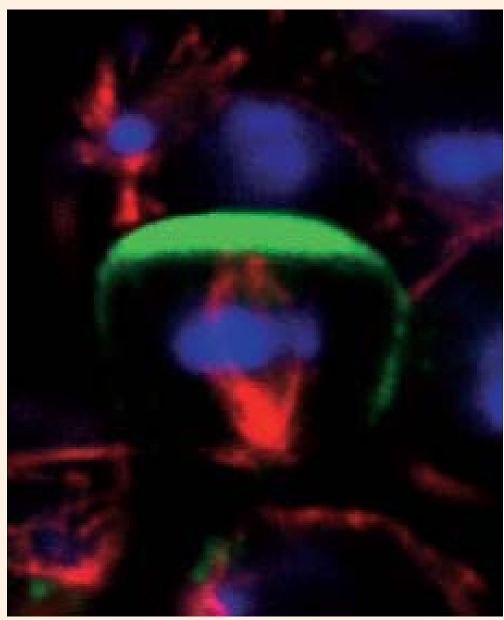
cell asymmetry

Fumio Matsuzaki
Ph. D.

Fumio Matsuzaki received his B. Sc. from the Department of Biochemistry and Biophysics at the University of Tokyo in 1979, and his doctorate from the same institution in 1984, for his work on the characterization of the erythrocyte cytoskeletal structure. He spent the period from 1984 to 1988 as a postdoctoral fellow, first in the Department of Cell Biology at the Tokyo Metropolitan Institute of Medical Science, then in the laboratory of Gerard Edelman at Rockefeller University. He returned to Japan in 1988 to take a position as a section chief in the Department of Molecular Genetics in the National Institute of Neuroscience. In 1998, he was appointed professor in the Institute of Development, Aging and Cancer at Tohoku University and remained there until taking his current position as group director at the RIKEN CDB.

Intracellular asymmetry

All the cells in the body originate from a single progenitor, and nearly all share an identical DNA code. How then do the vast numbers of distinct cell types in complex organisms differentiate from one another? The process of asymmetric division, in which regulatory factors in a single mother cell are distributed unequally to daughter cells produced by cell division, is a key to answering that fundamental question. This asymmetric distribution of regulatory factors allows resultant cells to assume different roles from each other in a process called fate determination. This process relies on the establishment of intracellular gradients of factors regulating the expression of genes, which results in differential patterns of gene expression in daughter cells when the cell undergoes mitosis. As it is the expression of genes, and not their simple presence or absence, which is primarily responsible for determining cell identity, controlled asymmetric division is critical to ensuring the development of appropriate numbers and types of daughter cells, and so, to the development of the organism as a whole.



In *Drosophila*, dividing neuroblasts localize the Miranda (green) / Prospero complex to be segregated into the daughter GMC.

Neural cell fate determination in *Drosophila*

Fumio Matsuzaki has dedicated his research to explicating the role of asymmetric division in determining cell identity, using the *Drosophila melanogaster* nervous system as a model. This system provides an attractive research platform for studying the development of cell diversity, as the nervous system features more cell types than any other organ system, and the fruit fly is highly amenable to genetic manipulation. With the advent of techniques for molecular analysis, Matsuzaki and others demonstrated that the fates of daughter neural cells are in large part determined by unequal distribution of factors within the mother cell during the process of cell division. Two factors in particular, known as Prospero and Numb, have been shown to localize to the basal side of the neuroblast (the neural progenitor cell) prior to its division. This polar distribution results in two daughter cells of distinct gene expression — a new neuroblast, and a smaller-sized ganglion mother cell (GMC) — in which Prospero and Numb are segregated in the GMC. In this process of asymmetric division, the neuroblast remains undiffer-

entiated and retains its multipotency, while the GMC becomes committed to generating neurons and glial cells. Further studies in Matsuzaki lab showed that Prospero is tethered to the basal cortex of the neuroblast by the molecule Miranda, and subsequently released into the newly-formed GMC. Mechanisms that polarize cells — underlying the asymmetric distribution of those molecules — have further been shown to have homologous counterparts in vertebrates, providing strong evidence for the evolutionary conservation of intrinsic signaling in the process of asymmetric cell division.

Coordinating asymmetric division

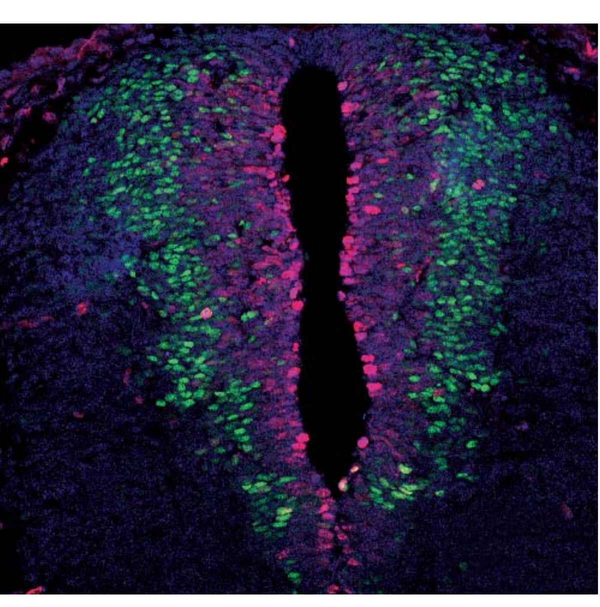
The basal localization of determinants is by itself not sufficient for the parental neuroblast to segregate cell fate determinants to the daughter GMC. It is also necessary for the division to occur along the axis of polar distribution of the Miranda-Prospero complex. This is achieved by the coordinated alignment of the mitotic spindle along the apicobasal axis. In the neuroblast, both the asymmetric localization of cell fate determinants and spindle orientation are governed by a multi-protein complex that

The fates of daughter neural cells are in large part determined by unequal distribution of factors within the mother cell during the process of cell division

creates intracellular asymmetry. This protein complex, named the "apical complex" for the site of its localization in the neuroblast, includes two different signaling complexes: atypical protein kinase C (aPKC) and heterotrimeric G protein. The Matsuzaki lab recently demonstrated that the two signaling pathways included in the apical complex play differential roles in the asymmetric segregation of the determinants. aPKC signaling is essential for the localization of the basal Miranda-Prospero complex, while the Pins molecule, known to be associated with an alpha subunit of the G protein in the apical complex, plays a critical role in orienting the mitotic spindle. Thus multiple intracellular signals operate coordinately and differentially to create intracellular asymmetry, and promote the asymmetric division of neuroblasts. The Matsuzaki lab is now confronting questions regarding the roles of extrinsic signals and intrinsic players, and the mechanism by which cells of different sizes are generated in the process of mitotic division.

Asymmetric cell sizes

This last question prompted investigations that led to the identification of a new regulatory function for G proteins, in *Drosophila* neuroblast cell division, a process in which the resultant neuroblast is much larger than the GMC. During mitotic cell division, chromosomes replicate within the mother cell and attach to spindles that draw them in opposite directions to ensure that a full complement of chromosomes is available to each daughter cell. The chromatids are drawn to the spindle poles through the action of molecular motors known as microtubules. In work carried out at the CDB, Matsuzaki's lab has found that Gβ protein signals are distributed unequally in neuroblasts and seem to restrict the development of microtubules, resulting in a shortening of the mitotic spindle on one side of the cell. These unequal spindle lengths cause the neuroblast to divide at a cleavage site that is off-center, and the sizes of the daughter cells reflect this imbalance, with the daughter neuroblast being more than twice the size of the GMC. Cells lacking the Gβ protein in question form a symmetrical mitotic spindle and produce daughter cells of equal size with the determinants being normally segregated. This provides a valuable platform for further studies in fields ranging from microtubule dynamics to animal morphogenesis.



In the developing mouse spinal cord, Prospero (green) is transiently expressed in newborn neurons immediately after birth from the mitotic neural progenitors (red).

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Publications

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Core Program

stem cell biology

Shin-ichi Nishikawa
M. D. , Ph. D.

Shin-ichi Nishikawa received his M. D. from the Kyoto University School of Medicine in 1973. He performed his internship and residency in the Department of Internal Medicine at the Kyoto University Chest Disease Research Institute, before taking an assistant professorship in the same department in 1979. He spent the period from 1980-82 at the University of Cologne Institute for Genetics (Germany) before returning to the Chest Disease Research Institute, where he was appointed associate professor in the Department of Microbiology in 1983. He moved to the Kumamoto University Medical School in 1987 to take a professorship in the Department of Immunology, and returned to Kyoto in 1993, as professor in the Department of Molecular Genetics at the Kyoto Graduate School of Medicine. He was appointed CDB group director in 2000.

Stem cell biology

Stem cells of various types, characterized by their abilities to self-renew and to generate more highly specialized cells indefinitely, are the key to replenishing the body's cells. The study of these cells stands at the heart of some of the central questions in development, but many aspects of stem cell biology, such as the limits (if any) of the ability to reprogram them to produce progeny cells of diverse lineages, and the identities of the intrinsic and extrinsic molecular factors that regulate a cell's decisions whether to maintain stemness or to commit to differentiation have yet to be resolved.

A stem cell's location and immediate environment can be important factors in defining its role. Recent findings that stem cells may arise from one tissue type and then later be directed to give rise to another strongly substantiate the model in which a stem cell's behavior and properties are influenced by external signals. By studying the microenvironments, known as 'niches,' in which stem cells originate, abide and proliferate, the Nishikawa research group seeks to gain insight into the identity and function of molecules involved in stem cell activity, and the potential of stem cell-based therapeutic applications.

Characterizing the stem cell niche

Working to isolate the defining characteristics of specific stem cell niches, the Nishikawa group has chosen melanocyte stem cells as one of the main model systems for their research, as melanocytes are readily identified by the presence of a melanocyte-specific promoter, *Dct*, and are amenable to experimental manipulations that allow specific cell types to be isolated from mixed populations. As the existence of niche cells and the identification of their characteristics can only be determined by first isolating the stem cells they support, Nishikawa's lab has developed an approach in which stem cells are dissected from their surroundings, dissociated and used to construct single cell libraries, a process involving the technically challenging initial step of isolating single intact hair follicles from the skin. Targeted GFP expression is next used to label the follicular melanocytes, making it possible to distinguish them from other cells. These cells are used to construct cDNA libraries of (GFP-positive) melanocytes from the bulge and matrix regions of

Melanocytes (marked red) found in each hair follicle of the mouse form a stem cell system within these minute organs. In this system, the most immature stem cell compartment is located in the upper region and the actively cycling compartment is located in the bottom of the follicle.

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the follicle, as well as all of the GFP-negative cells from the bulge and the subset of GFP-negative cells found specifically to adhere to bulge melanocytes.

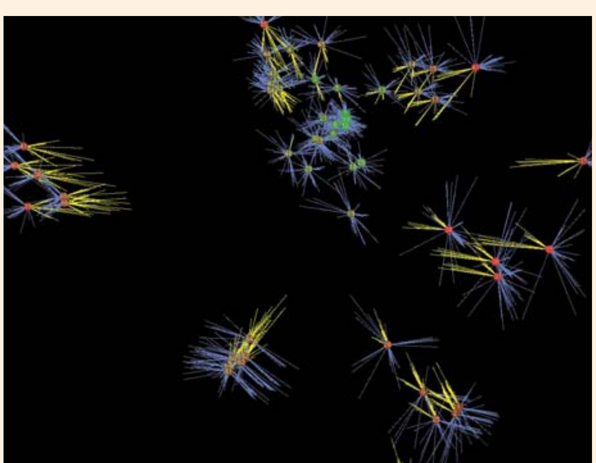
These follicular regions were selected based on the results of a previous study that showed that the bulge and matrix are the two main sites of localization of melanocyte stem cells, making them excellent sites for studies of niche properties. Each of these areas hosts a specific sub-population of stem cells. The matrix niche, the site of active hair growth and pigmentation, provides an environment in which amplifying progeny become committed to a stem cell fate, while the bulge serves as a reservoir for stem cells in their resting, or quiescent, state. Subtractive comparisons of cDNAs from stem cell, differentiated cell and 'niche-like' (keratinocyte) libraries revealed approximately 200 non-redundant genes, providing the lab with targets for further experiments in which the individual genes will be conditionally knocked out and the resulting phenotypes examined and analyzed. Other related studies, for which single cell libraries of other resting stem cells are now being constructed, will entail profiling gene expression in melanocytes and resting stem cells, which promises to provide a new resource for scientists investigating stem cells' genetic signatures.

Profiling gene expression in melanocytes and resting stem cells promises to provide a new resource for scientists investigating stem cells' genetic signatures

Differentiation of mesoderm and endoderm

In another ongoing project, members of the Nishikawa group are developing software to allow for improved DNA chip analyses and using it to build a database of the progressive intermediate states that endothelial cells pass through in the journey from ES (embryonic stem) to terminally differentiated cell. A number of mesoderm and endoderm cell lineages and their intermediates are being used, as these are available in pure cultures and their in vitro differentiation can be steered by the addition of the appropriate factors.

The mesendodermal lineage, which has been clearly defined at cell level by the Nishikawa group, includes a number of important cell types, including endothelial cells, the primary constituent cells in many important organs including the lungs, liver and pancreas. Mesendodermal differentiation is characterized by the activity of specific marker genes whose expression is switched on or off, depending on the pathway a cell has followed and the stage it has reached. Analysis of the stages in this stepwise process has also made it possible to develop a system for guiding differentiation from ES cell to endodermal, mesenchymal or mesodermal (paraxial and lateral) fates in vitro using combinations of extrinsic factors. By identifying the characteristic markers of cells at each step on each branch of the lineage tree and collecting samples to produce DNA arrays, the Nishikawa lab is working to build a database of cDNA profiles of the intermediate stages in ES cell differentiation in culture, which can be queried and visualized using software developed in the lab. Analyses of this cDNA chip database promise to lead to a better understanding of the process of endothelial differentiation, and of stem cell specification in general. This powerful new DNA analysis software application, which promises to make a great contribute to the harmonization of multiple DNA array databases, is being developed in collaboration with the Institute of Biomedical Research and Innovation, located adjacent to the CDB in the Kobe Biomedical Industry Project park.



Representation of the relationships between different cell types based on the expression of approximately 700 probe sets

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Nishikawa SI, Honda K, Vieira P and Yoshida H. Organogenesis of Peripheral Lymphoid Organs. *Immunol Rev* 195:72-80 (2003).

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Core Program

organogenesis and neurogenesis

Yoshiki Sasai
M. D. , Ph. D.

Yoshiki Sasai gained his M. D. from the Kyoto University School of Medicine in 1986, subsequently performing internships in general practice and emergency medicine. He received his Ph. D. from the same institution in 1992, for work on neural specific transcriptional regulators. In 1993, he took a postdoctoral fellowship in the De Robertis lab at the UCLA School of Medicine, remaining there until 1996 when he was appointed associate professor at the Kyoto University School of Medicine. He assumed a professorship at the Kyoto University Institute for Frontier Medical Sciences in 1998, and was appointed group director at the CDB in 2000. He serves on the editorial boards of Neuron, Development, Genesis and Developmental Dynamics.

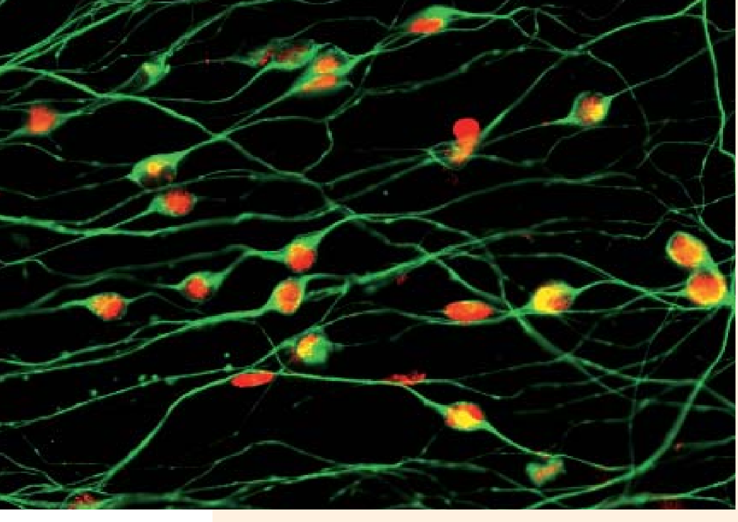
Induction and patterning of the nervous system

The interactions between cells from different germ layers and their distributions along body axes are established in early embryogenesis and serve to determine the spatial disposition and normal development of tissues and organs. The specification of the dorsal-ventral (back-belly) axis is significant in neural development in that the central nervous system forms on the dorsal side of the body in all vertebrate orders. This process is dictated by the effects of a number of signaling factors that diffuse from organizing centers and direct dorsal ectoderm to maintain a neural fate. These molecules, which include Noggin, Chordin, Follistatin and their homologs, participate in elaborate signaling networks in which factors collaborate and compete to initiate the embryonic nervous system.

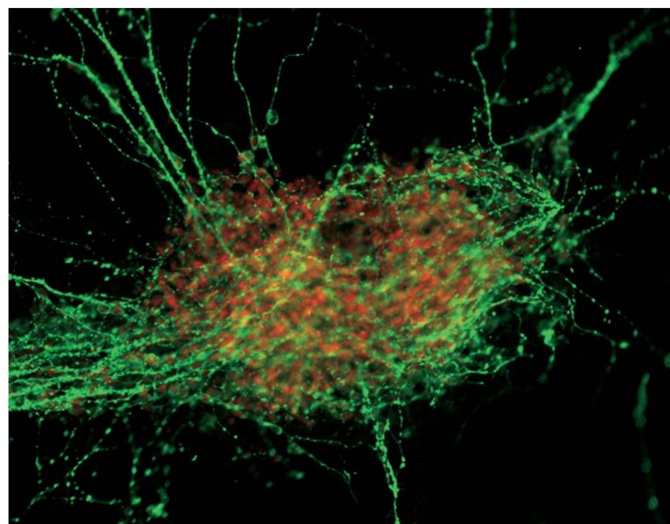
Beginning with the identification of the neural inducing factor Chordin in the early 1990s, Yoshiki Sasai's research has focused on explicating the molecular signaling mechanisms that enable the early embryo to organize itself into a complex, mature organism. Using the African clawed frog, *Xenopus laevis*, as a model in molecular embryological studies, Sasai and his group are engaged in clarifying the structure and extent of the signaling networks involved in setting up the dorsal-ventral axis and determining neural fate in the ectoderm, work which led to the recent identification of *Tiarin*, a novel dorsalization factor. The group is now also actively developing effective methods of inducing neuralization in mammals, work which has potential for application in the treatment of neurodegenerative disorders, such as Parkinson's disease.

Inducing neural differentiation in mammals

The Sasai lab previously reported the development of a technique for inducing the differentiation of neural cells by culturing mouse ES cells on plates of connective cells, called stromal cells. These cells have a strong neuralizing effect, termed SDIA (for Stromal cell-Derived Inducing Activity), inducing



Primate sensory neurons



Primate motoneurons

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nerve cells and precursors at high rates of efficiency. In 2003, the lab built on that previous work, setting out to address the question of whether the neural differentiation of ES cells could be guided with even greater precision and diversity by adding patterning factors — soluble molecules that work to determine the identities of cells in a developmental region — to the SDIA-cultured cells.

Although BMP signals have an inhibitory effect on neural development in earlier embryonic stages, they work to promote the neural crest at a later stage in development

Using BMP4, Sasai's group succeeded in causing the SDIA-treated ES cells to differentiate into cell types that normally derive from the neural crest, a developmental region that gives rise to nerve cells in the peripheral nervous system, including sensory, autonomic and enteric (gut) neurons. Although BMP signals have an inhibitory effect on neural development in earlier embryonic stages, the same signals work to promote the neural crest at a later stage in development. The group found that they were able to induce peripheral neural types that are naturally derived from the neural crest, including autonomic and sensory neurons and smooth muscle cells, when they treated the SDIA-treated cells with median concentrations of BMP4. A lower concentration prompts the same cells to differentiate into dorsal central nervous system neurons.

The neural crest arises from the dorsalmost region of the developing nervous system, and BMP4 is regarded as a 'dorsalizing' factor. Other factors have an antagonistic effect to BMP4, and cause the cells they act upon to take on a ventral character. Peripheral neurons are generally dorsal in their origins, while much of the central nervous system derives from more ventral developmental regions. Normal development involves the constant, dynamic interplay between patterning factors that steer neural precursors toward either a ventral or dorsal fate.

Sasai's group also investigated the effects of Shh (Sonic hedgehog), a factor that suppresses dorsal and promotes ventral development in vivo. As with BMP4, Shh provokes concentration-dependent differentiation in SDIA-cultured cells, analogous to its function in the body. A low concentration guides SDIA-derived neural precursors to develop into motor neurons, while higher concentrations bring about differentiation into neural types that in normal development would derive from the floor plate, the ventralmost region of neural development. Tests revealed that these SDIA and Shh-treated neurons display both the molecular markers and physiological attributes (such as directional axon guidance) characteristic of the same types of neurons in vivo, demonstrating that the cells produced by this method are structurally and functionally similar to natural floor plate cells.

Taken together, these findings show that SDIA-generated neural precursors respond to patterning factors with a versatility similar to that of their in vivo counterparts. Higher concentrations of the dorsalizing factor BMP4 induce increasingly dorsal neural types, while greater doses of Shh have the same effect in ventralizing neural precursors. This work represents the first efficient generation of such a broad spectrum of neuronal types from ES cells in culture, and the fact that the process has been demonstrated effective not only in mouse, but also in primate cells, marks a significant step toward the ability to induce specific types of neuron from ES cell-derived precursors that is one of the foremost challenges confronting researchers in regenerative medicine.

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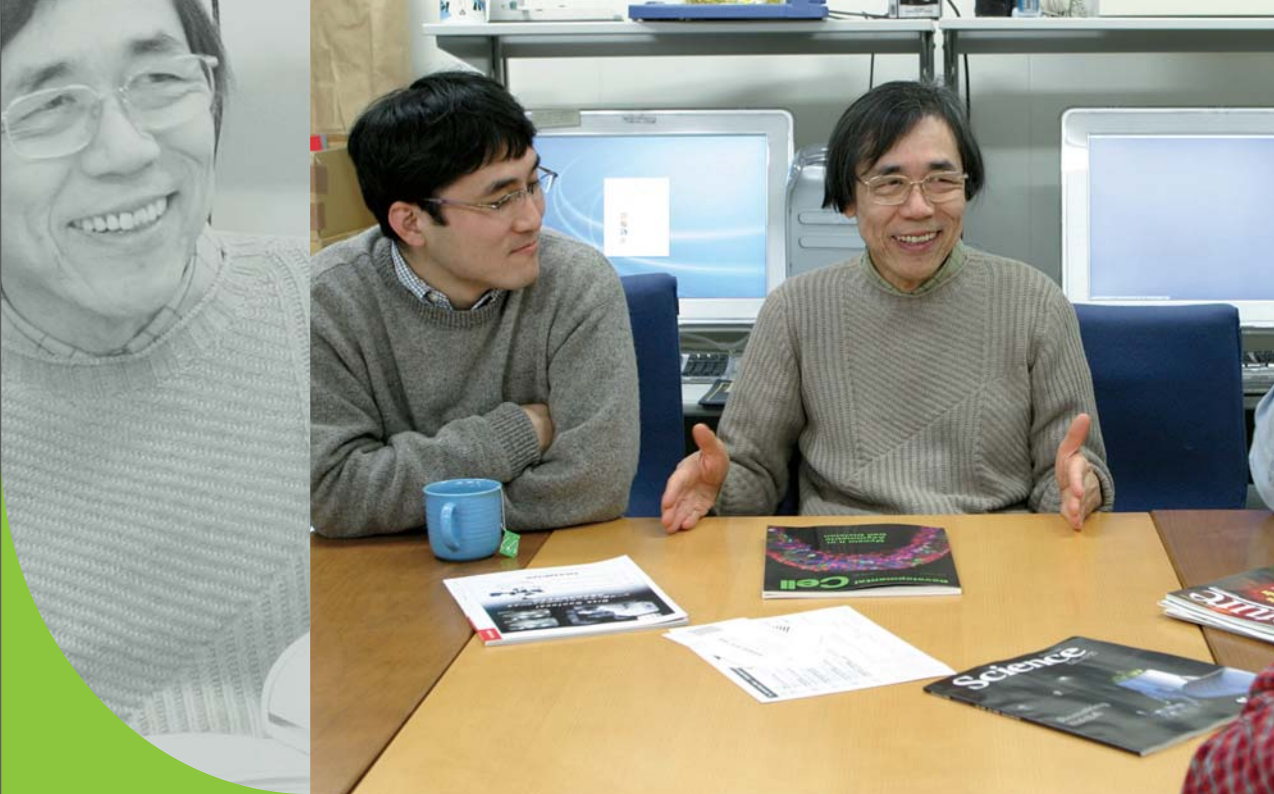


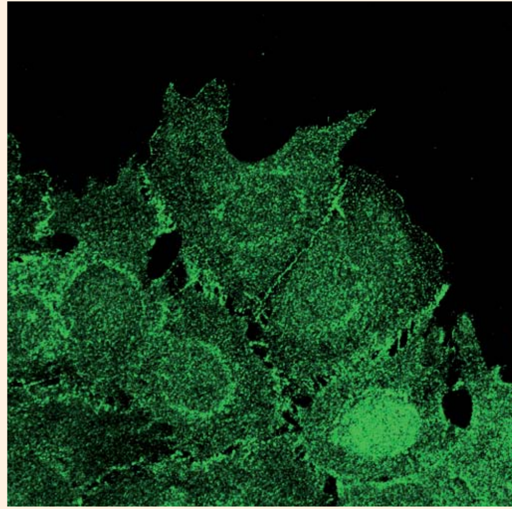
Core Program

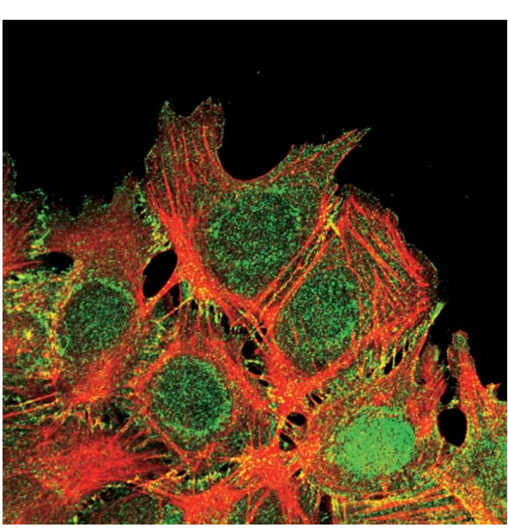
cell adhesion and tissue patterning

Masatoshi Takeichi
Ph. D.

Masatoshi Takeichi is director of the RIKEN Kobe Institute and the Center for Developmental Biology, as well as the Cell Adhesion and Tissue Patterning research group at the same institute. He completed the B. Sc. and M. S. programs in biology at Nagoya University before receiving a doctorate in biophysics from Kyoto University in 1973. After attaining his Ph. D., he took a research fellowship at the Carnegie Institution Department of Embryology under Richard Pagano. He then returned to Kyoto University, attaining a full professorship in the Department of Biophysics (1986-1999), before becoming professor in the Department of Cell and Developmental Biology in the Graduate School of Biostudies at the same university. He assumed his current positions at the CDB in 2000.

The development of multicellular systems and organisms depends on the ability of cells to congregate and cooperate in diverse ways. Individual cells must be able to identify each other and distinguish between diverse cell types. They need to be able to form bonds discriminatively, such that cells that must aggregate in order to form a tissue do so, while cells with different missions remain detached from one another. These functions of cell recognition and bonding are achieved by several types of molecules known as CAMs (for cellular adhesion molecules), which variously bind with partner molecules present on the surfaces of substrates, matrices and other cells. Since the discovery of cadherins, the role of the cadherin superfamily of molecules in regulating cell-cell adhesion has been studied extensively. These molecules form complexes with other intracellular factors to create bonds spanning membranes and intercellular spaces to join cells with other cells expressing similar cadherins. Cadherin bonds are dynamically stable — although the intercellular junctions they form persist in terms of the whole cell, the individual bonds are regulable and can respond to changes in cell state. Masatoshi Takeichi has been engaged in exploring the structure, function and biological implications of the cadherin superfamily since his discovery of the first cadherin molecules more than twenty years ago.





Fat1 cadherin (green) localizes at cell-cell contact sites (left), colocalizing with F-actin (red/yellow, right) in epithelial cells.

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Characterizing Fat1 function

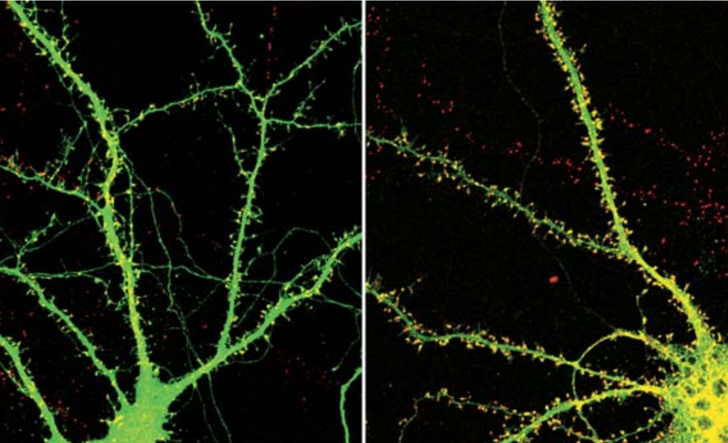
Cadherins do not act alone. Spanning both sides of the cell membrane, cadherin molecules bind with other cadherins of like type in the space between cells, while in the cytoplasm they form anchorage complexes with other proteins, such as various members of the catenin family, which in turn bind with molecules present within the cell and participate in important cell signaling networks. In 2003, the Takeichi lab investigated the function of a mammalian cadherin known as Fat1, which is found in the peripheries of cells. The group found that Fat1 acts to regulate the function of actin, a primary component of the cytoskeleton that functions in the control of cellular motility and changes in cell morphology. Among members of the cadherin family, Fat cadherins are distinguished by their unusually large number of extracellular repeat domains — nearly seven times the number found in classic cadherins — and by the uniqueness of the primary sequences of their cytoplasmic domains. Three Fat cadherins have been identified in mammals — Fat1, 2 and 3. It has been shown that the *Drosophila* homolog of these molecules plays roles in cell proliferation or in the determination of planar polarity, but the function of the vertebrate Fats has remained unknown.

Using RNA interference (RNAi) to knock down Fat1 function, researchers in the Laboratory for Cell Adhesion and Tissue Patterning discovered that Fat1 is required for the stable maintenance of tight associations between cells and the normal organization of actin. This inhibition of Fat1 also suppressed the formation of cell polarity, which is a general phenomenon induced in epithelial cell sheets during wound healing. Molecular studies uncovered an apparent partnership between Fat1 and Ena/VASP (vasodilator stimulated phosphoprotein) proteins; Fat1 colocalizes and binds with Ena/VASP proteins in filopodia, lamellipodia and cell junctions and the group's recent studies indicate that the Ena/VASP proteins may act as links in the molecular regulatory chain leading from Fat1 to actin, although the presence of other linkages was also suggested. The study's findings also suggest that Fat1 co-acts with the classic cadherins to reinforce or maintain cell-cell junctions, which would make this molecule a significant player on both sides of the cell membrane.

Contacts between neurons

Cadherin complexes are known to play diverse roles in a cell type-specific manner. In an ongoing study of cadherin-catenin function in interneuronal junctions, Takeichi's lab has found that αN-catenin, a form of α-catenin specific to the nervous system, is essential to the stabilization of dendritic spines. These projections are important for establishing and maintaining contacts between axons and dendrites, the two principal types of extensions from the neuronal cell body. Previous work showed that cadherins help to regulate the adhesion of dendritic spines to axons, which is a crucial step in synapse formation, a finding which was complemented by this year's elucidation of αN-catenin's role. The functional loss of this molecule resulted in dramatic increases in spine motility and shape alterations, while αN-catenin overexpression caused spines, which normally extend and retract intermittently, to turn over at a much lower rate, resulting in abnormal accumulations of mature spines over time. Taken together, these results suggest that αN-catenin is a critical agent in maintaining the appropriate balance between stability and turnover in synaptic contacts.

[Cadherins do not act alone]



Overexpression of αN-catenin in hippocampal neurons causes overproduction of dendritic spines (right). Left, control.

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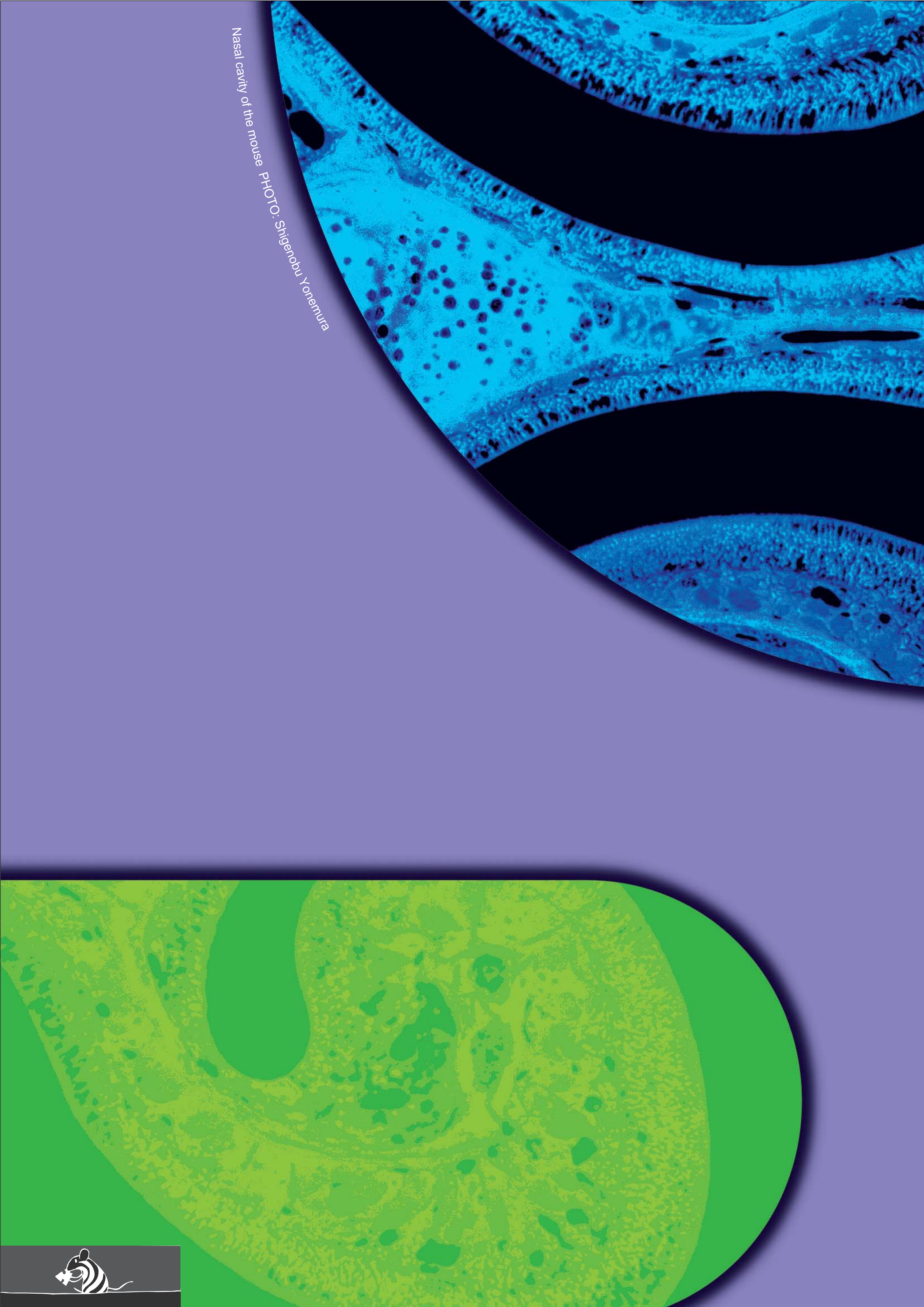
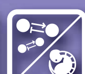

Nasal cavity of the mouse PHOTO: Shigenobu Yonemura

Creative Research Promoting Program

The Creative Research Promoting Program provides solid support to encourage relatively young researchers to carry out innovative and independent research plans. The teams are allowed a great deal of flexibility in regard to projects, budget use, and lab size. The program also places great emphasis on cooperation and international participation. It is hoped that this unique system will help to cultivate a new generation of leading researchers by fostering the creativity and originality of investigators in a bottom-up fashion.



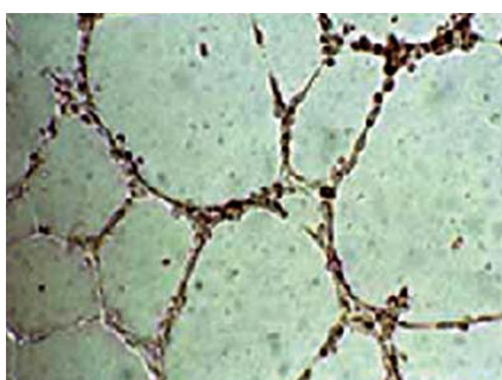
Creative Research Promoting Program

stem cell translational research

Takayuki Asahara
M. D. , Ph. D.

Takayuki Asahara received his M. D. from Tokyo Medical College in 1984, and performed residencies in cardiology and emergency medicine. He worked as a research fellow in cardiology at the Tokyo Medical College Hospital from 1989 to 1993, before moving to a fellowship in cardiovascular research at St. Elizabeth's Hospital in Boston (USA). He was appointed assistant professor at Tufts School of Medicine in 1995, and associate professor at the Tokai University Institute of Medical Sciences in 2000. In addition to his current position as CDB team leader, Dr. Asahara serves as director of Regenerative Medicine and Research at the Kobe Institute of Biomedical Research and Innovation, and Professor of Physiology at the Tokai University School of Medicine.

The emerging field of stem cell-based therapy continues to receive attention as one of the most promising frontiers of medical science. Building on previous work in which he identified bone marrow-derived endothelial progenitor cells (EPCs) and demonstrated their role in the generation of new blood vessels, Takayuki Asahara seeks to characterize adult stem and progenitor cells with even greater differentiative potential, and simultaneously to translate that research into clinically relevant advances.



Vaculogenesis achieved by endothelial progenitor cells

Blood vessels are formed by two distinct physiological processes in the adult body. In angiogenesis, new blood vessels are generated from pre-existing, differentiated endothelial cells. Vasculogenesis, on the other hand, involves the recruitment and differentiation of previously undifferentiated EPCs at the site of new blood vessel growth. These EPCs are themselves the progeny of adult stem cells known as hemangioblasts, which can be induced to demonstrate true pluripotency under the right culture conditions. The vascularization of regenerating tissue is a critical component of the natural healing process as well as fundamental to the recovery of blood vessels that have been damaged, blocked or lost, and the ability to promote and regulate the growth of new blood vessels using EPCs will provide impetus and new areas to explore for researchers and clinicians working to develop treatments for disorders of the cardiovascular and other systems.

Stimulating EPC accumulation

Researchers in the Asahara lab found that EPCs taken from human subjects and expanded ex vivo contributed to the vascularization of the damaged limbs of mice at higher efficiencies if the limbs were first injected with a factor known as SDF-1 (stromal cell-derived factor-1), a chemokine originally identified in mouse bone marrow and whose only known receptor is expressed in hematopoietic stem cells. The CXCR4 transmembrane receptor, present in CD34+ hematopoietic cells, shows a strong attraction to SDF-1 and is believed to help recruit stem cells to sites in need of vascular replacement or repair.

This belief was borne out by the EPC transplantation study, in which the researchers first isolated large numbers of human EPCs for transplantation into mouse models of limb ischemia — localized oxygen starvation of tissue resulting in damage to blood vessels. The transplanted human EPCs were found to improve neovascularization in the ischemic mouse limbs, as well as to reduce the secondary effects of prolonged ischemia, which include necrosis and autoamputation. The incorporation of the transplanted EPCs at ischemic sites was determined by measuring the uptake of labeled human

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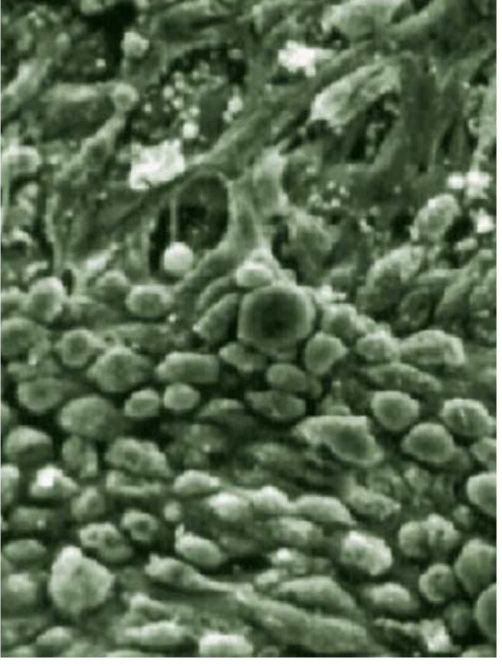

A colony of endothelial progenitor cells on the surface of an injured blood vessel

progenitor cells in tissue sections from the SDF-1 and uninjected groups, which showed that the EPCs accumulated at nearly two times the rate in the limbs which had been injected with SDF-1 compared to that in the control group. This represents an important first proof-of-principle demonstration that the homing of EPCs to a damaged area can be augmented by an extrinsic factor.

Targeted and autologous transplantation

EPCs represent an extremely promising new mode of promoting the therapeutic growth of blood vessels, but significant obstacles must be overcome before that promise can be realized. Previous studies in rats have used quantities of EPCs that would require impractically large supplies of peripheral blood to derive equivalent amounts of EPCs for use in humans, and the possibility of rejection of blood from non-self sources by the immune system

The vascularization of regenerating tissue is a critical component of the natural healing process

remains another important potential barrier. Members of the Asahara team sought to address these questions by developing a system for the local, rather than systemic, transplantation of EPCs freshly isolated from the host. These studies were conducted using a swine model of myocardial (heart muscle) ischemia, from which small quantities of blood were drawn to allow the isolation and expansion of EPCs, identified by the presence of the marker molecule CD31. Catheters were used to inject similar quantities of either CD31+ or CD31- cells, or of a control solution containing no cells, directly into ischemic sites in the hearts of pigs, to test the effects of the concentrated delivery of autologous EPCs. The results showed that the targeted transplantation of CD31+ hematopoietic cells reduced ischemic damage and promoted neovascularization, resulting in improved cardiac function, while pigs receiving CD31- or control solutions experienced no such benefits. A complementary study involving the targeted transplantation of human CD34+ cells into ischemic rat hearts showed that non-autologous cells can also incorporate into damage sites and differentiate into functioning endothelial cells, even when the transplanted quantities are one-twentieth the amounts used in similar previous experiments.

Clinical work

In addition to his role as team leader of the CDB Laboratory for Stem Cell Translational Research, Asahara also serves as director of the regenerative medicine program at the neighboring Institute for Biomedical Research and Innovation. In its role as a translational research lab, Asahara's team is beginning clinical tests of the therapeutic uses of EPCs in the treatment of Buerger Disease (thromboangiitis obliterans), an inflammatory condition involving the obstruction of small and medium-sized blood vessels in the limbs, and linked with heavy tobacco use. Plans are in place to expand the patient population of this preliminary study into the therapeutic transplantation of EPCs, which may offer a new mode of treatment for this disease which at present frequently necessitates the surgical amputation of ischemic digits or limbs.

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Creative Research Promoting Program

neuronal differentiation and regeneration

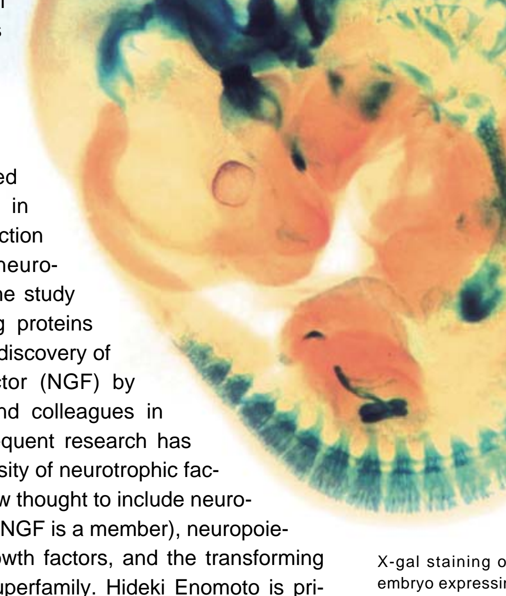
Hideki Enomoto
M. D. , Ph. D.

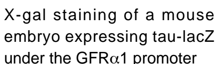
Hideki Enomoto received his M. D. from the Chiba University School of medicine in 1988, and his Ph. D. from the same institution in 1996 for his work in the molecular cloning of the human DAN gene. After serving as the Chief of the Department of Pediatric Surgery, Matsudo Municipal Hospital in Chiba, Japan, he moved to the Washington University School of Medicine, St. Louis in 1997, to work as a research associate studying the physiological roles of the GDNF family of ligands in neural development and function. He returned to Japan to take a position as a team leader at the CDB in 2002.

Neurotrophic factors

The early stages of neural network development see the growth of a number of neurons in excess of the number that will ultimately populate any given region. Neurons that have formed synaptic connections establish what is known as a trophic interaction in which the target tissue provides the neuron with chemical signals necessary for its survival and continued function. The supply of such signals is limited, and neurons which fail to compete successfully for these factors undergo apoptosis, a controlled die-off that establishes an appropriate balance in the neural population.

The target-derived chemical signals in the trophic interaction are known as neurotrophic factors. The study of these signaling proteins dates back to the discovery of nerve growth factor (NGF) by Levi-Montalcini and colleagues in the 1950s. Subsequent research has revealed the diversity of neurotrophic factors, which are now thought to include neurotrophins (of which NGF is a member), neuropoietins, fibroblast growth factors, and the transforming growth factor-β superfamily. Hideki Enomoto is primarily interested in this last group, the GDNF Family Ligands (the GFLs) in particular. This family of neurotrophic factors includes four known members — GDNF (for Glial cell line Derived Neurotrophic Factor), Neurturin, Artemin and Persephin. The GFLs signal via receptor complexes formed by the RET receptor tyrosine kinase and one of four co-receptors, GRFα1-4. In vitro, these four receptors show differential affinities for specific GFLs, with GFR?1 showing the greatest ability to interact with the range of GFL family members.




X-gal staining of a mouse embryo expressing tau-lacZ under the GFRα1 promoter

GDNF ligand-receptor pairings

Enomoto studied phenotypes of mice in which individual GFL, GFRα or Ret genes are disrupted and found that, in receptor-ligand interactions in vivo, there are affinitive pairings between GFRα1-GDNF, GFRα2-NRTN and GFRα3-ARTN. The loss of GDNF-GFRα1 system had the strongest developmental effects, causing defects in enteric, autonomic, sensory and motor neurons as well as failures in the development of the kidney, and resulting in lethality by the P0 stage of development, birth. Similar neuronal populations are affected by Neur-

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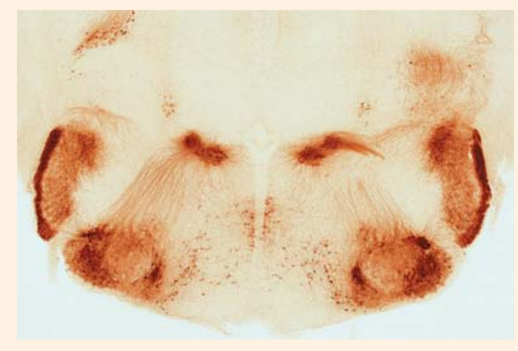


GFP staining of RET-expressing motor and sensory neurons in the brainstem of RET-GFP mice

turin or GFRα2 deficiency, but the phenotypes are less severe, with kidneys forming normally and indefinite effects on sympathetic and motor neural development. Analysis of the consequences of these mutations in developing enteric and parasympathetic neurons suggests that GDNF-GFRα1 functions in neural precursors to regulate fundamental neurodevelopmental processes such as migration and proliferation, while the later expression of Neurturin-GFRα2 in target tissues serves to maintain previously established neurons.

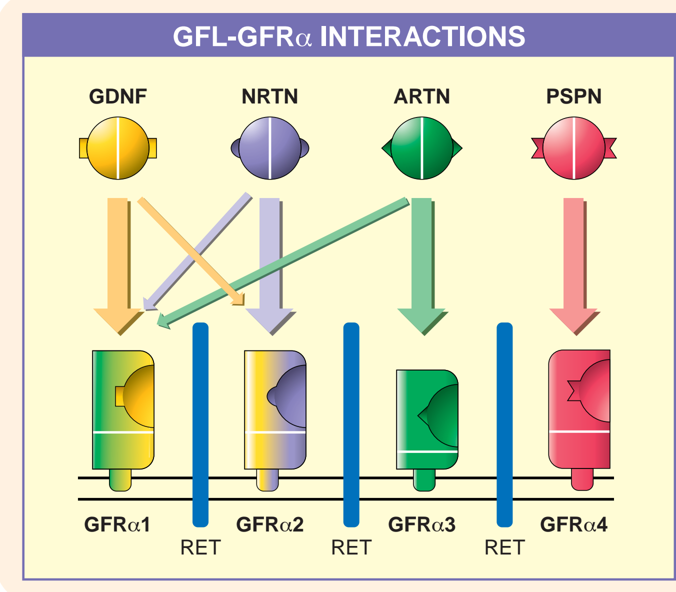
Artemin-GFRα3 signaling seems to operate in a more specific subset of cells, sympathetic neurons, with no apparent function in enteric or central neural development. The sympathetic nervous system arises from the neural crest and follows a stepwise developmental pathway that directs its neurons to establish their appropriate functional roles and positions throughout the body. In a series of signal-directed decisions, sympathetic precursor cells migrate and differentiate into neurons soon after they undergo lineage commitment and restriction to a catecholaminergic (sympathetic neurotransmitter-producing) fate. Enomoto found that Artemin is expressed in blood vessels and attracts growing axons emanating from sympathetic ganglion that expresses RET. This interaction is crucial for sympathetic axons to follow vascular pathways and to reach the final target tissues. The fourth GFL-receptor pairing of Persephin and GFRα4 is known to be expressed ubiquitously at low levels, but its developmental activity remains obscure.

In related work, members of the Enomoto lab studied the roles of RET and TRKA (neurotrophin receptor) in sympathetic neurogenesis and maintenance. Both RET and TRKA are expressed in developing sympathetic neurons, but the balance between these two receptor types shifts over time, with RET predominating during earlier phases of axon growth and migration, while TRK assumes a more important role in ensuring the perinatal survival and maintenance of these cells.

Studies of RET signaling are of particular interest for their value in biomedical research

Studies of RET signaling are of particular interest for their value in biomedical research. Hirschsprung's disease, a congenital disorder afflicting about 1 in 5,000 newborn children in which enteric neuron precursors fail to colonize the distal part of the gut resulting in a loss of bowel motility, chronic constipation and bowel obstruction, is caused by a loss-of-function mutation in the *Ret* gene. Hyperactivation of RET results in abnormal development of the neural crest, a developmentally transient structure that normally gives rise to neurons, glia, endocrine cells and mesenchymal cells, and has been linked to the oncogenesis of endocrine tumors, such as thyroid carcinoma and pheochromocytoma.

In the future, the Enomoto research team plans to conduct systematic analyses of RET-bearing neurons to achieve a better understanding of the molecular mechanisms involved in RET and GFL-receptor signaling. One immediate focus will be on investigating roles for RET, GDNF and GFRα1 in the postnatal maintenance of motor, sensory and dopaminergic neurons, using a Cre-Lox conditional knockout strategy. By providing insights into the functions of GFL-specific neurotrophic factor signaling pathways, Enomoto hopes to contribute to the development of stem cell-basedtherapies for nervous system disorders.



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Creative Research Promoting Program

neural network development

Neural networks

Neural network development involves the orchestration of complex processes from neuronal differentiation to axon growth to the formation of synapses. The mechanisms by which neuronal cells are able to identify and form synapses with appropriate partners, and the ways that circuits formed from interconnecting neurons are able to demonstrate adaptive plasticity are questions of bewildering intricacy and depth.

Chihiro Hama is working to address these questions through investigations into the neural network of the fruit fly *Drosophila*, a system which, in spite of its comparatively simple structure, demonstrates the capacity for instinctive behaviors, learning and memory. While there are profound differences in degrees of network sophistication and cell type diversity between *Drosophila* and more highly evolved organisms such as mammals, they nonetheless share a large number of homologous genes important in neural development. Using a research strategy of large-scale mutant screening in the fly, the Hama research team seeks to open new windows into the genetic bases of neural circuitry.

Slender lobes

The mushroom body, containing on the order of 2000 neurons, is the functional center for olfactory leaning and memory in *Drosophila*. Minako Orihara in the Hama lab has identified the gene *slender lobes* (*sle*), whose homozygous mutation results in sterile flies with thinner axonal bundles and reduced neuron counts in the mushroom body, a phenotype attributable to the failure of flies lacking the *sle* gene to sustain the normally high rate of cellular proliferation by mushroom body neuroblasts (neural progenitor cells).

The protein encoded by the *sle* gene is found in the nuclei of a variety of cells, including neuroblasts, and is abundant in the perinucleolar region during interphase. Genetic analyses have revealed that a majority of interphase nucleoli in the mutant are shifted to a particular state of assembly that transiently or rarely occurs in the wild type. Electron microscopy has showed that the nucleoli in mutant neuroblasts are unusually densely packed, and are separated from the perinucleolar heterochromatins that surround or tightly associate with nucleoli in wild type. Similar phenotypes were also observed in neurons that are quiescent during mitosis and DNA

Mushroom body in the *Drosophila* brain

Chihiro Hama
Ph. D.

Chihiro Hama received his B. Sc. and M. Sc. from the University of Tokyo Department of Biophysics and Biochemistry and was awarded a Ph. D from the same institution in 1985 for his work on the regulation of plasmid Collb DNA replication by inc and repY. He spent the period from 1985 to 1988 as a post-doc in the laboratory of Thomas Kornberg at the University of California, San Francisco before returning to Japan to continue his post-doctoral work at the National Institute of Neuroscience, NCNP, Tokyo. He advanced to section chief in the Department of Molecular Genetics in 1991, and remained at the NCNP until 2001 when he was appointed to his current position at the CDB.

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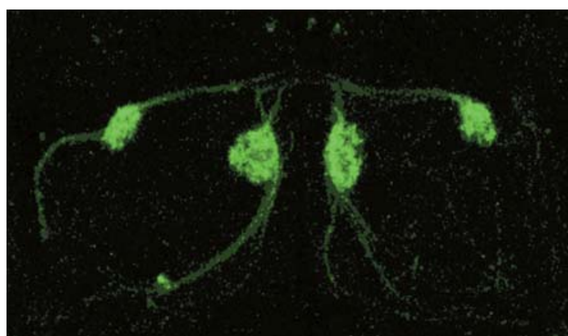
synthesis. The Hama lab's studies indicate that Sle, a novel perinucleolar protein, may modulate nucleolar assembly and consequently promotes the proliferation of neuroblasts.

Olfactory axon guidance

The *Drosophila* olfactory sensory system comprises around 1,300 neurons that are positioned in the head appendages and project into the antennal lobe of the brain, which contains 43 glomerular synaptic clusters of sensory neurons and interneurons. Each individual sensory neuron expresses only one or two types of the approximately 60 known types of odorant receptors, and all neurons expressing the same odorant receptor project to the same glomerulus. The Hama lab is interested in resolving the mechanisms by which these specialized neurons are able to identify and home to specific targets over considerable distances with such great accuracy during the organization of the olfactory network. This research seeks both to clarify the roles of known genetic elements and to uncover new pieces needed to solve this intricate puzzle through screening for new mutations that affect the projection of olfactory neurons.

Keita Endo in the Hama lab has developed a system to generate mutant flies and screen them for olfactory neuron projection phenotypes by visualizing specific projection patterns. The first step of this approach takes advantage of an enhancer trap line that enables the forced expression of marker genes in specific subsets of olfactory sensory neurons, making it possible to visualize the extension of their axons toward the glomeruli. The second phase entails the production of genetically mosaic flies in which specific olfactory neurons are homozygous for mutations in otherwise heterozygous animals. This screening approach has already yielded approximately 70 mutant lines exhibiting phenotypes in which axons mistarget, overshoot, arborize improperly or fail to project entirely. Some of these mutations are now being mapped on the chromosome by SNP analyses and the use of chromosomal deficiencies.



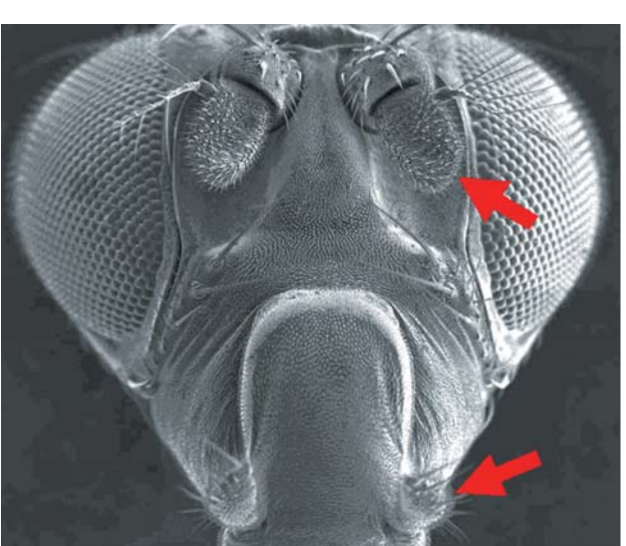
Targeting of olfactory sensory neurons into specific glomeruli in the antenna lobes

Vesicle transport in neural development

All cells contain cargo-bearing compartments known as vesicles. These vesicles function to deliver the macromolecules that serve the cells as membrane components or secreted signals, and the metabolite products of the cellular digestive process to the rest of the cell, where they are stored or used to fuel

The Hama lab is interested in resolving the mechanisms by which olfactory neurons identify and home to specific targets

activity. Microtubules serve as 'railways' for vesicle transport; their ends anchor to sites on the cell cortex where the vesicles fuse. It is likely that the extrusion of cell membrane at given sites, such as takes place in axon extension, requires vesicles to be transported along microtubule in a specific orientation. To clarify how vesicle transport is involved in neural development, members of the Hama lab are now analyzing *Drosophila* orthologs of genes encoding proteins known to be involved in the regulation of vesicular traffic in budding yeast. Hama hopes to elucidate the roles of these proteins in neurite extension, branching and guidance, and synapse formation as well, by conducting conditional RNAi studies using a DNA-based transgenic approach.



Antenna segment (top arrow) and maxillary palp (bottom arrow); olfactory sensory apparatuses in *Drosophila*

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Creative Research Promoting Program

vertebrate axis formation

Masahiko Hibi
M. D. , Ph. D.

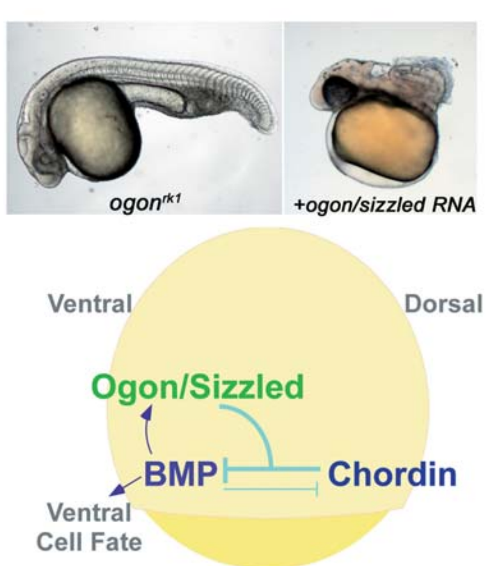
Masahiko Hibi received his M. D. from Hiroshima University, School of Medicine in 1988, and his Ph. D. from the Osaka University Institute for Molecular and Cellular Biology in 1992. From 1992 to 1995, he worked as a postdoctoral fellow in Michael Karin's lab in the University of California, San Diego Department of Pharmacology, then returned to Japan to take an assistant professorship in the Division of Molecular Oncology at Osaka University Medical School. He was appointed associate professor in the same division in 1999, where he remained until he assumed his position as team leader at the RIKEN CDB.

Vertebrate embryogenesis begins with a seemingly formless egg that, through processes of division, growth, differentiation, migration and rearrangement, rapidly gives rise to a highly organized structure characterized by a number of definitive axes. This transformation is particularly striking and rapid in the zebrafish (*Danio rerio*), which in the space of a single day develops from an ovum to a recognizably vertebrate body laid down in respect to multiple polar vectors. The speed of its growth and reproduction, coupled with its amenability to genetic studies and the revealing pellucidity of its embryo, make this organism an ideal system for the study of axis formation in early vertebrate development.

Regulating the dorsal-ventral axis

Masahiko Hibi's laboratory focuses on the molecular genetic cascade of events stemming from organizing centers in the zebrafish embryo. These centers, which emerge soon after fertilization, play pivotal roles in setting up positional axes; Hibi's lab is particularly interested in the formation of the dorsal-ventral (back-belly) axis, an essential step in the establishment of the body plan. An embryonic region known in zebrafish as the dorsal organizer guides dorsoventral patterning by generating inductive signals, including inhibitors of BMPs and Wnts (molecules which ultimately determine dorsal and ventral fates). These molecules play roles in a complex network of axis-determining factors, and the interplay between dorsalizing and ventralizing factors is central to the formation of the dorsal mesoderm and endoderm, and the neuroectoderm.

In work published in 2003, the Hibi lab analyzed and isolated the responsible gene for a mutant named *ogon*, which displays ventralized phenotypes, and found that the *ogon* locus encodes a zebrafish homologue of the protein Secreted Frizzled (Sizzled), which functions as negative feedback regulator of BMP signaling. While numerous mutations are known to result in dorsalized phenotypes, *ogon* is only the second zebrafish mutant reported to show clearly ventralized phenotypes, such as expanded ventral tail fins, blood, pronephron, and other posterior structures. These patterns are similar to the phenotypes observed in mutations of the gene for the BMP antagonist, Chordin, which suggested that Ogon might also be involved in BMP inhibition.



ogon mutant and ogon-overexpressing embryos. Ogon functions as a negative feedback regulator of BMP signaling

Hibi and colleagues set out to clone the ogon gene (which is also known as *mercedes* and *short tail*), in order to determine its precise relationship with Chordin, and the molecular bases for its action in regulating the establishment of the dorsal-ventral axis. The results of that positional cloning showed that *ogon* encodes a homolog of the Secreted Frizzled (Sizzled) protein, which has similarities to the Wnt receptor, Frizzled. The canonical Wnt pathway is known to play a role in axis formation, but it has been suggested that *ogon*/sizzled influences the dorsal-ventral axis independently of its effect on Wnt. To test this idea, members of the Hibi lab compared the activity of Ogon/Sizzled with that of Wnt inhibitors in ectopic expression studies, and found that the effects of Ogon/Sizzled misexpression resembled those of BMP, but not Wnt, inhibitors, suggesting an exceptional mode of action.

Organizing centers, which emerge soon after fertilization, play pivotal roles in setting up positional axes in the zebrafish embryo

They next looked at the possible involvement of Chordin (which is known to be essential in the process of dorsalization) in Ogo/Szl's effects by injecting *ogon*/*sizzled* RNA into mutants (*chordino*) lacking a functional *chordin* gene. This failed to rescue the ventralized phenotype of homozygous *chordino* mutants, indicating that Ogon/Sizzled activity depends on Chordin. The detailed functional relationship between these two dorsalizing factors remains to be worked out, but these initial findings suggest that Ogon/Sizzled augments Chordin activity either by inhibiting a Chordin inhibitor, by upregulating Chordin activity, or by making BMP more sensitive to Chordin's inhibitory effects.

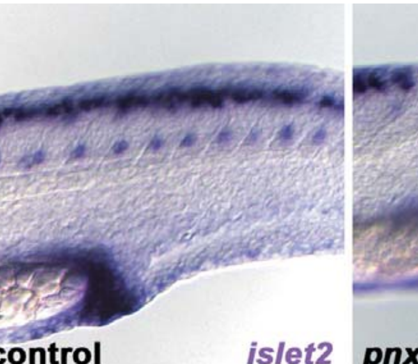
The maternal-effect mutant *tokkaebi* displays a completely ventralized phenotype.

Neurogenesis

Vertebrate neuronal tissues are generated in a stepwise fashion. These steps include neural induction, antero-posterior patterning, and neurogenesis. In amphibian and teleost (bony fish) embryos, neuroectoderm is induced by BMP inhibitors derived from organizing centers. Induced neuroectoderm is by default anterior in character, but a subset in some tissues is subsequently subject to posteriorizing transformations. The hindbrain and spinal cord, which are posteriorized regions, are specified by a signal from a region known as non-axial mesendoderm, while anterior neuroectoderm that does not receive this posteriorizing signal develops into fore- and midbrain. After these initial processes of neural induction and patterning, neurogenic regions, the domains in which neurogenesis takes place, are established.

The Hibi lab has been working to identify genes involved in the control of this patterning and neurogenesis, focusing on two genes in particular: the zinc finger gene *fez-like* expressed in the forebrain, and the posterior neuron-specific homeobox gene *pnx*. In collaborative work, they showed that *fez-like* is required for the formation of hypothalamic monoaminergic neurons in zebrafish. These neurons produce neurotransmitters such as dopamine and serotonin, which are centrally important to the regulation of mood, behavior, endocrine and cognitive functions in humans.

A separate study identified a homeobox gene, *pnx*, involved in the development of posterior neurons. This gene, which is regulated by a signal from the posteriorizing non-axial mesendoderm and Notch signaling, acts as a transcriptional repressor. Misexpression of *pnx* results in an increase in neural precursor cells and neurons, while its repression caused reductions in certain populations of posterior neurons, indicating that the gene participates in posterior neurogenesis.



Pnx is required for the formation of motoneurons.

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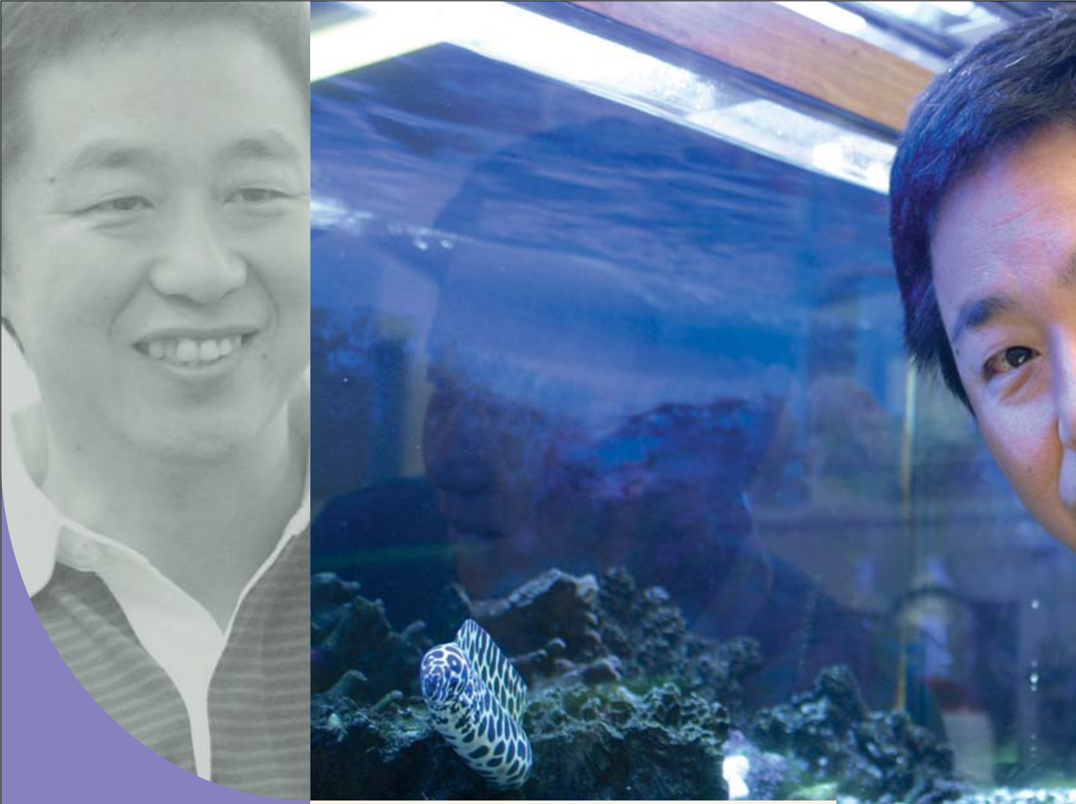

Creative Research Promoting Program

positional information

Shigeru Kondo
Ph. D.

Shigeru Kondo received his doctorate from the Kyoto University Faculty of Medicine in 1988. He spent the period from 1989 to 1990 as a postdoctoral fellow in Masami Muramatsu's lab at the University of Tokyo, before taking an overseas fellowship at the Basel University Biocenter under Walter Gehring. He returned to Kyoto University in 1994 as assistant professor, where he remained until 1998, when he was appointed professor in the Tokushima University Faculty of Integrated Arts and Sciences. Kondo was appointed CDB team leader in 2001.

The patterning of the natural world

The question of how complex patterns arise from seemingly disorganized or formless initial structures represents an intriguing challenge to mathematicians, physicists, chemists and biologists alike. Theoretical work indicates that the mechanisms underlying pattern formation are similar in both biological and non-biological systems, and a number of mathematical models capable of describing pattern generation in chemical media have been proposed, but the greater complexity of living systems has made it much more difficult to demonstrate a mathematical basis for biological patterns. In 1952, the British mathematician Alan Turing proposed a simple mathematical equation capable of generating a wide range of patterns commonly found in the natural world, such as stripes, spots and reticulations. This model, known as the reaction-diffusion model, demonstrates that the interaction between a local activator and a long-range inhibitor can give rise to various periodic structures in response to differences in their individual diffusion rates.

Shigeru Kondo is interested in demonstrating the mathematical basis of pattern formation in development, and using mathematical models as predictive tools to aid in the identification of genes and molecules involved in the generation of spatial structures. Research in the Kondo lab focuses on skin surface and morphogenetic patterning, both of which feature prominent examples of periodic structures that can be described in terms of standing and moving waves.

Traveling wave mice

In research published this year, Kondo identified a mathematical similarity between a well-characterized reaction that produces wave-like patterns in chemical media and a pattern-forming phenomenon in the skin of a mutant mouse. These mice, which have defects in a gene responsible for hair follicle development, develop bands of darkened skin that traverse the body surface in waves. Kondo, working in collaboration with researchers from the Medical School of Mie University, showed that these traveling waves of skin coloration are strikingly similar to nonlinear waves produced by the Belousov-Zhabotinskii (BZ) reaction in chemical systems, suggesting a shared underlying principle. The research began when Kondo learned of a mutant strain of mouse with an unusual striped phenotype. The mutation, a splicing defect in the *Foxn1* (*Whn* or *nude*) gene, causes hair follicle development to terminate just after skin pigments begin to accumulate. The immature follicles die off and are quickly replaced by a new hair cycle. The cyclical nature of

Traveling wave skin pattern of the newly isolated mutant traveling wave (tw) mouse. Each wave arises at the base of the forelimb, and moves like a wave spreading on the surface of water.

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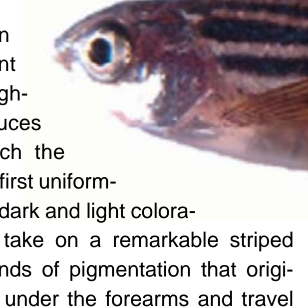
this follicular attrition and the subsequent re-activation by neighboring follicles produces a phenotype in which the mouse's skin color at first uniformly oscillates between dark and light coloration, then begins to take on a remarkable striped appearance, with bands of pigmentation that originate from the region under the forearms and travel across the body surface in all directions. These waves first appear at about three months after birth, and continue to arise and propagate throughout the life of the animal.

Kondo identified similarities between wave-like patterns observed in chemical media and a pattern-forming phenomenon in the skin of a mutant mouse

By tracking the movements of traveling waves in the *Foxn1* mouse skin pattern, Kondo ascertained that their formation was fundamentally similar to that of waves generated by the BZ reaction. Although this finding does not directly identify the specific molecular mechanism at work in the *Foxn1* phenotype, mathematical analysis suggests a candidate molecule, Shh, which satisfies the necessary criteria for a molecule key to traveling wave pattern formation. And, as many other species demonstrate similar striping mechanisms, this report may help to provide biologists with a mathematical model to explain this skin patterning phenomenon.

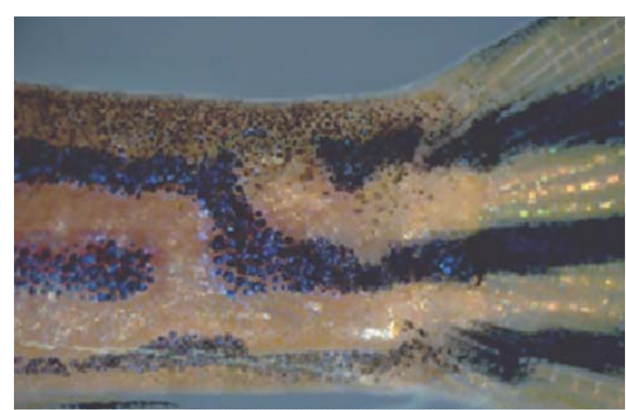
Turing mechanism in zebrafish stripes

The zebrafish (*Danio rerio*) is strikingly patterned in bands of light and dark, lending it the zebra-like appearance for which it is named. This striped effect is achieved by the tightly regulated distribution of three types of pigment cells (chromatophores) in the hypodermis — xanthophores, which produce yellow pigment, melanophores, which produce black, and iridophores, which have a glittering appearance. Iridophores are evenly distributed, but xanthophores and melanophores appear in mutually exclusive bands, which appear as stripes in the adult fish. Noting resemblances between zebrafish stripe development and mathematical patterns generated by the Turing mechanism, Kondo speculated that a reaction-diffusion effect might be at work. To test this hypothesis, he used lasers to ablate specific populations of pigment cells and observed their re-growth. When a band of xanthophores or melanophores is ablated, small precursor cells are generated to replace the cells that have been lost. The initial number of replacement cells that arise is in excess of the final number, indicating that some of



The zebrafish skin pattern is generated by combinations of cells producing various pigments

the precursors die off during the process of regeneration. And while in principle any type of chromatophore can grow at any location in the skin, xanthophore bands regenerate only xanthophores and melanophores only melanophores, suggesting that cells of both types inhibit the growth of their counterparts. Using mathematical simulations, Kondo developed a model to account for this banded skin patterning, in which chromatophores of each type produce both short-range inhibitors of pigment cells of the same type and long-range inhibitors of the complementary cell type. When mathematical simulations using inhibitors of varying strengths of diffusion rates are performed, this double negative feedback relationship gives rise to a range of Turing patterns similar to skin patterns seen in nature, a finding that provides new avenues of inquiry for investigators studying the molecular mechanisms of pattern formation in biological systems.



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Creative Research Promoting Program

evolutionary morphology

Shigeru Kuratani
Ph. D.

Shigeru Kuratani received his M. S. and Ph. D. from the Kyoto University Department of Zoology. He spent the period from 1988 to 1991 working in experimental embryology in the Department of Anatomy at the Medical College of Georgia before moving to the Biochemistry Department, Baylor College of Medicine, where he was engaged in molecular embryological research. He returned to Japan in 1994 to take a position as associate professor at the Institute of Medical Embryology and Genetics in the Kumamoto University School of Medicine. He moved to Okayama University to assume a professorship in the Department of Biology in 1997, where he remained until he was appointed team leader at the CDB.

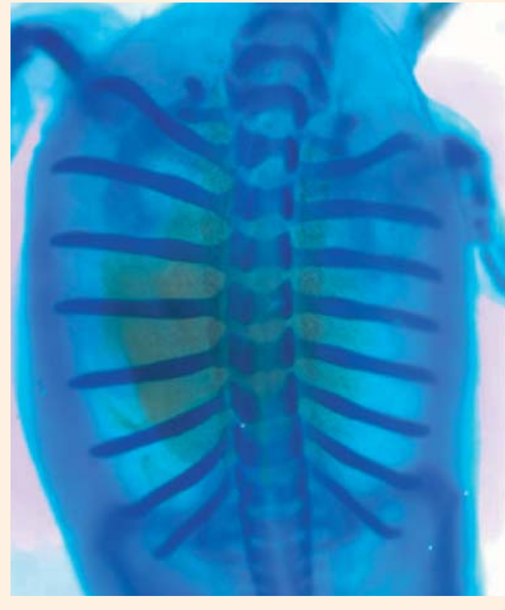
Evo-devo

Darwin conceived evolution as a process driven by the interplay between random mutation and natural selection, but the advent of molecular developmental biology in the 1980s led to the recognition that inherent genetic constraints also have a role in defining the trajectories that evolutionary processes are most likely to follow. The homeobox *(Hox)* genes, a developmentally important set of genes provide one instantiation of this concept; they play central roles in regulating the morphological development of organisms ranging from yeast and plants to animals from every branch of the phylogenetic tree and share a highly conserved domain (the 180-base-pair homeodomain) that indicates a non-random mode of evolutionary selection.

Combining experimental and analytic techniques from molecular biology, phylogenetics and comparative morphology, Shigeru Kuratani seeks to deepen the understanding of the part played by developmental biological mechanisms, such as the *Hox* genes, in the divergence of species. His approach involves examining related genes in phylogenetically distinct animals to uncover the ways in which context affects gene expression, and thereby influences body development. By comparing the molecular bases of the emergence of discrete structures, Kuratani hopes to illustrate the means by which developmental mechanisms mediate the translation of changes in the genome to changes in morphology.

The turtle's shell

The abrupt advent of the turtle's shell, or 'carapace,' which appeared with few recognizable precursors, provides a remarkable exception to the general rule of gradual evolution in incremental steps. Such a phenomenon is difficult to explain by mutation and selection alone, as it is improbable that the genomic alterations necessary to produce such a dramatically new bodily structure would be achievable by the introduction of new genes in the short timeframe of the turtle's emergence. Evo-devo (evolutionary developmental biology) theory predicts instead that the genes responsible for carapace development actually belong to a set of genes shared by groups related to the turtle, but which function distinctly in the unique context of the turtle's molecular-genetic network. This would also explain the independent appearance of the carapace at other points in evolu-



Removal of carapacial ridge results in a characteristic fan-shape splaying of turtle ribs.

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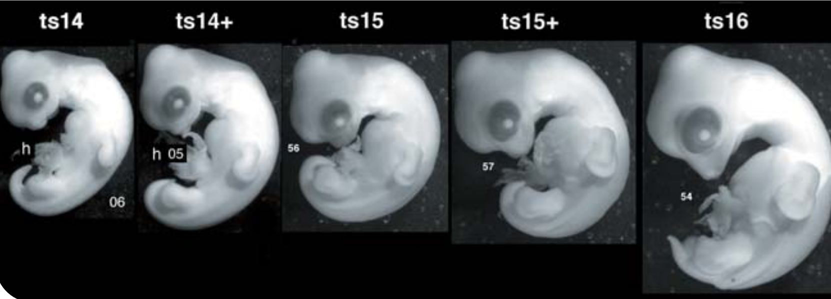
tionary history, as the underlying genetic elements are presumed to be conserved, making carapace formation one of the trajectories available to the process of morphological development.

In other closely related species, ribs are entirely internal and reside interior to the scapulae (corresponding to the shoulder blades in humans). In the turtle, however, the rib bones grow exterior to the scapulae to form the external carapace. Using cross-species explantation, cDNA library and cell sorting studies, researchers in the Kuratani lab have identified a number of developmental switching points that enable this exteriorization of this portion of the turtle endoskeleton. Their findings indicate that alterations in a number of essentially conserved genes, rather than genetic novelty, have resulted in the turtle's unique anatomical phenotype.

Hindbrain evolution

In vertebrates, the hindbrain is a segmented structure, subdivided into clearly demarcated units called rhombomeres, which generate specific sets of neurons. The lancelet *Amphioxus*, a more primitive chordate, however, lacks this hindbrain segmentation. The lamprey, a jawless fish that arose in the interval between non-vertebrate chordates (such as *Amphioxus*) and gnathostomes (jawed animals), provides a relevant model for studying the emergence of the hindbrain developmental plan.

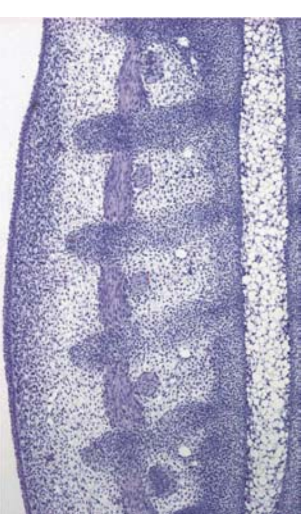
In work published in 2004, investigators in the Kuratani lab labeled reticulospinal and branchial motor neurons (which derive from rhombomeres) to reveal the neuronal organization of the hindbrain of the Japanese lamprey, *Lethenteron japonicum*, and studies the expression patterns of rhombomere-specific genes. They found that lamprey reticular neurons develop in conserved rhombomere-specific positions, similar to those observed in the gnathostome zebrafish. Interestingly, in lamprey the positions of other sets of hindbrain neurons — the trigeminal and facial motor nuclei — do not map neatly to rhombomeric borderlines, as they do in gnathostomes. Rather, the trigeminal-facial nerve originates in the middle of rhombomere 4, in the region of expression of the lamprey *Hox* gene *LjHox3*. When retinoic acid (which is known to alter *Hox* gene expression and associated developmental programs) was introduced to the developing hindbrain region, it caused positional shifts of both *LjHox3* expression and branchiomotor nuclei, but no apparent changes in segmentation or the positions of reticular neurons.



Embryonic developmental stages in Chinese soft-shelled turtle

Nature prefers to repurpose or tinker with existing genes rather than to introduce novelties ex *nihilo*

These findings indicate that, in the lamprey, hindbrain neural identities and rhombomeric segmentation are governed by independent mechanisms, providing strong counter-evidence to one prevailing model that suggests that the establishment of neuronal identity is a *Hox*-dependent process. Based on their discoveries, Kuratani et al. offer an alternate model in which the positional concurrence between *Hox* expression, rhombomere identity and specific subsets of hindbrain neurons is the result of a convergent process in which originally independent mechanisms became linked over evolutionary time.



The rib grows laterally towards the edge of the body in the turtle embryo.

Studies such as these underscore a pair of evo-devo precepts: that molecular designs capable of supporting viable ontogenies tend to act as magnets for convergent evolution, and that Nature is parsimonious with her creations, preferring to repurpose or tinker with existing genes rather than to introduce novelties *ex nihilo*.

Publications

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Creative Research Promoting Program

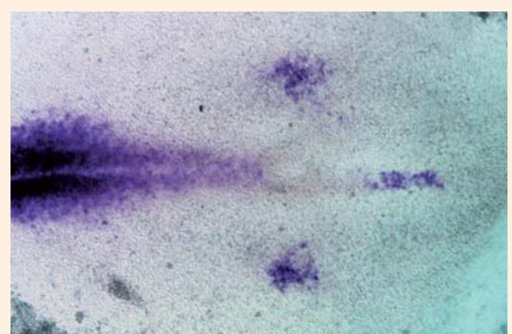
sensory development

Raj Ladher
Ph. D.

Raj Ladher received his B. Sc. in biochemistry from Imperial College, London in 1992 and his Ph. D. in developmental biology from the National Institute for Medical Research in 1996, for his thesis on the cloning of the *Xenopus* homeobox gene, Xom, under the supervsion of Jim Smith. He worked as a research associate at King's College, London from 1996 to 2000 in the lab of Pip Francis-West, and first came to Japan during that period as a visiting scientist at the Tokyo Medical and Dental University. In 2000, he moved to the University of Utah as a postdoctoral fellow in Gary Schoenwolf's laboratory, and was appointed team leader at the RIKEN CDB in 2002.

Developing sense organs

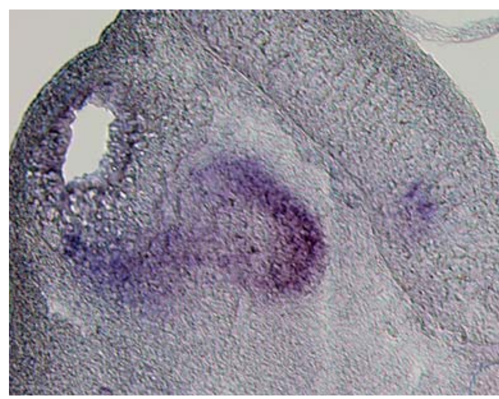
The embryo provides many examples of elegant micro-engineering, wedding intricate organic structures with specialized cellular function. The sensory organs provide a particularly good example of this sophistication. By initiating in the correct place, differentiating into the correct subtypes and finally integrating with structures of diverse origin to form a sensory complex, these structures permit the animal to apprehend its environment by processing a range of physical and chemical stimuli. The development of these systems involves an extraordinary level of coordination between cells and emergent tissues at very early stages of embryogenesis. Research in Raj Ladher's lab focuses on the induction of the inner ear and the peripheral olfactory system. The chick embryo is used as a model system, chosen for its tractability to classical embryological techniques and the opportunities it affords for investigating the processes of developmental program initiation, cell differentiation and the integration of discrete cellular motifs in structurally and neuronally specialized organs.



Expression of Fgf-19 is in purple. Shown is a section through a stage 22 embryo. Fgf-19 is expressed in the condensing acoustic ganglia at these stages, suggesting a role in the later development of the inner ear as well as in its induction.

Signals in inner ear induction

Sense organs originate in placodes, thickened regions within the early ectoderm. The inner ear arises from the otic placode, a cluster of cells which ultimately gives rise to the mature inner ear. Raj is studying the inductive factors that dictate the location and timing of these developmental processes. In a series of tissue manipulation experiments conducted previously, he identified a trio of signaling factors — FGF-8, FGF-19, and Wnt-8c — each of which localizes in a separate germ layer, and which cooperate to induce inner ear development. In this network, it appears that FGF-8, expressed in the endodermal layer, induces the expression of FGF-19 in the immediately overlying mesoderm. FGF-19 in turn induces Wnt-8c in the neuroectoderm, triggering a complex regulatory loop in which FGF-19 and Wnt-8c maintain each other's expression for the



Fgf-19 is expressed in the mesoderm of the chicken embryo from early stages.

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Sensory cellular differentiation

The conversion of uncommitted precursor cells in the otic placode to differentiated, functional otic subtypes is very precisely timed. If it occurs prematurely, the resulting organ is underdeveloped, with too few cells forming the adult structure. If it happens too late, there is a danger that these cells will still be immature and unable to fulfill their needed roles in subsequent developmental processes. An example of this temporal regulation is seen in the wiring of the inner ear to the brain, which requires the formation of neurons from the otic placode. If these cells are not formed when this process occurs, the function of the auditory system is compromised. But what is the nature of this control? By using embryological studies, combined with gene manipulation and ex-ovo culture, Raj's lab is addressing this question, finding the basis for this precise regulation in the functional characteristics of specific signaling molecules. Their control and effects on the development of the inner ear represents one of the most exciting aspects of research in Raj's lab, with implications in regenerative technologies and tissue engineering-based therapeutics.

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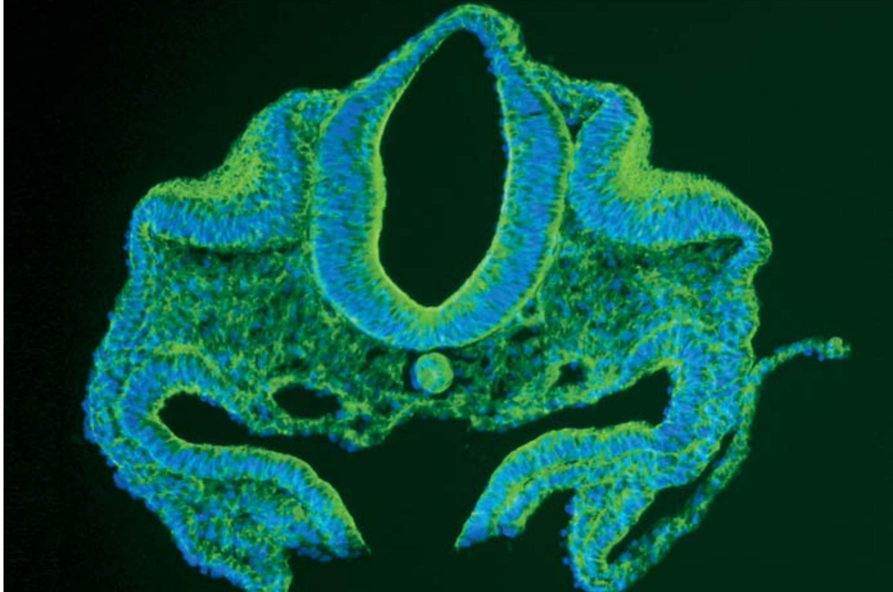
duration of early ear development. Signaling interactions, acting upon the cell surface, trigger a series of transduction events that are interpreted in the nucleus of the responding cell as transcription factors. Raj's lab is characterizing the transcriptional response to the different signals that act to collectively specify the inner ear. By understanding their function, Raj hopes to be able to determine a mechanism of molecular synergy.

Morphogenesis of the otic placode

Although the adult inner ear is located in the interior of the animal it is nonetheless an ectodermal structure, originating from a placode embedded within the outermost layer of the embryo. Through a process known as invagination, the otic placode pinches off from the ectoderm. This represents one of the first cellular consequences of inner ear induction, and Raj has focused on this process to understand the functional significance of signaling factors and their transcriptional responses. Working from the hypothesis that extracellular cues choreograph this complex dance of cells, modifying their shape, motility, growth and the location of subcellular components, Raj hopes to be able to reconcile the events that occur outside the cell with the biological changes occurring within.

Raj hopes to reconcile the events that occur outside the cell with the biological changes occurring within

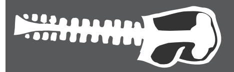


Nuclei marked using DAPI (blue), microtubules marked using phalloidin (green). A section through a chick embryo at stage 17 shows the morphogenesis of the otic placode during invagination. This results from a dynamic rearrangement of subcellular components.

Development of other sensory organs

As well as the inner ear, Raj's lab is also investigating the development of other sensory organs, in particular the nose and the eye. Using embryological manipulations, the Lab for Sensory Development is mapping the tissues that are responsible for the formation and differentiation of these structures. Though still in their infancy, these research projects do indicate that tissue interactions distinct from each other and from those operating to induce the inner ear are responsible for the control of their development.

In the future, the Ladher lab looks to extend its research scope to other model systems, with the goal of determining whether some as-yet unidentified general mechanisms are at work in sensory organogenesis.



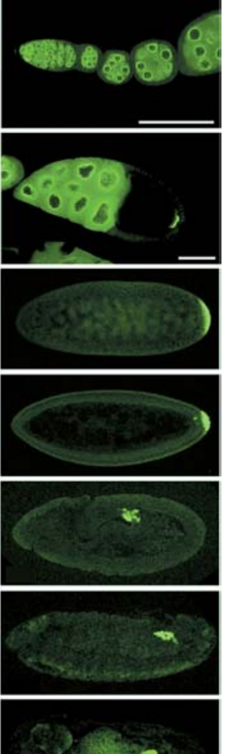
Creative Research Promoting Program

germline development

Akira Nakamura
Ph. D.

Akira Nakamura received both his baccalaureate and his Ph. D. from the University of Tsukuba. He spent a year as a post-doctoral fellow at the same institution before moving to the Department of Biology at McGill University in Montreal in 1995 to work as a post-doc under Paul Lasko. He returned to Japan in 1997 as a research associate at the University of Tsukuba. He was appointed assistant professor in the university's Gene Research Center and Institute of Biological Sciences in 2000, and began a three-year term as a PRESTO researcher in the Japan Science and Technology Corporation (JST) in December 2001. He was appointed CDB team leader in March 2002.

Germ cells are the only cell types capable of transmitting genetic information across generations, and their formation is characterized by unique developmental processes as well. In many types of animals, including the Drosophila fruit fly, the formation and differentiation of germ cells is controlled by mRNAs and proteins localized in a specific cytoplasmic region within eggs, called germ plasm. Germ plasm mRNAs are translated in a spatiotemporally regulated manner, but the means by which the germ plasm is formed and achieves this controlled translation remain largely unknown. Akira Nakamura studies the establishment of the *Drosophila* germ line as a model of the processes of germ plasm formation and differentiation, as well as for the insights this system can provide into the general mechanisms of mRNA localization and translation, which are important to a number of other developmental processes including asymmetric cell division, cell migration and axis formation and likely synaptic plasticity.



Drosophila germ plasm and germ cells visualized using GFP-Vasa fusion protein.

Translational repression

RNA activity during *Drosophila* oogenesis involves a number of sequential processes. The *Drosophila* oocyte shares cytoplasm with neighboring nurse cells via an incomplete cell membrane, allowing mRNAs and proteins from the nurse cells to be transported to the oocyte in the form of ribonucleoproteins. Following their export from the nurse cell nuclei, mRNAs are translationally repressed, or 'masked,' and transported to specified regions of the oocyte, where they establish fixed and precise localizations and regain their ability to undergo translation. In one example of this critically important regulation, the translation of the RNA for the maternal gene *oskar*, which has critical functions in embryonic patterning and the formation of germline cells, is repressed during its transport to the posterior pole of the oocyte. This transcript-specific repression is known to be mediated by the protein Bruno, which binds to the 3' UTR of *oskar* mRNA, but the underlying mechanisms have remained obscure.

Following their export from the nurse cell nuclei, mRNAs are translationally repressed, or 'masked,' and transported to specified regions of the oocyte

In recent work, the Nakamura lab demonstrated that an ovarian protein, Cup, is another protein required to inhibit the premature translation of *oskar* mRNA, and that Cup achieves this by binding to a second protein, eIF4E, a 5' cap-binding general translation initiation factor. The binding with Cup prevents eIF-4E from binding with a different partnering molecule,

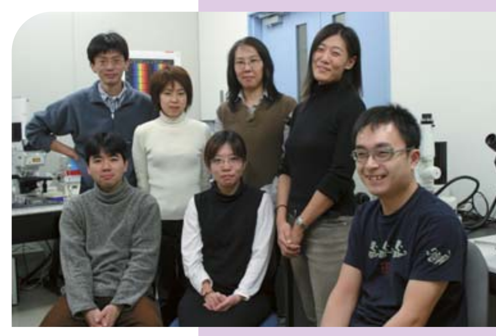
eIF4G, and thereby inhibits the initiation of translation. Findings that a mutant form of Cup lacking the sequence with which it binds eIF4E failed to repress *oskar* translation in vivo, that Cup interacts with Bruno in a yeast two-hybrid assay, and that the Cup-eIF4E complex associates with Bruno *in vivo* suggest that these three proteins form a complex that achieves translational repression by interactions with both the 3' and 5' ends of the *oskar* RNA. A similar model of protein interactions is observed in the translational repression of the *cyclin-B1* RNA in the *Xenopus* African clawed frog, indicating that this paradigm of translational repression through the 5' /3' interactions is conserved across species.

Nakamura next intends to look into the means by which the repressor effects of the eIF4E-Cup-Bruno complex are alleviated at the appropriate developmental stage, after the *oskar* ribonucleoprotein complex has reached and anchored to its appropriate destination at the pole of the oocyte.

previously unexpected roles for a maternally supplied factor involved in lipid signaling, which the team is now working to characterize in more detail. Mutation of the underlying gene results in a phenotype similar to that of the *Pgc* mutant, in which germ cells fail to migrate to the embryonic gonads. Nakamura is now investigating the roles of both genes in the maintenance and migration of germ cells.

Development of the *Ciona intestinalis* germline

In addition to their investigation of fruit fly germline development, scientists in the Nakamura lab are also beginning investigations using the ascidian, *Ciona intestinalis*, a member of the chordate lineage from which all vertebrates arose. Commonly referred to as sea squirts, these animals provide a good model for studying vertebrate evolution. Soon



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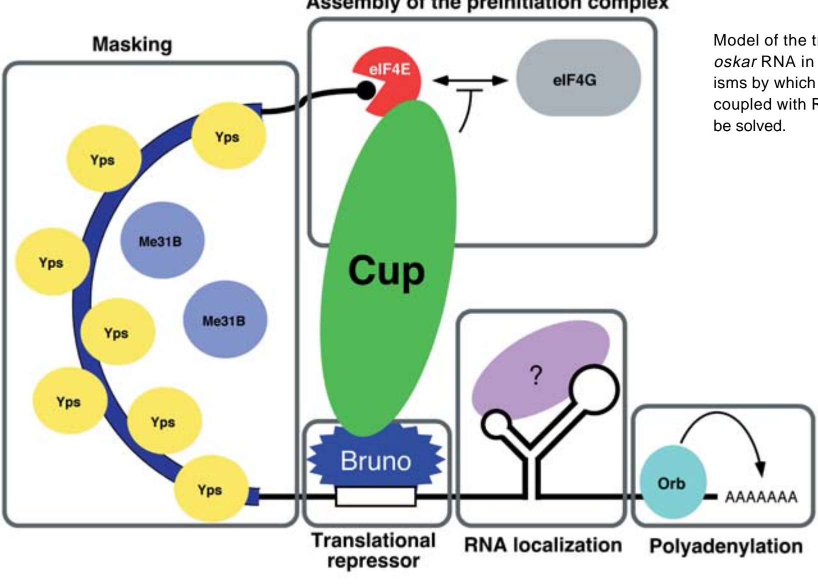

Model of the translational repression of *oskar* RNA in oogenesis. The mechanisms by which translational repression is coupled with RNA localization remain to be solved.

Polar granule maintenance

Polar granules are large complexes of ribonucleoproteins that store RNAs and proteins required for the formation of germ cells in the fruit fly. A non-coding *Polar granule component* (*Pgc*) RNA has been shown to be important in the maintenance of these germ plasm organelles; in *Pgc* antisense knockdown embryos, germ cells are formed but subsequently degenerate, which has led to the hypothesis that *Pgc* serves as a form of supportive scaffold maintaining the structural integrity of polar granules. The Nakamura lab is now exploring *Pgc* function in more detail by isolating a complete loss-of-function mutant, allowing for more specific analyses of the gene's role. They also plan to look for the gene's functional domains, using *Pgc* orthologs from other species of *Drosophila* as a basis for comparison.

In other ongoing research, members of the Nakamura lab are using forward-genetics techniques to search for novel genes involved in germ cell development. Early results of those studies have revealed

after fertilization, the ascidian egg develops into a small free-swimming tadpole possessing a notochord, an evolutionary forebear of the spinal cord, and a rudimentary nervous system, both of which are lost when it enters its immobile adult stage.

Nakamura seeks to analyze the regulatory mechanisms of germline development in *Ciona*, which are interesting in that while several lines of evidence indicate that germ cells are formed from maternally derived germ plasm, it also appears that germ cells can be regenerated after metamorphosis, suggesting that two independent mechanisms may be at work. In research conducted in collaboration with Suma Aqualife Park, a local municipal aquarium, Nakamura's team will explore the genetic regulation of ascidian germline development by selecting candidate genes from EST and genome databases (a draft sequence of the *Ciona* genome is available), confirm the spatial and temporal patterns of their expression and characterize promoter regions and *trans*-acting factors.

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Creative Research Promoting Program

chromatin dynamics

Jun-ichi Nakayama
Ph. D.

Jun-ichi Nakayama received his bachelor's, master's and doctoral degrees in bioscience from the Tokyo Institute of Technology, the last in 1999 for his work on the cloning and characterization of mammalian telomerase components. He spent the period from 1999 to 2001 as a postdoctoral researcher at the Cold Spring Harbor Laboratory in Shiv Grewal's lab, before returning to Japan in December 2001 as a PRESTO researcher in the Japan Science and Technology Corporation (JST). He was appointed team leader in the RIKEN CDB Laboratory for Chromatin Dynamics in 2002.

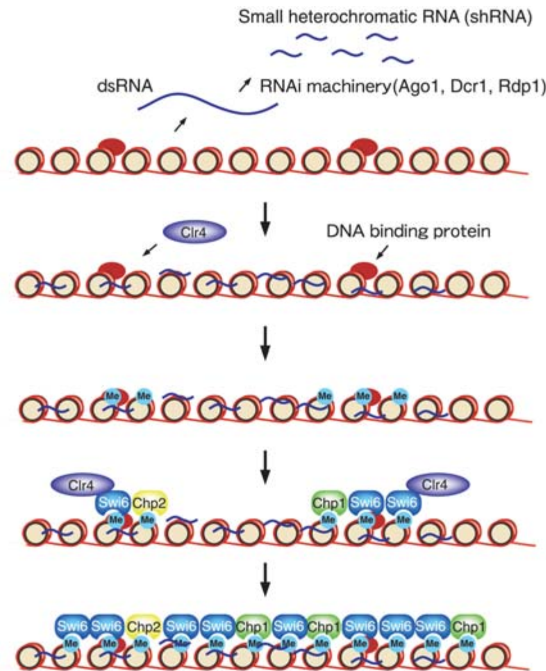
Modifications to the nuclear DNA-protein chromatin complex are central to the epigenetic regulation of gene transcription, an activity that must be maintained and propagated across mitotic cycles and throughout the development of the organism proper in order for cells to establish and maintain their identities. Chromatin occurs in highly-condensed and less spatially concentrated states, known as heterochromatin and euchromatin, respectively. Heterochromatin regions have fewer genes overall than euchromatic stretches of the genome, and many of the genes that are found there remain unexpressed. However, heterochromatin functions as more than a locked closet to store stretches of unused DNA; in fact, there appear to be a number of functionally distinct types of heterochromatin serving in a spectrum of developmentally important capacities, from the transcriptional regulation of cell-type specific genes to genomic self-defense by compartmentalizing and neutralizing foreign mobile genetic elements that might otherwise interfere with proper gene function. Heterochromatin also functions in two genetically silent chromosomal regions: telomeres, which play key roles in replicative senescence and cancer, and centromeres, the linchpins of mitosis.

Using the fission yeast *Saccharomyces pombe* as a model system, Jun-ichi Nakayama focuses on investigations of heterochromatin dynamics, modifications to the DNA-packing proteins called 'histones' in particular, and the molecular mechanisms that allow such chemical states to be heritably transmitted. Histones are the primary protein constituents of the nucleosome, the most basic unit of chromosomal organization, which provides highly compact but readily accessible packaging for a cell's gene-encoding DNA. The amino-terminal tails of histones protrude from the nucleosome and are subject to covalent modifications including phosphorylation, acetylation, and methylation. These histone modifications affect higher-order chromatin structure and influence gene expression.

Heterochromatin also functions in telomeres, which play key roles in replicative senescence and cancer, and centromeres, the linchpins of mitosis

In previous studies, Nakayama demonstrated that heterochromatin protein binding states play a role in the regulation of gene silencing. Nakayama performed detailed analyses of the binding states of Swi6, a homolog of the mammalian HP-1 heterochromatin protein at the silent mating-type (mat) locus of the fission yeast. The results of that study showed that Swi6 protein is a dosage-critical component involved in imprinting the mat locus. This binding of Swi6 is maintained both across mitotic cell cycles and intergenerationally, as it is propagated through meiosis as well. Nakayama's study also showed that the deacetylation and subsequent methylation of a specific histone H3 lysine residue are essential to this process. Chemical modifications to histones are fundamental epigenetic processes that can act to switch the expression of a target gene on or off.

Nakayama has now linked Swi6 function to a number of other chromodomain proteins in fission yeast that seem to act in a stepwise and context-sensitive fashion to silence genes in the process of



A model of heterochromatin formation in fission yeast

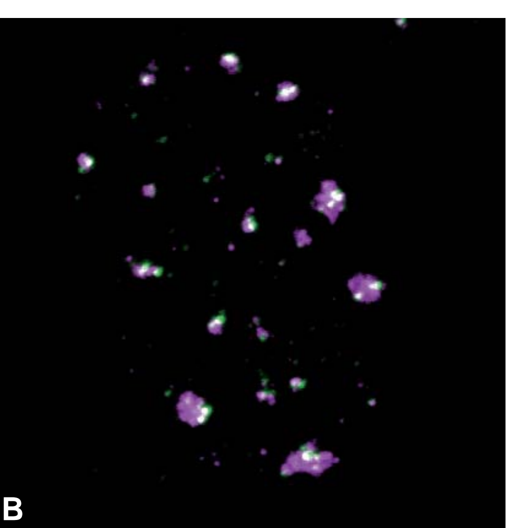
heterochromatin assembly. The number of species of these proteins, which possess characteristic SET or chromodomain sequence motifs, is much smaller in yeast than it is in human, making S. pombe an apt model for studying the basic means by which these molecules respond to the methyl modifications to histone residues. The methylation of specific sites on the histone H3 catalyzed by a SET domain-containing methyltransferase provides epigenetic markers that allow chromodomain proteins to bind the histone and direct it toward eu- or heterochromatin assembly or to initiate developmentally regulated gene silencing.

The Nakayama lab has shown the histone methyltransferase Clr4 to be essential for heterochromatin assembly as an upstream element that helps Swi6 to localize correctly. Two other chromodomain proteins, Chp1 and Chp2, have also been implicated in heterochromatin formation and function. Disruption of the gene encoding Chp1causes defects in centromeric silencing and higher mitotic loss rates in minichromosomes, while *Chp2* mutations result in weak silencing defects in three heterochromatic regions: centromeres, telomeres and the mat locus.

The group has demonstrated that Swi6, Chp1 and Chp2 localize at three heterochromatic regions (centromere, telomere and mat locus), and that this localization is clearly dependent on H3-lys9 methylation mediated by Clr4. Following on a series of experiments in which the gene for each protein was disrupted, Nakayama has developed a model in which Chp1 function is specific to the establishment and spreading of heterochromatin, while Chp2 and Swi6 form a dimer that supports the stable maintenance of heterochromatin. They also found that, among three heterochromatic regions, centromeres are more dynamic and require establishment steps.

Nakayama is also interested in how chromodomain proteins function in higher eukaryotic cells. In previous study, Nakayama found an evolutionally-conserved chromodomain protein is a stable component of histone deacetylase complex in fission yeast. Using mammalian cells, he is now investigating the function of a chromodomain protein which has been linked to cell senescence and development.

Through his work on heterochromatin histone modifications, Nakayama has uncovered potentially important new roles for proteins in the establishment, maintenance and transmission of epigenetic information. These findings show that the definition of a gene as a simple string of DNA nucleotides needs to be expanded to include the action of proteins in the functional genetic unit. In the future, Nakayama plans to perform more detailed analyses of the molecular mechanisms that underlie epigenetic function, as well as studies in higher organisms and epigenetic gene expression in developmental processes.



Localization of a chromodomain protein (green) and phosphorylated form of RNA polymerase II (magenta) in HeLa cell.

Localization of a chromodomain protein (green) and SC-35 (magenta) under alpha-amanitin treatment in HeLa cell.

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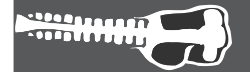


Creative Research Promoting Program

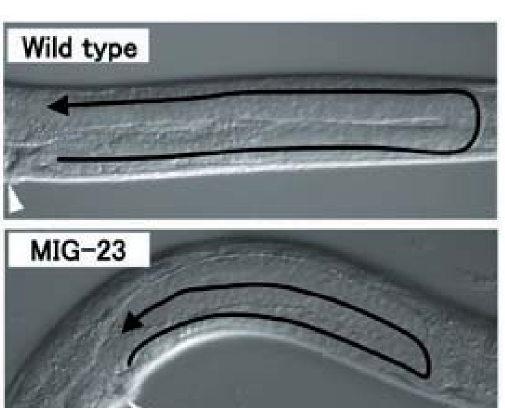
cell migration

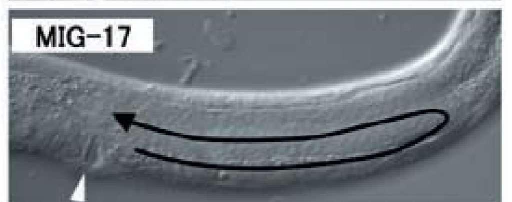
Kiyoji Nishiwaki
Ph. D.

Kiyoji Nishiwaki received his B. Sc. from Osaka City University and M. Sc. from Osaka University where he worked on the molecular biology of the yeast *S. cerevisiae*. He joined NEC Corp. in 1986 as a researcher in the Fundamental Research Laboratory studying the molecular genetics of *C. elegans*. He left NEC in 1992 to work as a visiting researcher at Johns Hopkins University, then returned to the company in 1993 to continue his work on nematode development. He was awarded a Ph. D. by Osaka City University for work on the molecular analysis of *C. elegans* embryogenesis in 1994. He remained at NEC until receiving an appointment as team leader at the RIKEN CDB.

Cell migration and organogenesis

Cell division, cell migration and changes in cell shape are coordinated to generate complex tissue and organ systems during animal development and regeneration. One important aspect of this minutely regulated process is the movement of cells in sheets, an activity orchestrated by mechanisms which remain poorly understood. During organogenesis, sheets of cells migrate and spread over the underlying tissues while maintaining selective adhesive bonds with cells of similar type, and make their way to destinations in the body, following molecular guidance cues generated by mechanisms that remain enigmatic. Kiyoji Nishiwaki studies gonadogenesis in the nematode *C. elegans* as a model system to improve the understanding of the molecular bases of cell migration during organ development. By developing a clearer picture of evolutionarily conserved mechanisms, this research promises to provide insights into cell migration and organ development in humans, and contribute to the understanding of human diseases involving disturbances in coordinated cellular movement.





The U-shaped gonad in the wild type becomes abnormal in *mig-23* and *mig-17* mutants due to the misdirected migration of distal tip cells.

Guidance of the gonad

Interactions between the basement membrane of the migrating gonad and that of the body wall are keystone processes in gonadal development. The *C. elegans* gonad is U-shaped, the result of the directed migration of cells known as distal tip cells present at the leading ends of the developing gonad in larvae. The gene *mig-17*, which Nishiwaki previously showed to be essential to this guided movement, is expressed in muscle cells and secreted into the body cavity where it binds to the migrating gonadal tip cells. Distal tip cells in worms engineered to lack the MIG-17 protein fail to steer cell migration properly, resulting in abnormal development and a 'meandering' gonad phenotype. However, the means by which MIG-17 coordinates the timing and direction of the developing gonad's progress though the larval body has remained unknown.

MIG-17 is an ADAM (a disintegrin and metalloprotease) protease, a family of secreted or membrane proteins first identified as components of various snake venoms, where they have hemorrhagic and

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anti-coagulatory effects. ADAM family proteases have been implicated in a number of human physiological and pathological processes, including blood coagulation disorders, rheumatoid arthritis and asthma. These proteins act to proteolytically release membrane-bound growth factors or to disrupt components of extracellular matrices such as collagen and proteoglycans (which help to build skin and soft tissue).

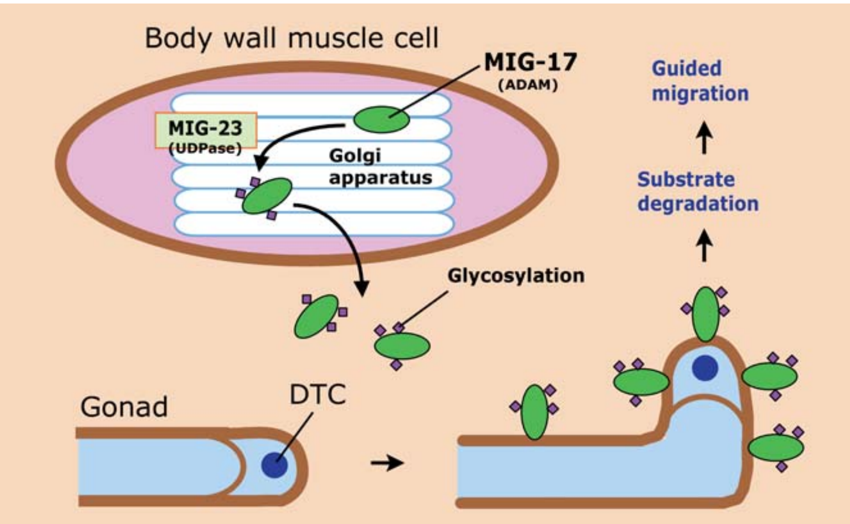
This research promises to provide insights into cell migration and organ development in humans

Nishiwaki's team has analyzed a number of mutations that result in aberrant gonadal phenotypes similar to those seen in *mig-17* mutants, including the mutation of the gene that encodes MIG-23. This protein was found to be structurally related to nucleoside diphosphatases (NDPases), glycosylating proteins found in the Golgi apparatus, the intracellular processing center where proteins are modified, sorted and released from the cell. Glycosylation involves the addition of a sugar to a site on a protein chain, a post-translational modification whose roles are incompletely understood, but which is thought to serve a regulatory function in some signaling molecules. On further investigation, MIG-23 mutants showed reductions in NDPase activity. The wild-type MIG-23 protein successfully rescued yeast mutants for NDPase, lending solid support to the case for the NDPase nature of MIG-23.

The similarity between the MIG-23 and MIG-17 phenotypes suggested that the mutation of MIG-23 might result in abnormal MIG-17 glycosylation. Using gel electrophoresis, Nishiwaki and colleagues demonstrated that MIG-17 molecules in MIG-23 mutants had lower than normal molecular weights consistent with the loss of glycosylation, while immunoblotting studies confirmed that in these mutants MIG-17 failed to bind sugars at the normal levels. MIG-17 molecules designed with deficient glycosylation sites also failed to localize at the gonad and showed no ability to direct its migration. Genetic analyses revealed that MIG-23 is expressed in muscle cells, which reconciles well with MIG-17's muscle cell origins. Taken together, these results indicate that MIG-17 molecules are secreted from muscle cells after glycosylation by MIG-23, a modification which enables their recruitment to the migrating gonad.

Questions and implications

Although Golgi body NDPases such as MIG-23 are capable of modifying a number of other secreted proteins, the Nishiwaki study indicates that the effects of the NDPase dysfunction are especially prominent in the abnormal glycosylation and function of an ADAM family protease, suggesting that glycosylation may play a more important role in the function of proteins in this family than it does in that of other secreted proteins. ADAM family proteases are known to be involved in a number of human diseases, and the question of whether glycosylation defects play a role in their pathogenesis merits further investigation. Research such as this provides insights into basic biological mechanisms that may one day make it possible to treat a range of health conditions by restoring normal ADAM protease glycosylation in cases in which it is aberrant.



MIG-17 is glycosylated by MIG-23 and secreted from the muscle cells to localize on the gonad surface where it is required for the migration of distal tip cells.

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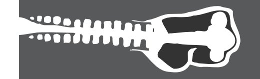


Creative Research Promoting Program

pluripotent cell studies

Hitoshi Niwa
M. D. , Ph. D.

Hitoshi Niwa received his M. D. from Nara Medical University in 1989, and his Ph. D. in medical physiology in 1993 from the Kumamoto University Graduate School of Medicine for his work in gene trap methods. From 1993 to 1994, he worked as a research associate in the Department of Developmental Genetics at the same university, before taking a postdoctoral fellowship with Austin Smith at the University of Edinburgh Centre for Genome Research. He returned to Japan in 1996 as a research associate in the Department of Nutrition and Physiological Chemistry at the Osaka University Graduate School of Medicine, where he remained until taking his current position at the RIKEN CDB.

Self-renewal and pluripotency

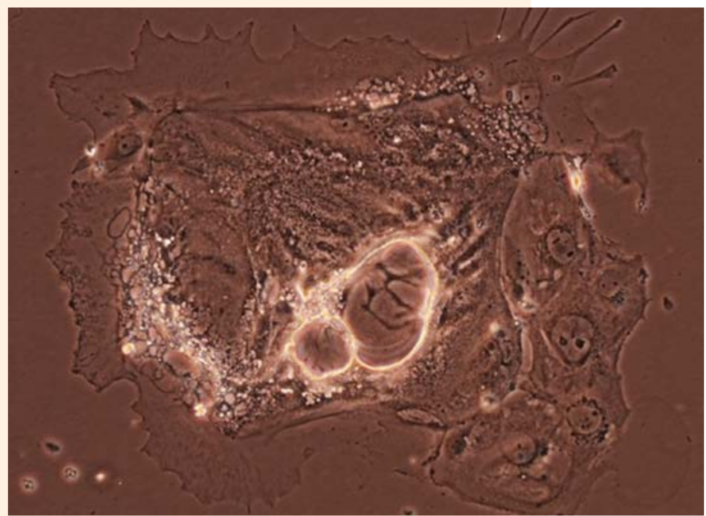
Their ability to self-renew indefinitely and to differentiate into cells of all three germ layer types (a differentiative capacity termed 'pluripotency'), makes embryonic stem (ES) cells one of the most promising subjects of study in regenerative medicine, as well as an attractive model system for research into a spectrum of developmental processes. However, the mechanisms by which ES cells are able to maintain these capabilities are incompletely understood, and a better understanding of the stemness of these cells will be necessary in order to be able to take best advantage of their remarkable properties.

Two of the biggest challenges that now face stem cell research are the determination of the factors that allow ES cells to generate limitlessly self-renewable progeny, and the identification of molecules that direct the dividing ES cell to produce daughter cells of specific types. Hitoshi Niwa's research addresses both of these challenges, with the aims of developing solid scientific foundations and reliable technologies to support this exciting field of biomedicine.

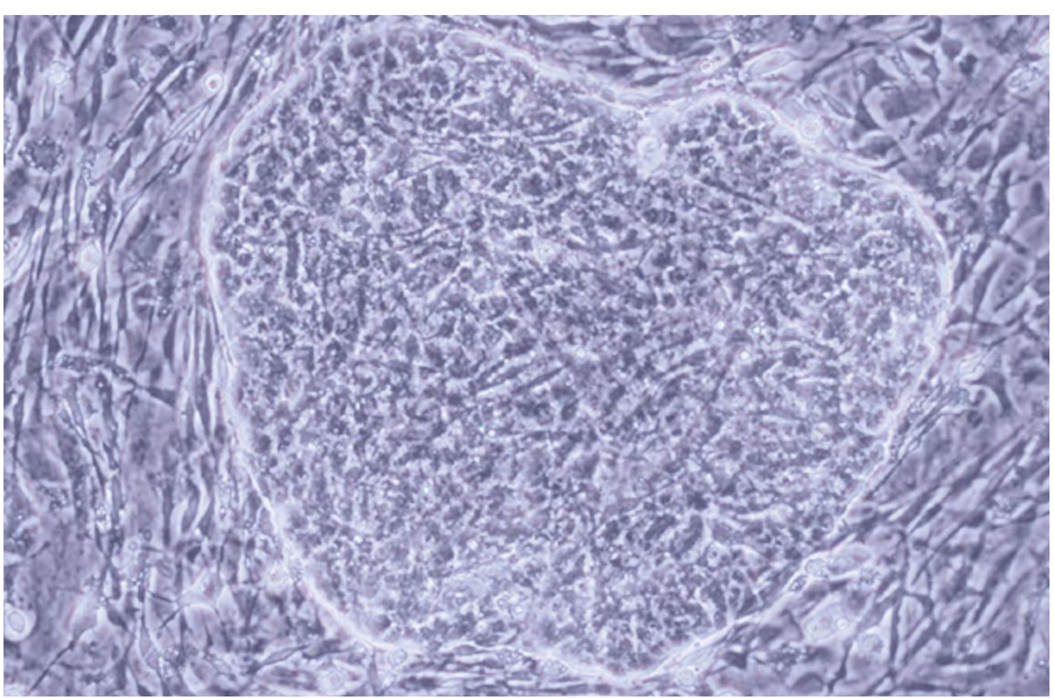
Inducing differentiation

The development of methods by which undifferentiated ES cells can be prompted to commit to a specific cell lineage is a field of central importance to the stem cell research community. Studying factors identified in work on knockout mice, the Niwa research team has been engaged in the analysis of transcription factors with potential roles in the development of extraembryonic cell lineages, extraembryonic endoderm and trophectoderm. Extraembryonic endoderm derivatives include the parietal and visceral endoderm, which give rise to the yolk sac covering embryos in utero during development, and trophectoderm derivatives, the source of the placenta that sustains the developing mammalian embryos.

In previous research, the Niwa lab showed that the overexpression of GATA transcription factors such as Gata-4 or Gata-6 resulted in the specific conversion of undifferentiated ES cells into extraembryonic endodermal cells, and that the exogenous



Terminally differentiated trophoblast giant cells derived from ES cells, showing large, flat morphologies and polyploidy



Trophoblast stem (TS) cells, derived from ES cells, grown on feeder cells in the presence of recombinant FGF4

expression of either of these factors simultaneously induced the expression of the other from the endogenous gene. New work suggests that the homeobox gene *Cdx-2* can serve as a similar trigger for the induction of trophectoderm from ES cells expressing low levels of the pluripotency maintaining gene, Oct 3/4. Using regulable activation of Cdx-2 in vitro, Niwa successfully induced ES cells to differentiate into trophoblast stem cells. These results, in combination with the GATA study, have led to the development of a model in which Oct 3/4, when maintained at an appropriate mid-range level, inhibits the differentiation of ES cells into either the trophectodermal or primitive endodermal lineages by suppressing Cdx-2 and Gata-6, respectively.

Maintaining pluripotency

The POU-family transcriptional regulator Oct 3/4 was the first factor found to elicit multiple differentiative outcomes dependent on its expression level. ES cells expressing median levels of Oct 3/4 maintain their pluripotency, while its overexpression results in differentiation into primitive endoderm and mesoderm and its inhibition causes the cells to take up a trophectodermal fate. These findings established Oct 3/4 as a primary regulator of pluripotency in ES cells, but the function of this regulator in the commitment of more specific lineages remains an open issue.

In addition to its function as a maintainer of pluripotency, Oct 3/4 may also play a second key role by regulating neuronal differentiation

In 2003, the Niwa lab contributed to a study that demonstrated a role for Oct 3/4 in neurogenesis promoted by stromal cell derived inducing activity (SDIA, see p. 18-19 for a description of this phenomenon). The researchers found that SDIA acts to maintain Oct 3/4 expression in ES cells, which appears to be important as a promoter of the differentiation of ES cells into neural lineages. ES cells from which Oct 3/4 had been deleted lost their ability to differentiate into neurons, while heightened levels of Oct 3/4 intensified the neurogenic effects of SDIA. This finding raises the possibility that, in addition to its function as a maintainer of pluripotency, Oct 3/4 also plays a second key role by regulating neuronal differentiation in a concentration-dependent manner.

ES cell growth in culture

This goal of controlling culture conditions to achieve specific outcomes is important to the growth of ES cells in vitro as well. The Niwa lab is working to develop a serum- and feeder-cell-free system for culturing mouse ES cells, which necessitates developing a detailed picture of both the extrinsic factors and the intrinsic networks that function in these cells in culture. It is known that a single ES cell in isolation will fail to proliferate, while colonies of such cells grow normally. It is also known that even isolated single cells can be induced to proliferate if the culture medium from a larger ES cell colony is transferred to the single cell's plate, suggesting that ES cells produce a growth-stimulating factor that acts by community effect. Niwa has developed an assay system to isolate candidate molecules for this putative 'stem cell autocrine factor' (SAF), and is actively pursuing the characterization of the most promising candidates. The results of these analyses should improve the ability of researchers to grow ES cells in culture, facilitating the study of these fascinating and potentially revolutionarily important cells.

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Publications

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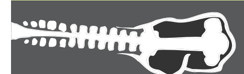


Creative Research Promoting Program

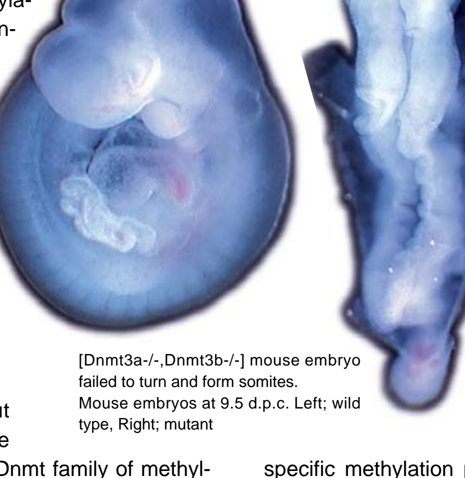
mammalian epigenetic studies

Masaki Okano
Ph. D.

Masaki Okano received his baccalaureate and master's degrees from Kyoto University, and his doctorate in biochemistry from the Research Division in Agriculture at the same institution in 1994. He spent the period from 1994 to 1995 as a research fellow in cell biology at the Nagoya University BioScience Center before moving to Massachusetts, where he worked as a research fellow in medicine at Harvard Medical School and Massachusetts General Hospital from 1995 to 2002, serving as an instructor in medicine at Harvard for the last two years of that period. He was appointed team leader at the RIKEN Center for Developmental Biology in 2001, and returned to work in Japan full-time in 2002.

The methylation of mammalian DNA plays an important role in determining gene expression. The number and locations of methyl tags provide molecular icons marking genes as inactive, or in some cases, active. Certain types of protein recognize and bind to the tagged DNA, affecting the expression patterns of the genes it encodes. During embryonic development, DNA methylation patterns are established in a sequence of steps involving both the removal of methyl tags at the preimplantation stage and the establishment of new sets of tags, a process known as de novo methylation, which occurs after implantation and again during gametogenesis. These methylation patterns can subsequently be maintained and transmitted across generations by maintenance methylation.

Masaki Okano's research concentrates on the mechanisms by which DNA methylation is established and maintained throughout development, focusing on the properties and activity of the Dnmt family of methyltransferases, which includes Dnmt1, Dnmt3a and Dnmt3b. He particularly focuses on working out and clarifying the roles of de novo methyltransferases in embryogenesis and cell differentiation, seeing these enzymes as necessary agents in maintaining the proper balance between stability and plasticity in the expression of specific genes.



[Dnmt3a-/-,Dnmt3b-/-] mouse embryo failed to turn and form somites.
Mouse embryos at 9.5 d.p.c. Left; wild type, Right; mutant

Dnmt3a and *Dnmt3b*

Methylation lays down developmentally significant gene expression patterns that tend not to alter once established. In previous work, Okano showed that *de novo* methylation in mice required the expression of a pair of methyltransferase genes, *Dnmt3a* and *Dnmt3b*, at a very early stage of embryonic development, as well during gametogenesis. Prior to this finding, it was believed that a related gene, *Dnmt1*, was solely responsible for the methylation of DNA. Using *lacZ* reporter gene studies, Okano showed that these genes are expressed in early embryonic and ES cells, suggesting that they function in the epigenetic reprogramming of DNA methylation states. Based on these findings, it is now known that *Dnmt1* functions primarily in methylation maintenance, which is necessary for the stable inheritance of tissue-specific methylation patterns, but that *Dnmt3a* and *Dnmt3b* are the principal determinants in initiating DNA methylation. Recent work has also shown that isoforms of Dnmt3a and Dnmt3b function as maintenance methyltransferases in mouse embryonic stem cells, indicating a broader role for these proteins.

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As de novo methylation activity takes place mainly in embryonic stem (ES) cells, early postimplantation embryos and gametogenesis, ES cells provide a highly suitable experimental model system for investigating the molecular mechanisms underlying the establishment of new methylation patterns. Okano is studying the consequences of conditional knockout and overexpression of methyltransferase genes on mouse development using ES cells and transgenic mice, in the hopes of uncovering links with tumorigenesis, congenital nervous system defects and abnormal cell differentiation.

Developmental roles of methylation

Knockout mutations of *Dnmt3a* and *Dnmt3b* result in a spectrum of organic, neurological and spermatic defects. *Dnmt3b* mutants generally die by embryonic stage 16.5 due to widespread defects affecting organ development and general growth. Loss of *Dnmt3a* function produces less pronounced effects; embryogenesis appears normal, but most mutants die by the fourth week after birth and show abnormal growth, intestinal motility and spermatogenesis. The deletion of both genes results in developmental arrest at around E8.0, prior to somitogenesis, and early embryonic lethality by E9.5. These loss-of-function defects indicate that the establishment of the DNA methylation state plays an important part in many aspects of mouse development, an area the Okano lab will continue to explore.

> **The establishment of the DNA methylation state plays an important part in many aspects of mouse development**

Abnormal methylation has been implicated in a number of human congenital anomalies as well. ICF (Immunodeficiency-Centromeric instability-Facial anomalies) syndrome, which is characterized by immune deficiency, heterochromatin instability and defects in facial development, results from a mutation in the gene encoding Dnmt3b. A mutation in the gene encoding the methyl-cytosine binding protein, MeCP2, is also known to cause Rett syndrome, a neurological disorder that occurs almost entirely in females. In this form of mental retardation, development appears normal until the child is 6-18 months old, after which the child loses communication skills and develops disturbances in gait and use of the hands and slowed growth of the head.

In the future, the Okano lab will study how epigenetic reprogramming works at a molecular level. By identifying the genes involved in *de novo* DNA methylation, they hope to discover the means by which it is determined what genes will be methylated. Comparisons of the expression patterns of such genes across germ layers, or in normal mice against clones created from somatically derived cells should lead to new insights in the context-specificity of the DNA methylation process as well as the roles of methylation in development, health, and regeneration.

Dnmt3b expression in the embryonic ectoderm and the chorion shown by IRES-βgeo marker driven by the endogenous Dnmt3b promoter.

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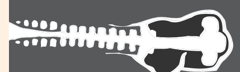

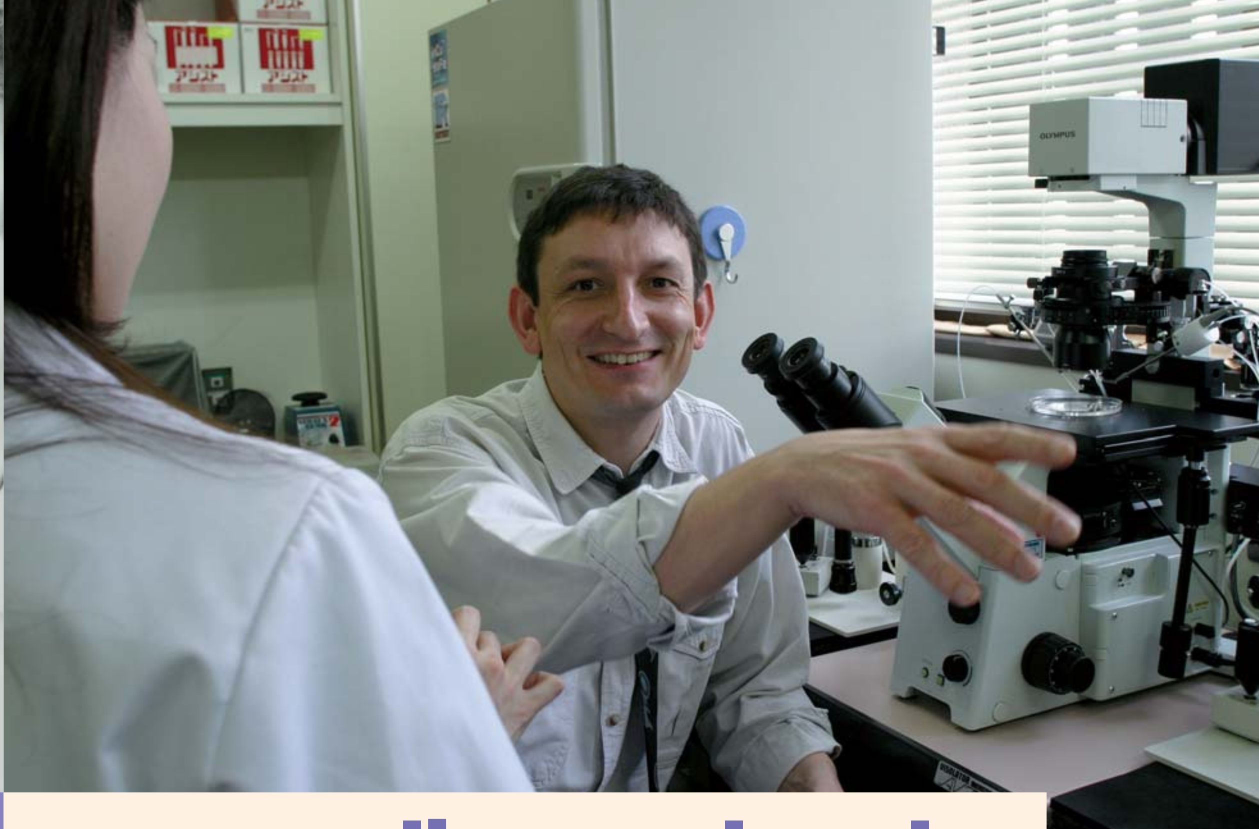

Creative Research Promoting Program

mammalian molecular embryology

Tony Perry
Ph. D.

Tony Perry received his B.Sc. in Microbiology from the Department of Bacteriology at the University of Bristol, England and his doctorate from the University of Liverpool four years later. In 1989 he became a postdoctoral fellow working on epididymal sperm maturation at Bristol University and in 1996 won a European Molecular Biology Travel Fellowship to work in the laboratory of Ryuzo Yanagimachi on the mechanism of oocyte activation, which remains one of his research interests. From there, Dr. Perry moved first to the Rockefeller University and then to the company Advanced Cell Technology, working primarily on novel methods of genome manipulation. In 2002 Dr. Perry took his present position as Team Leader of the Laboratory of Mammalian Molecular Embryology at the RIKEN CDB, where he works on mechanisms in mammalian preimplantation embryos.

By mechanisms still unknown, fertilization achieves the transformation of two highly specialized cells — a sperm and an unfertilized egg (oocyte) — to a state of totipotency: a single-cell embryo that can give rise to an entire individual. The first moments of this remarkable process are known as oocyte activation, an intricate orchestration of sub-cellular events that include all the checks and balances that presage healthy growth of a new embryo. Oocyte activation can be observed at the light microscope level as dramatic change in the morphology of the newly fertilized egg and provides an incredible read-out of the underlying molecular mechanisms. The Laboratory of Mammalian Molecular Embryology combines molecular and cellular biology with piezo-actuated micromanipulation of mouse (*Mus musculus*) gametes and embryos to study oocyte activation and its developmental consequences.

Interactions between gamete cytoplasms

Microinjection of an isolated sperm head into an oocyte can result in the birth of normal young. Thus, the sperm head — the part of the head that lies beneath its membranes — apparently contains all of the paternal information sufficient for full development. Tony Perry is systematically elucidating interactions between sub-membrane sperm head components and the oocyte at fertilization, particularly during the moments soon after the sperm has entered the oocyte. The Perry lab's interests center on attributing molecular identities to the proteins involved in these interactions and characterizing them functionally.

This task is large, because beneath the membrane of a sperm head reside macromolecular complexes that include a nucleus (containing the paternal genome and associated proteins) and a surrounding cytoplasmic matrix, the perinuclear matrix (PNM). It has been estimated that the PNM comprises some 230 protein species. Because it is juxtaposed to the inner leaflet of enshrouding sperm head membranes, the PNM rapidly comes into contact with the oocyte cytoplasm at fertilization and is an immediate source of paternally-contributed molecules that potentially modulate development. However, owing in part to its size and complexity, its excoriation and disassembly of the PNM takes hours to complete within the oocyte activation period. During this time, it is likely to release molecules that may be placed in one of several categories, according to whether they (i) have an immediate function in neoembryogenesis, (ii) are stored for later use, or (iii) are targeted for rapid proteolytic degradation. Each category implies an intimate series of interactions with the surrounding cytoplasm. However, to date, few proteins of the PNM have been described, and the function of almost none of them is known. This represents an extraordinary gap in the understanding of a conserved biological structure.

An early model of the sperm-borne oocyte activating factor

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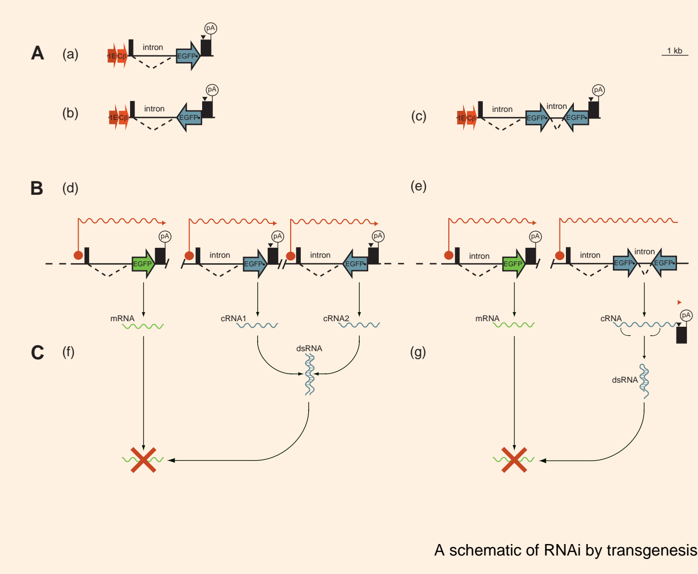

A schematic of RNAi by transgenesis

Molecular identities of PNM proteins

Protein id	Role	Reference
Calicin (PT60)	F-actin-interacting; provides rigidity	Longo et al., 1987
Cylicin I	Role unknown	Hess et al., 1993
Cylicin II	Role unknown	Hess et al., 1995
Arp-T1	Actin-related; role unknown	Heid et al., 2002
Arp-T2	Actin-related; role unknown	Heid et al., 2002
SubH2Bv	Histone H2B variant	Aul and Oko, 2002
PERF15	Lipophilic transport protein-related; role unknown	Oko and Morales, 1994
Stat4	Jak-Stat signaling; role unknown	Herrada & Wolgemuth, 1997
c-Yes	Protein tyrosine kinase ; role unknown	Leclerc and Goupil, 2002
PT32	WBP2, a c-Yes BP; role unknown	Gong et al., 1997
p30	Calmodulin BP; role unknown	Leclerc and Goupil, 2000

The Perry team's analysis of sperm-oocyte interactions at fertilization begins with demembranated sperm that are subsequently exposed to standardized conditions that recapitulate key aspects of the oocyte cytoplasm. Proteins solubilized in this way can be injected into eggs, typically by piezo-actuated microinjection, to probe their function. Such functional analysis is coupled to molecular analyses that span several disciplines. The proteins can be purified and identified; Perry's team employs chromatographic and state-of-the art 2D electrophoretic methods to this end. Antibodies raised against peptides identified in this way are used to localize the proteins before, during and after fertilization; antibody function can also be assayed by microinjection. Owing to dissimilarities between frog and mammalian oocytes and the paucity of material obtainable from the latter, genetic methods are used to identify mouse oocyte proteins that interact with the sperm proteins that have been characterized. Collectively, these studies hold forth the promise of enabling the identification of oocyte signaling pathways and processes that become operational at fertilization and establish what roles they play in subsequent development. Such roles may not be restricted to short-term development, as a growing body of evidence indicates that they have far-reaching consequences, even after the resulting adult is in old age.

Novel methods of genetic modulation using gametes

Utilizing the integrated molecular, cellular, and embryological approach from the work in his laboratory, Perry expects to gain insights that will enable us to genetically alter embryos in new ways. He has developed a novel method of genome manipulation known as metaphase II (mII) transgenesis. This efficient method of transgenesis works when oocytes (normally arrested at mII) are co-injected with a nucleus and transgene (tg) DNA. Typically, sperm heads are depleted of their membranes by detergent extraction prior to mixing with tg DNA and co-injection. This has several advantages over lentiviral methods of tg introduction; cloning and propagation in viral vectors is not required and the method lends itself to tgs in the megabase range, which is too large for lentiviral delivery. Perry and members of his lab are adapting the method to facilitate high throughput targeted knock-out and -down phenotypes. Developing these approaches is an important supplementary part of their work to facilitate the molecular dissection of sperm protein function in the context of embryos and whole animals.

To date, few PNM proteins have been described, and the function of almost none of them is known

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Creative Research Promoting Program

embryonic induction

Hiroshi Sasaki
Ph. D.

Hiroshi Sasaki received his B. Sc. in zoology from the University of Tokyo, and his master's and Ph. D. in developmental biology from the same institution. He worked as a research associate in Atsushi Kuroiwa's lab at Tohoku University from 1990 to 1992, and in Brigid Hogan's lab at Vanderbilt University from 1992 to 1994. He returned to Japan to take a position as assistant professor at Osaka University beginning in 1995, where he remained until his appointment as team leader of the Laboratory for Embryonic Induction at the RIKEN Center for Developmental Biology.

Signaling centers in mammalian development

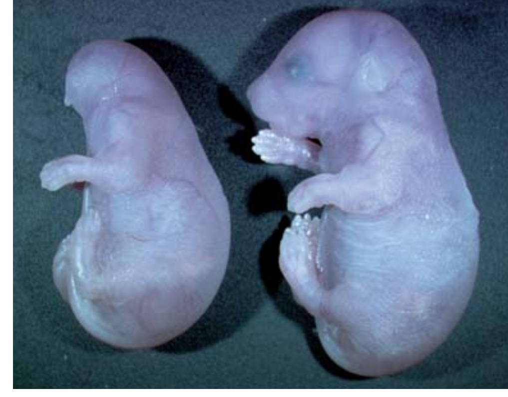
The earliest stages of mouse embryogenesis play out over the course of a few days as a series of dynamic transformations from a simple, nearly featureless blastocyst to a recognizable vertebrate body by the end of gastrulation. This rapid and striking transition is achieved by the highly coordinated differentiation of cells toward specific fates, a feat orchestrated by a number of organizing loci, collectively known as 'signaling centers.' These centers produce diffusible proteins that serve to regulate embryonic development, guiding neighboring cell communities to take up the roles they will play in the adult animal, or steering them away from inappropriate fates.

These recent findings suggest a new model to explain node induction

Hiroshi Sasaki's research team investigates the network of signaling centers that work to induce normal development in mammals. The node, a center responsible for induction of body parts in the mouse, is a particular focus of Sasaki's research. He has identified a number of transcription factors involved in the formation and the function of the node in previous studies, and he remains intent on solving questions of how these centers are formed, and how they set courses for the developing embryo to follow.

Activation of nodal differentiation

Foxa2 (also known as Hepatocyte Nuclear Factor 3β, HNF3β) is a highly-conserved transcription factor required for the formation of embryonic signaling centers in the mouse. The Sasaki lab is engaged in an analysis of molecules that activate *Foxa2* expression in both node and notochord. Members of the Sasaki team previously identified the key regulatory element of the *Foxa2* enhancer, CS3, and have now determined that this element binds to a member of the TEAD/TEF family of proteins, four transcription factors found in both mice and humans, which share a common DNA-binding domain. TEAD proteins are widely expressed in the mouse embryo and their activity is regulated by interaction with multiple transcription factors and co-activators. Complementary studies of TEAD activation showed that increases or decreases in TEAD activation resulted in corre-



headshrinker mutant lacks head induction by the signaling center, and shows headless phenotype at birth (left).

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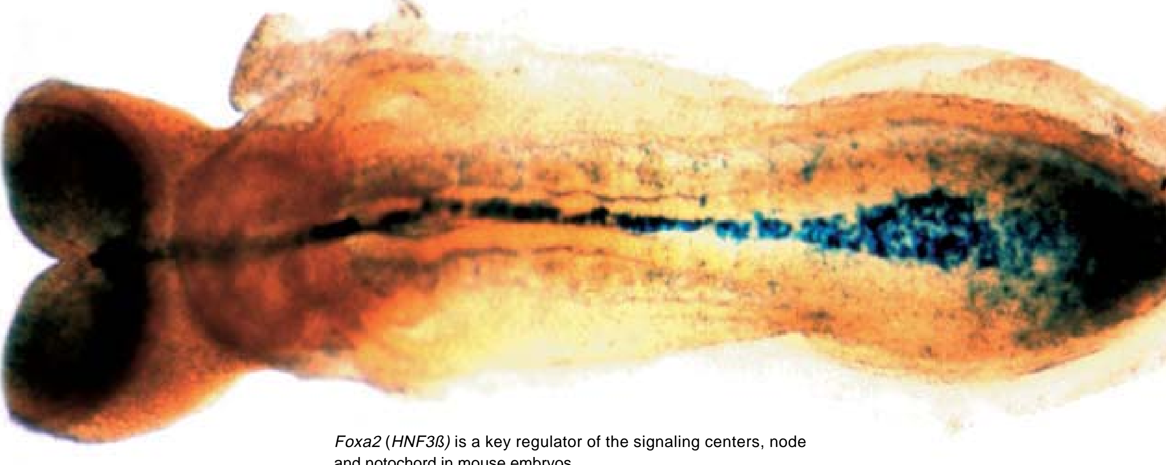
sponding increases or decreases in the area of *Foxa2* expression. *The Foxa2* enhancer element CS3 (which binds with TEAD) functions downstream of Wnt, a known node-inducing signal and the synergistic activation of *Foxa2* by TEAD and Wnt has also been demonstrated in zebrafish, indicating that this pathway has been conserved in at least some other vertebrate taxa as well. A second signaling factor known to work in node induction, Nodal, has also been linked to TEAD activity. These recent findings suggest a new model to explain node induction, in which TEAD complements the function of multiple activating co-factors from primary signaling centers by activating the enhancer element CS3 to induce *Foxa2* expression.

headshrinker

Although a variety of mutations can cause the head to fail to develop properly, it is rare for embryos in which the head is entirely lacking to develop to full term. However, a limited number of such headless birth phenotypes have been reported, all of which have been linked to mutations in node-related genes, such as *Lim1* and *Otx2*. The fortuitous discovery of a previously unknown headless phenotype in the mouse prompted Sasaki to investigate the genetic cause of this mutation, which he named headshrinker (*hsk*), and to study the gene's role in the induction of the head. Gene expression analysis revealed losses in the expression of important head-organizing genes in *hsk* mutants, resulting in the impaired function of the late-stage head organizer or the prechordal plate, a fore/midbrain-inducing mesenchymal mass that forms at the anterior end of the developing embryo.

Northern blotting showed reduced levels in the expression of the mRNA for the gene *Ssdp1* (a sequence-specific, single-stranded-DNA-binding protein) in *headshrinker* mutants, and the researchers found that they could rescue the headless phenotype by using *Ssdp1c*DNA. *Ssdp1/hsk* encodes a protein that binds with Ldb1, which itself binds LIM-homeodomain proteins and other developmentally important transcription factors. Sasaki proposes a model for the interaction of these three co-factors in which Sspd1 enhances the activation of target genes by the Lim1-Ldb1 complex. In an analysis using a labeled reporter gene, Sspd1 alone showed almost no activity, but boosted the activity of both Lim1 and Ldb1 on co-expression, and had the strongest effect when all three factors were present. In addition to the lethal loss of the head, *hsk* mutants exhibit a range of other developmental abnormalities, implicating Ssdp1 as a co-activator molecule with multiple and diverse embryogenetic roles.



Foxa2 (*HNF3ß)* is a key regulator of the signaling centers, node and notochord in mouse embryos.

Sasaki continues to pursue a deeper understanding of the structure and function of signaling centers using an array of approaches and techniques. Other ongoing work in the lab is aimed at isolating cells from transgenic mice engineered to express *LacZ* in the node and notochord, which promises to speed the identification of new genes specifically expressed in those regions.

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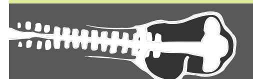


Creative Research Promoting Program

cell fate decision

Hitoshi Sawa
Ph. D.

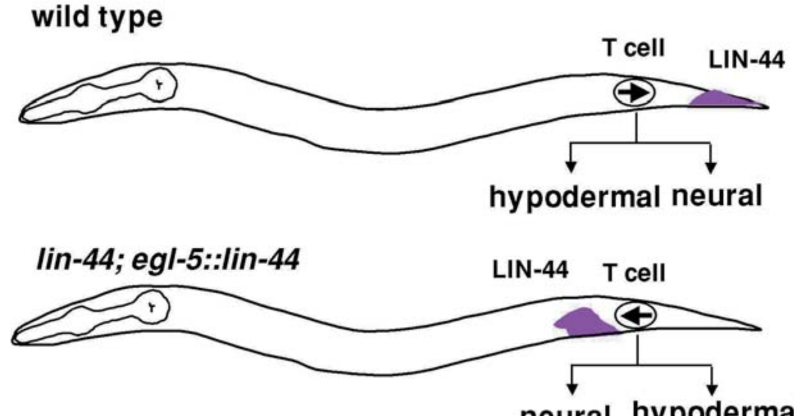
Hitoshi Sawa obtained his baccalaureate, master's and doctoral degrees from Kyoto University in the period from 1982 to 1991. He worked as a postdoctoral fellow at the California Institute of Technology on a Human Frontier Science Program grant during the period from 1991 to 1994, then at the Massachusetts Institute of Technology from 1994 to 1997 under support from the Howard Hughes Medical Institute. He returned to Japan in 1997 to continue his work at Osaka University, first as a postdoc, then as researcher funded by the Japan Science and Technology Corporation PRESTO program. He took his current position at the RIKEN CDB in 2001.

Asymmetric cell division and cell diversity

The animal body is constructed from the myriad and multiform progeny cells from a single fertilized egg. Asymmetric cell division, in which a single mother cell splits to generate daughter cells with discrete characters, is the fundamental process by which cellular diversity is achieved, but remains imperfectly understood at the molecular level. Using the nematode *C. elegans* as a model system, Hitoshi Sawa is engaged in the study of the genetic and molecular interactions that underlie asymmetric division, and the related phenomenon of cellular polarity, in which the contents of individual cells are distributed unevenly, giving distinct characters to daughter cells on division. He seeks also to resolve the ways in which transcription-regulating complexes integrate the intracellular signaling networks that collaborate to help determine cell fate.

T cell polarity

The *Wnt* pathway represents one of the most highly conserved and widely adapted signaling systems in biology, playing developmentally critical roles in the regulation of cell fate, proliferation and polarity in species from worm to human. Wnt signaling, in fact, transpires along multiple divergent routes. In the canonical Wnt pathway in *Drosophila* (the species in which it was first described), Wnt binds to the transmembrane receptor Frizzled, which activates a series of molecules and molecular complexes within the cell, thereby preventing the degradation of β-catenin and allowing it to accumulate and signal transcription factors that act on the pathway's ultimate downstream gene targets. Studying the effects of *lin-44* (a *Wnt* homolog) on asymmetric division in the nematode, the Sawa team ectopically expressed and examined its effects on the division of T cells, which form a diverse lineage of hypodermal and neural cells arising from a single progenitor. In normal development, the anterior daughter of the parent T cell produces hypodermal cells, while the posterior daugh-



Ectopic expression of LIN-44/Wnt anterior to the T cell strongly reverses the polarity of T cell division.

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ter produces neural cells. In *lin-44* mutants, however, the opposite is often the case, with anterior daughters frequently giving rise to neural cells, and posterior daughters generating cells in the hypodermal lineage. This phenotype is attributable to a reversal in parent cell polarity, which occurs in about 70% of *lin-44* mutants. Misexpression of *lin-44* at the anterior of the T cell in *lin-44* mutants caused a significant enhancement of the reversal phenotype; 97% of such mutants showed switching of fates in T cell daughters. These results demonstrate that the orientation of T cell polarity is regulated by the LIN-44 signal.

Sawa is engaged in the study of the genetic and molecular interactions that underlie asymmetric division and cellular polarity

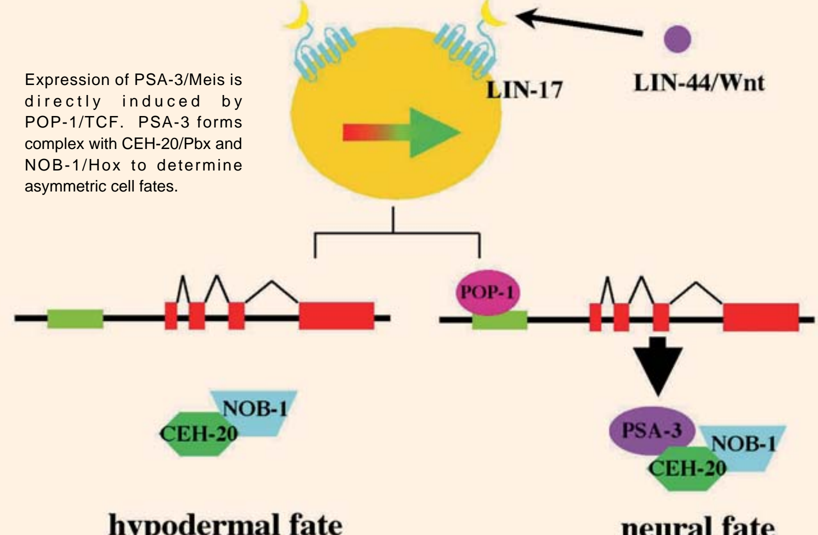
A pathway homologous to the Wnt cascade exists in *C. elegans*, and it has been suggested that a number of downstream factors corresponding to many of the most prominent *Wnt* signaling components also play roles in roundworm asymmetric cell division. POP-1, the *C. elegans* homolog of TCF/LEF-1, a transcription factor acting downstream of Wnt, has recently been shown to be required for asymmetric T cell division. In the canonical Wnt pathway, TCF/LEF-1 is a target of β-catenin, but the direct involvement of β-catenin in *C. elegans* asymmetric cell division has never clearly been demonstrated. Researchers in the Sawa lab found that *wrm-1*/β-catenin mutants show defects in asymmetric T cell division, similar to those observed in *lin-17* mutants, suggesting that that the asymmetric cell division is controlled by β -catenin.

Factors in asymmetric T cell division

Additional work has also focused on T hypodermal cells, which in normal development divide asymmetrically to produce non-identical daughter cells of neural or hypodermal fate. Recent work by the Sawa team has shown that three genes, *psa-3*, *ceh-20* and *nob-1* (homologs of *Meis*, *Pbx* and *Hox*, respectively) are involved in this asymmetric T mitosis, as mutations in any of these genes result in the abnormal loss of asymmetry in cell divisions, in which the posterior daughters of the T cell are hypodermal rather than neural in character.

Investigations into the role of PSA-3 uncovered that expression of PSA-3 is stronger in the posterior T cell daughter. *psa-3* expression is dependent on a consensus binding site within its promoter region for the transcriptional factor POP-1/TCF, which has previously been reported to be required for asymmetric T cell division, suggesting that the *psa-3* gene is a direct target of *Wnt* signaling and functions as a cell fate determinant in asymmetric cell division. The asymmetry of PSA-3/Meis expression is also perturbed in *lin-44*/*Wnt* and *lin-17*/*Frizzled* mutants, corroborating the argument for a downstream role for *psa-3* in the Wnt pathway.

Expression of *psa-3*/*Meis* in the T cell lineage is also markedly reduced in *nob-1*/*Hox* and ceh-20/Pbx mutants, indicating that, NOB-1/Hox and *CEH-20*/*Pbx* also act in the regulation of *psa-3*/*Meis* expression. Taken together, these data indicate that the expression of *psa-3*/*Meis* is controlled by binary mechanisms; Wnt signaling establishes cellular polarity, while the Hox-Pbx complex regulates positional identity. In other metazoans, both Meis and Pbx are known to bind directly as co-factors to Hox, raising the possibility that 20/Pbx and NOB-1/Hox proteins may form a ternary complex with PSA-3/Meis in *C. elegans* as well. In vitro studies show that PSA-3/Meis interacts directly with CEH-20/Pbx and NOB-1/Hox, lending support to the model in which PSA-3/Meis functions as a cell fate determinant by forming a complex with CEH-20/Pbx and NOB-1/Hox in asymmetric cell division of the T cell.



Expression of PSA-3/Meis is directly induced by POP-1/TCF. PSA-3 forms complex with CEH-20/Pbx and NOB-1/Hox to determine asymmetric cell fates.

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Creative Research Promoting Program

functional genomics

Asako Sugimoto
Ph. D.

Asako Sugimoto received her B. Sc. degree from the Department of Biophysics and Biochemistry in the University of Tokyo School of Science in 1987, and her doctorate from the same institution in 1992. She worked as a postdoctoral fellow in Joel Rothman's laboratory in the University of Wisconsin – Madison from 1992 to 1996, before returning to Japan to assume an assistant professorship at the University of Tokyo. She remained in that position until 2002, pursuing concurrent work as a Japan Science and Technology Corporation PRESTO researcher from 1997 to 2000. She was appointed team leader at the RIKEN CDB in 2001.

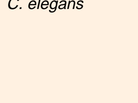
Interactions between genes at the network level are of fundamental importance in instructing the development and function of multicellular organisms. While the characterization of genomes at the level of the solitary gene remains an important challenge in many yet unsequenced organisms, the analysis of how genes function in networks is now in increasing demand for those genomes that are already available. In the post-genome era, scientists seek to understand the roles of genes in evolution through comparative genomics studies as well as to characterize genes in isolation and understand their functions in the context of interactive networks, a field of investigation known as functional genomics.

Asako Sugimoto has adopted the nematode *Caenorhabditis elegans* as an experimental model to take advantage of its tractability to the systematic functional analysis of its genome using unique high-throughput screening techniques. By studying the interactions between the approximately 19,000 genes predicted for *C. elegans*, the Sugimoto research team seeks to identify the means by which sets of genes working in combination help to establish and direct developmental processes. The lab also looks to take the findings from these studies as a base for advancing the understanding of developmentally important mechanisms. Sugimoto hopes that by opening windows into the role of networked genes in guiding development in a simple worm, new light will be shed on universal mechanisms in the genetic regulation of the developmental program.

Caenorhabditis elegans
(adult hermaphrodite)

RNAi-based profiling of gene function

The wild-type nematode is built from precisely 959 somatic cells, yet this simple organism exhibits a wide range of the specialized cell types, such as muscles and neurons, that characterize more highly derived species. And, thanks to the complete knowledge of the lineage of every cell in the *C. elegans* body, the differentiative pathway of every one of those cells can be followed from its origin in the fertilized egg to its role in the fully-grown adult. The amenability of this worm's sequenced genome to reverse-genetic techniques has also served to make it one of the standard model organisms in the world of genetics research. The discovery in the late 1990s that the introduction of double stranded RNA (dsRNA) could be used to



Embryogenesis of
C. elegans

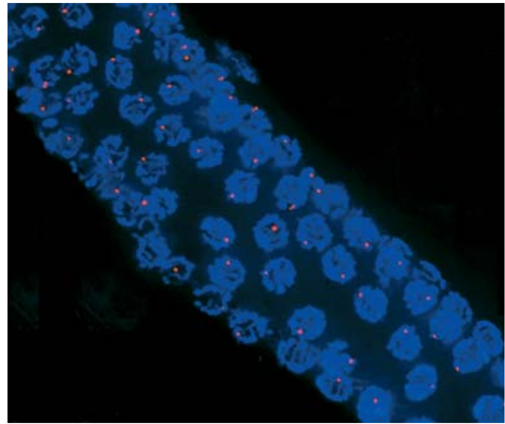
knock down the expression of specific genes in the nematode has only added to its appeal, giving scientists the ability to inhibit gene function without disturbing its underlying DNA. Sugimoto has refined this technique of RNA interference (RNAi) by developing a method in which nematodes directly uptake dsRNA in solution. This process of 'RNAi by soaking' offers greater efficiency and ease-of-use than other RNAi methods and has enabled systematic high-throughput studies of gene suppression to be conducted more rapidly than ever before possible.

Starting with a cDNA library of approximately 10,000 genes expressed in developmental processes, the Sugimoto lab used RNAi to knock down each gene's function and has started to construct a database in which the resulting phenotypes are sorted by developmental outcome. To date, nearly 6,000 phenotypes have been categorized, and it was found that loss of function in more than 25% of these genes resulted in lethal, morphologically altered or sterile phenotypes. Many lethal phenotypes result in developmental arrest and death at very early stages, which typically prevents the study of the underlying gene's function in later development. By exposing worms to RNAi-inducing dsRNA at the L1 stage of larval development, Sugimoto is able to study the post-embryonic function of genes that are lethal when knocked down in embryos. This is critically important to the understanding of genetic networks in post-embryonic development, as more than one out of every ten genes analyzed by the lab so far is essential for embryogenesis. Sugimoto's studies to date show that approximately 50% of all such genes play post-embryonic roles as well, supporting the concept that a great number of genes play multiple roles at different stages in development.

Phenotype analysis

The data sets produced by high-throughput phenotype analyses can be formidably large and unwieldy, and the lack of standardized descriptive terms and categories can limit the accessibility to the wealth of information they contain. To improve the value, ease-of-use and distributability of her RNAi phenotyping results, Sugimoto has developed a taxonomic system and nomenclature to enable the precise and consistent description of embryonic and post-embryonic phenotypes using a checklist of more than 50 identifying traits, such as abnormalities in cell division, cellular differentiation, organogenesis, morphogenesis, growth, and movement. She plans to make these RNAi phenotype profiles available to the *C. elegans* research community, in the hopes of establishing a universal taxonomy for phenotype analysis. Phenotypes described using this system can be subjected to hierarchical clustering analysis, which makes it possible to categorize genes based on relatedness between phenotypes. The genes clustered by this method are likely to be involved in the same developmental process, thus this analysis will provide pivotal information to uncover genetic networks involved in the regulation of development. Members of the Sugimoto team are now utilizing these RNAi analysis data to investigate developmental processes such as microtubule dynamics in mitosis, morphogenesis, and programmed cell death.

Sugimoto has developed a taxonomic system and nomenclature to enable the precise and consistent description of embryonic and post-embryonic phenotypes



Germ cells in meiotic prophase I.
(Blue: DNA, Red: A specific locus on Chromosome V)

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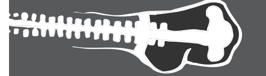
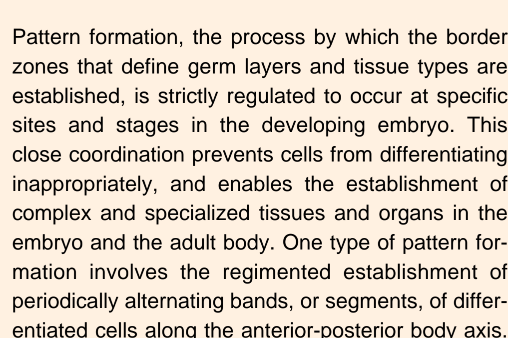


Creative Research Promoting Program

body patterning

Yoshiko Takahashi
Ph. D.

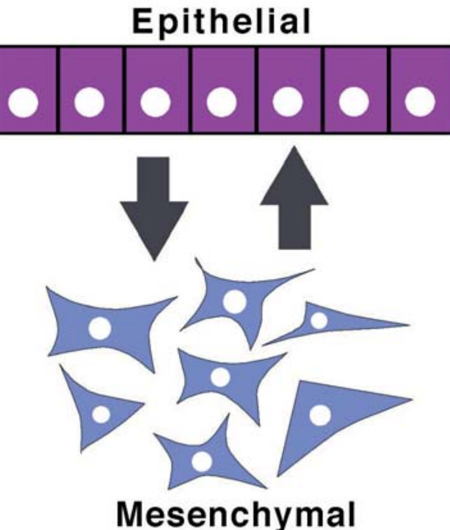
Yoshiko Takahashi received her B. Sc. from the University of Hiroshima, before moving to the Kyoto University Department of Biophysics where she received her master's and doctoral degrees in developmental biology. She pursued consecutive postdoctoral fellowships in developmental biology at the Institut d'Embryologie du CNRS (1988 to 1991), the University of Oregon Institute of Neuroscience (1991 to 1993) and Columbia University (1994). She returned to Japan as an associate professor at Kitasato University in 1994, where she worked until 1998, when she took an associate professorship at the Nara Institute of Science and Technology Graduate School of Biological Sciences. She was appointed team leader at the RIKEN CDB in 2001.



Pattern formation, the process by which the border zones that define germ layers and tissue types are established, is strictly regulated to occur at specific sites and stages in the developing embryo. This close coordination prevents cells from differentiating inappropriately, and enables the establishment of complex and specialized tissues and organs in the embryo and the adult body. One type of pattern formation involves the regimented establishment of periodically alternating bands, or segments, of differentiated cells along the anterior-posterior body axis. The results of this process of segmentation can be observed in repeated orderly structures such as vertebrae, ribs and spinal ganglia. In vertebrates, one form of segmentation takes place in the embryonic region known as the 'somitic mesoderm,' and involves the formation of transient segment-organizing bodies called somites.

Somites comprise both epithelial and mesenchymal cells, two cell types that vividly demonstrate the range of possibilities in cellular function and morphology

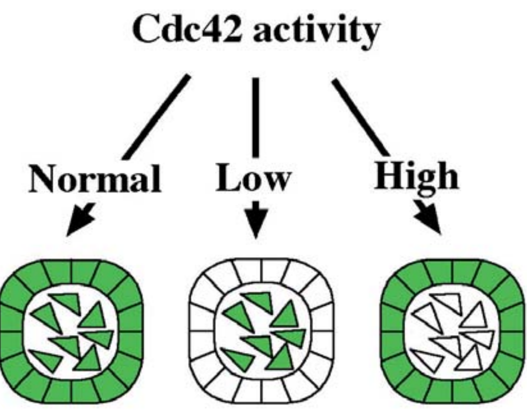
Yoshiko Takahashi's research involves molecular and cellular analyses of segmentation and somitogenesis in the chick embryo, which are invaluable for the insights they provide into how populations of cells are able to cleave at specific boundary lines and reorganize into highly ordered and reiterative patterns. In the early chick embryo, two strips of unsegmented paraxial mesoderm, collectively referred to as the 'segmental plate,' flank the neural tube, which runs along the anterior-posterior axis. Somites arise one by one from this segmental plate in a head-down direction, directed by the inductive activity of a putative regulatory region Takahashi has termed the 'segmenter.' Recent work in her lab is aimed at explicating transitions in cell structure and type that take place in nascent somites once their initial boundaries have been established.



Transitions between epithelial and mesenchymal states are pivotal to early morphogenetic processes.

Epithelial-mesenchymal transitions in somites

Somites comprise both epithelial and mesenchymal cells, two contrasting cell types that vividly demonstrate the range of possibilities in cellular function and morphology. Epithelial cells, which in adults form the skin and the linings of many organs, adhere to each other tightly and have polarized structures with distinct basal and apical sides. By comparison, mesenchymal cells are less rigidly structured and form looser aggregations. However, these two cell types are by no means isolated or independent from one another; indeed, a great many developmental and physiological phenomena rely on interactions between epithelium and mesenchyme. An extreme example of their inter-relatedness is the ability of cells of one type to convert into the other, in a process known as epithelial-mesenchymal transition. Such transitions (which can proceed in either an epithelial-to-mesenchymal or the reverse direction) are not only essential to normal embryonic development and organogenesis, they also play roles in wound healing and the pathogenesis of cancer.

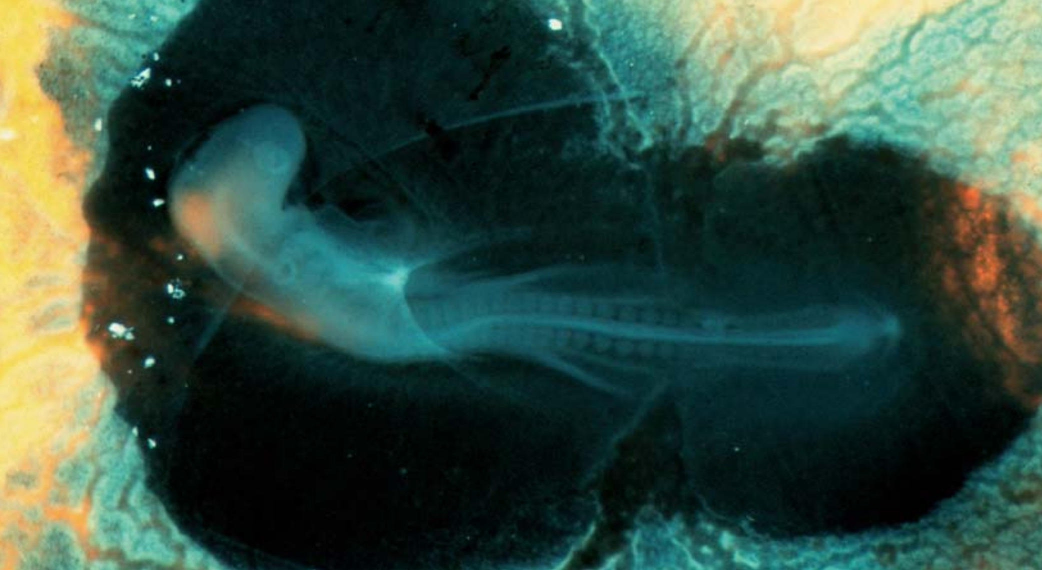


Cdc42, a Rho family GTPase, plays an important role in mesenchymal to epithelial transitions in somitogenesis.

After the boundaries of a somite have been established, the mesenchymal cells in the border region undergo a transition, acquiring the polarized structure and properties of epithelial cells. This results in a somite body in which a core of mesenchymal cells is circumscribed by an outer layer of cells of epithelial character. The Takahashi lab seeks to clarify the molecular mechanisms that regulate this mesenchymal-epithelial transition, and is concentrating on the roles played by members of the Rho family of GTPase proteins in this process. These proteins, notably Rho, Rac1 and Cdc42, are known to play roles in cytoskeletal rearrangement and cell locomotion in vitro, cycling between GDP-bound inactive and GTP-bound active states, and triggering downstream effectors linked to the cytoskeleton when activated.

Now, studies in the Takahashi lab are beginning to reveal additional functions for Rho family members in the context of somitic development. Both the inhibition of Rac1 and the overexpression of Cdc42 were found to induce the conversion of epithelial cells to mesenchyme, while interference with Cdc42 function caused mesenchymal cells to take on epithelial characteristics. These roles seem to be at least partly specific to somites; neither Cdc42 nor Rac1 was found to be involved in epithelial-mesenchymal transitions in epiblast cells. These findings suggest that the mesenchymal-epithelial transition that occurs during somitogenesis is determined by relative Rac1 and Cdc42 activity levels, with higher levels of Rac1 inducing an epithelial fate, while higher levels of Cdc42 tip the balance in the mesenchymal direction. She now seeks to further characterize the induction of the activity of these Rho family members in the earliest stages of the establishment of the somite by segmenter activity.

Takahashi emphasizes that what drew her to science and maintains her interest are the opportunities and challenges it offers to those willing and able to learn from natural phenomena. Her interests are diverse and far-ranging; she chooses to pursue avenues that others may have overlooked, and encourages her students and colleagues to do the same.



2-day embryo showing somites beautifully arrayed in pairs along the anterior-posterior axis

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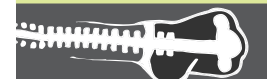


Creative Research Promoting Program

genomic reprogramming

Teruhiko Wakayama
Ph. D.

Teruhiko Wakayama received his B. Sc. and M. Sc. from Ibaraki University, and was awarded a Ph. D. in reproductive biology from the University of Tokyo Department of Veterinary Anatomy in 1996. He received a postdoctoral fellowship from the Japanese Society for the Promotion of Science in 1996 and spent the next three years at the Yanagimachi lab in the University of Hawaii Medical School, where he succeeded in producing the world's first cloned mouse. He was appointed to an assistant professorship at the same institution in 1998, and moved to Rockefeller University as a research assistant professor in 1999. He spent a year as a researcher at Advanced Cell Technology before returning to Japan to take his current position at the RIKEN CDB.

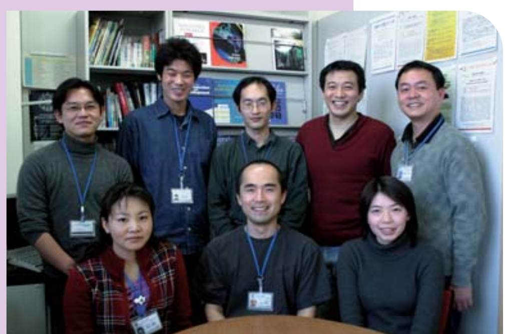
The experimental cloning of animals has a history that extends back more than fifty years, to when Briggs and King successfully produced tadpoles by transplanting the nuclei from embryonic cells into frogs' eggs whose own nuclei had been removed. At the time, cloning was used not as a technique to be studied for its immediate application, but as a means of testing a fundamental question of reproductive biology: whether the processes of fertilization and later development, in which the body's cells become more specialized and functionally distinct, involves a loss of genetic information, or whether all cells retain the full set of genetic code, even after differentiation has proceeded. The success of these initial experiments in cloning by nuclear transfer demonstrated conclusively that cells do maintain intact genomes even after differentiation, as the genetic code in a specialized cell's nucleus is sufficient to instruct an egg into which it is transplanted to give rise to a normal individual.

Although questions of whether genetic information is lost or irreversibly altered during development were laid to rest, new questions arose to take their place. For Teruhiko Wakayama, the most intriguing issue raised by the ability of animals to be cloned using the nuclei of specialized somatic cells (which form the body and, unlike sperm and eggs, cannot normally produce a new individual), is that of reprogramming, the processes by which the genome receives new sets of coding instructions enabling it to order the development of all the cells in a new individual while remaining intact and fundamentally unchanged in each of those cells. The Wakayama lab studies mammalian cloning and fertilization with the same basic goal as drove Briggs and King in their nuclear transfer studies a half century ago: to answer central questions in the biology of animal reproduction.

Wakame, the first mouse cloned at the RIKEN CDB

Cloning efficiencies

In all species and in all experimental methods tested to date, cloning by nuclear transfer has consistently low efficiency rates — generally below 5% of enucleated eggs fertilized by nuclear transfer go on to develop into live-born offspring. Many hypotheses have been pro-



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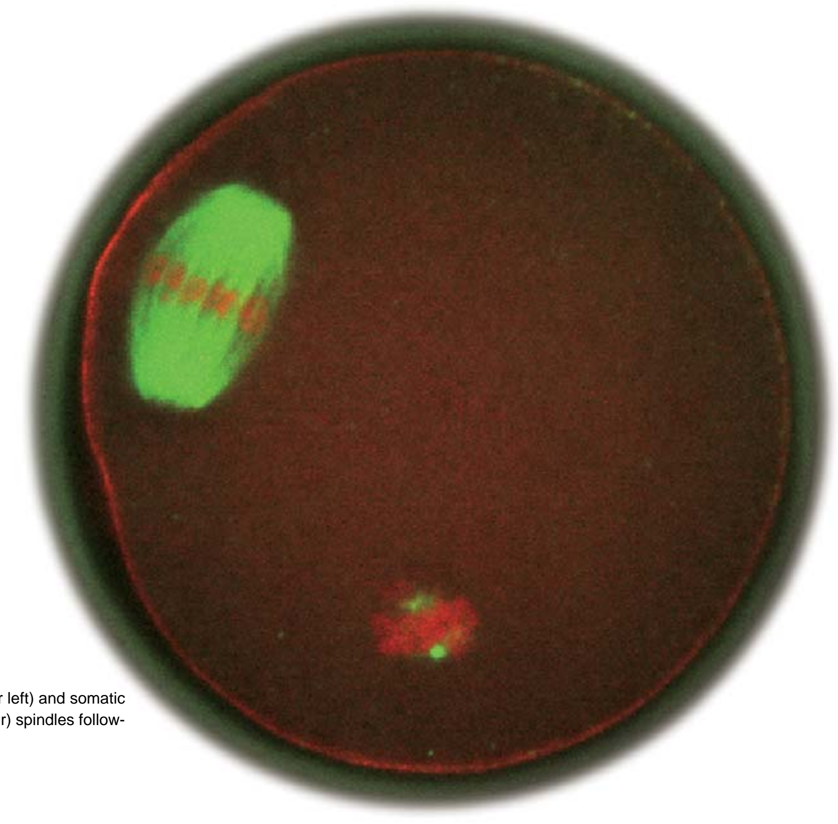

Oocyte (large, upper left) and somatic (small, bottom center) spindles following nuclear transfer

posed to explain the inefficiency of this procedure, while accounting for the fact that cloning is not altogether impossible. It has been suggested that the process of removing the nucleus from an oocyte (an unfertilized egg) or the absence of chromosomal information during the several hours in which the egg is missing a nucleus may somehow damage or cause the loss of factors that would normally act to reprogram the genetic information in the fertilizing (or transplanted) cell's nucleus. To test this possibility, Wakayama re-ordered conventional cloning methodology by first transferring nuclei from cumulus cells into oocytes whose own nuclei were still present, and only then removing the mitotic spindle derived from the native nucleus. These experiments resulted in the generation of live offspring at a rate of efficiency similar to that of standard cloning by nuclear transfer, providing evidence that tends to counter the hypothesis that the temporary absence of a nucleus is responsible for the poor developmental prospects of NT oocytes.

Many hypotheses have been proposed to explain cloning's inefficiency, while accounting for the fact that it is not altogether impossible

Scientists in the Wakayama lab have adopted similar approaches to the study of fertilization — experiments that involve the substitution of components or the reordering of natural sequences of events to test for specific biological function. In nature, fertilization occurs after a mature spermatozoon fuses with and activates an oocyte, but some laboratory techniques achieve fertilization by artificially activating the egg and then injecting a spermatid, which is an incompletely maturated sperm cell. Such techniques are known to be less efficient than fertilization using mature spermatozoa, but the reason for this lower developmental competency remains unknown. The Laboratory for Genomic Reprogramming is now comparing in vitro fertilization using spermatozoa and spermatids under controlled conditions in an effort to identify the differences between these stages in the developing sperm cell. Future research will look at changes in epigenetic modifications to the sperm genome over time as a possible explanation for their disparate potentials.

New technologies

Sperm preserved for use in experiments and in vitro fertilization is traditionally frozen in liquid nitrogen at extremely low temperatures (around -190˚C). This cryopreservation allows the sperm to be maintained viably for very long periods, but requires expensive facilities and some degree of technical skill in handling. Oocytes and fertilized eggs are also extremely labile, and must likewise be stored cryogenically. But these requirements tend to limit the access of germ cells to researchers unequipped with liquid nitrogen facilities, preventing the spread of the technology and the development of research using these cells. Wakayama hopes to develop new, less expensive and less technically demanding methods for the storage and maintenance of germ cells for experimental use. Recent tests using a modified commercially available culture medium showed that under the right conditions spermatozoa can be preserved for long periods, up to 70 days, at 4 degrees Celsius, a temperature that can be maintained using ordinary and inexpensive refrigeration equipment. This new preservation method opens up opportunities in animal breeding and reproductive biology research to scientists working under limited budgets, which Wakayama hopes may help to make these fields of science more accessible to developing countries and smaller labs.

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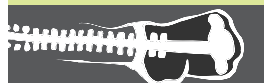

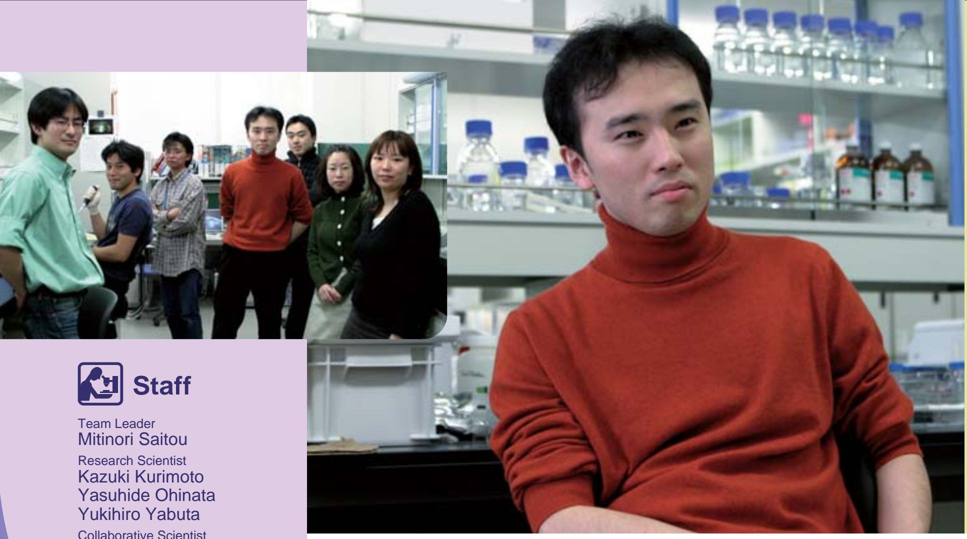

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mammalian germ cell biology

2003 New Faces

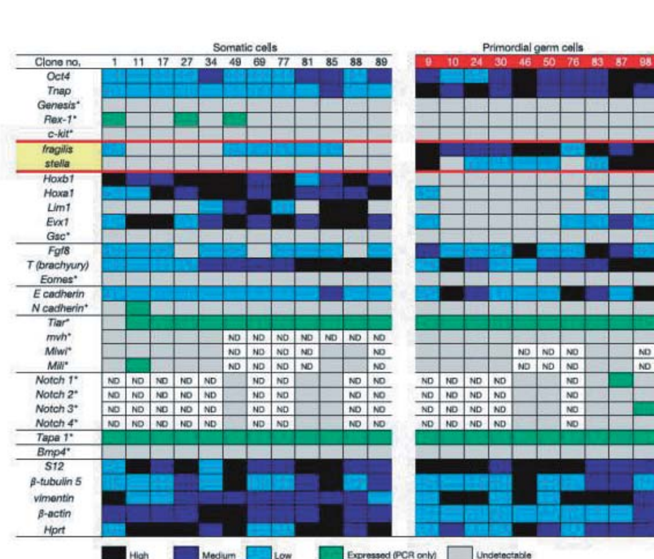
Mitinori Saitou
M. D. , Ph. D.

Mitinori Saitou received his MD from the Kyoto University Faculty of Medicine in 1995, and was awarded a Ph D in 1999 for his study of the structure and function of mammalian tight junctions under Prof. Shoichiro Tsukita in the Kyoto University Graduate School of Medicine. After a brief fellowship in the same department, he moved to the Wellcome Trust/Cancer Research UK Institute in 2000, where worked as a postdoctoral research associate in Azim Surani's laboratory, focusing on the origin and properties of the germ line in the mouse. He remained there until 2003, when he returned to Japan to take up his current position as head of the CDB Laboratory for Mammalian Germ Cell Biology. He received a three-year grant from the Japan Science and Technology Corporation (JST) PRESTO program that same year.

A case could be made for the statement that the ultimate role of all of the other types of cells in the body is to ensure that germline cells — sperm and eggs — are able to fulfill their task of conveying genetic information from one generation to the next. But despite this centrality, many aspects of germ cell biology have been, and remain to this day, poorly understood. There has been, however, progress in deepening this understanding, particularly in regard to the mechanisms by which germ cells are specified and segregated from the larger population of somatic cells early in development. This is achieved by different means in different species. In some animals, germ cell fate is predetermined from the very onset of development, in others, including all mammals, all cells in the early embryo have the potential to take on a germline fate, but only a small percentage of them actually do.

Mitinori Saitou studies the ways in which germ cell fate is specified in mammalian embryonic development, using the mouse as a representative model. He joins the CDB from Azim Surani's lab at the Wellcome Trust/Cancer Research UK Institute, where he worked on the single cell analysis of primordial germ cells, research that ultimately yielded a number of molecules that act to distinguish cells on the pathway to differentiation into germ cells. The first of these molecules, fragilis, is the product of a gene found to be strongly and specifically expressed in cells en route to assuming a germ cell fate, the result of inductive signaling from cells lying outside the embryo proper. A second such protein, stella, acts even more specifically, and is expressed only in a core subset of *fragilis*-expressing primordial germ cells. The co-expression of *fragilis* and *stella* serves to distinguish nascent germ cells from the somatic cell community in the developing embryo, and their activity is thought to play a part in the gene network that maintains germ cell characteristics and suppresses those of the somatic lineage. Single cell analysis has also revealed that repression of *Hox* genes and maintenance of genes linked to pluripotency, such as *Oct4*, are the two major events that occur during germ cell lineage-restriction.

Although progress has been made, much about the specification of the germline has yet to be explained. The identification of *fragilis* and *stella* now offers a means to identify incipient germ cells, but the initial molecular trigger for germ cell determination and key (epi-) genetic properties that determined germ cells acquire are still unknown. The Saitou lab plans to develop comprehensive gene expression analysis at the single cell level to search for the molecular mechanisms that underlie this process, and to identify the external factors that instruct a pluripotent cell to take on a germ cell fate. The lab also hopes to develop technologies to maintain immature oocytes (egg cells), and to induce their maturation in vitro, thereby enabling oocyte-mediated gene transfer techniques.



Gene expression profiles of BMP4 negative single PGCs and neighbouring somatic cells

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Ikuko Tada

systems biology

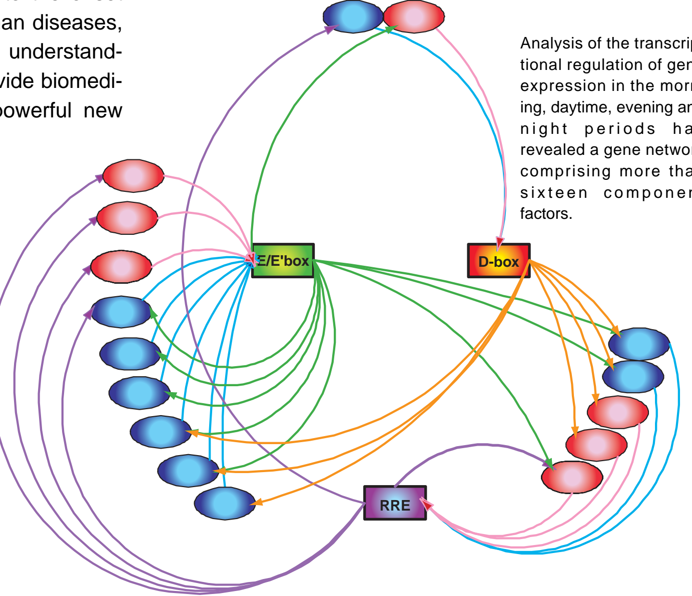
2003 New Faces

Recent large-scale efforts in genome-sequencing and expression analysis have produced an embarrassment of riches for life science researchers - biological data can now be accessed in quantities that are orders of magnitude greater than were available even a few years ago. This burgeoning set of raw data has not, however, necessarily led to equally explosive advances in the understanding of the relationships between its component parts. The need for integration has set the stage for the advent of systems biology, in which discrete biological processes and phenomena are approached as complex, interactive systems. Hiroki Ueda sees systems biology research as a multi-stage process, beginning with the identification and analysis of individual system components and their networked interactions, and leading to the ability to control existing systems and design new ones based on an understanding of structure and underlying principles.

The Ueda lab has taken the mammalian circadian clock as a relatively simple and self-contained initial model for the study of a biological system. In addition to its advantages as a basic research model, the function of the circadian clock is intimately involved in the control of metabolic and hormonal cycles, and its dysregulation is linked to the onset and symptomatology of numerous human diseases, including sleep disorders. An improved understanding at the system level promises to provide biomedical and clinical investigators with a powerful new arsenal to attack these conditions.

To address complex and dynamic biological systems such as the circadian clock, it is necessary to make comprehensive and precise measurements of the system's dynamics and to work out the organization of its underlying gene network. The Ueda lab has conducted a genome-wide screen and statistical analysis of gene expression to work out the clock-controlled genes that are rhythmically expressed in the central (suprachiasmatic nucleus; SCN) and peripheral (liver) circadian clocks. This phase of the study required the development of a genome-wide promoter database, which the Ueda lab will make available to all researchers at the CDB. Subsequent phases involved determining gene transcription start sites across the entire genome, predicting the regulatory sequences involved in time-specific transcription, and studying their actual functions in vitro using a high-throughput real-time monitoring system for luciferase-tagged transgenes. Analysis of the transcriptional regulation of gene expression in the morning, daytime, evening and night periods revealed a gene network comprising sixteen inter-regulating activators and inhibitors of time-linked gene expression.

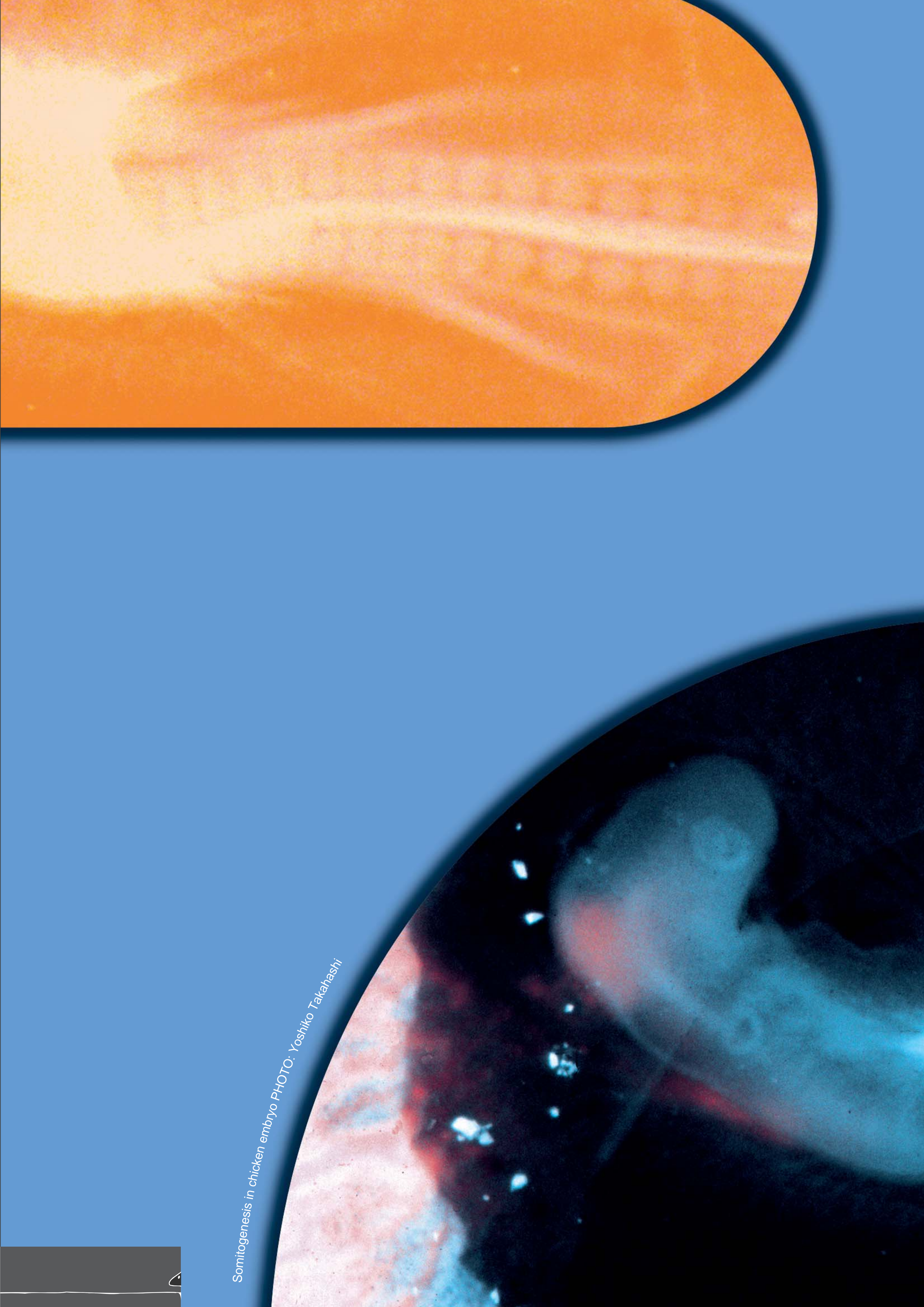
The initial success of the systems approach to the mammalian circadian clock has been encouraging, and the Ueda lab now seeks to apply similar genome-wide, high-throughput technologies to more involved and elaborate developmental processes. Guiding that research will be Einstein's (and the system biologist's) dictum to "Make everything as simple as possible, but not simpler."



Analysis of the transcriptional regulation of gene expression in the morning, daytime, evening and night periods has revealed a gene network comprising more than sixteen component factors.

Hiroki R. Ueda
Ph. D.

Hiroki R. Ueda received his MD from Faculty of Medicine, the University of Tokyo in 2000, and will receive PhD from Graduate School of Medicine, the University of Tokyo in March 2004. While an undergraduate student, he worked as a research assistant on a biological simulation system project at Sony Computer Science Laboratories. While a graduate student, he next went on to work, first as a researcher (2000) and then as a group leader (2002), at Yamanouchi Pharmaceutical, on a project studying biological clock mechanisms in fly and mouse. He was appointed team leader at the CDB in 2003, and will assume this position full time in April 2004.



Somitogenesis in chicken embryo PHOTO: Yoshiko Takahashi

Supporting Laboratories

The supporting laboratories offer technical services, develop new technology, and conduct independent research projects. Their services are available to all CDB research groups and teams.

Supporting Laboratories

cellular morphogenesis

Shigenobu Yonemura

Ph. D.

Shigenobu Yonemura received his B. Sc., M. Sc. and Ph. D. from the University of Tokyo, earning his doctorate in 1988 for thesis work under Prof. I. Mabuchi. He spent a year as a postdoctoral fellow at the same institution before moving to pursue a fellowship at Johns Hopkins University from 1989 to 1990. He returned to Japan as an assistant professor in the Department of Cell Biology at the National Institute for Physiological Sciences, where he remained until 1995. He joined the Kyoto University Faculty of Medicine the same year, first as an assistant professor, then as a lecturer in the Department of Cell Biology, a position he retained until his appointment as CDB team leader in 2001.

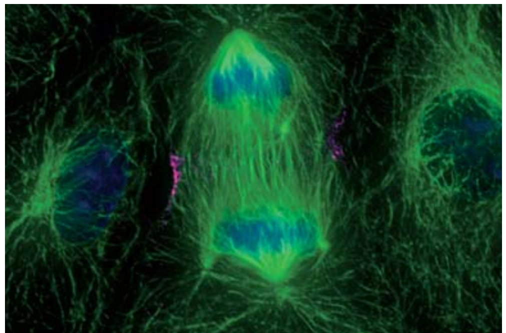
Cellular morphology is defined by interactions involving mechanical and osmotic stresses from the cell's external environment, its permeable membrane, its viscous cytosolic content, and an internal framework comprising pre-stressed tubular struts, tensile cables and a lattice-like filamentous cortex. Microtubules are one important and extensively studied component of this cytoskeletal structure, acting both as internal stress-bearing braces and pathways to guide intracellular transport. A second critical form-giving element, actin filaments, are most prevalent in cellular peripheries, where they provide a stable but regulable meshwork enabling the cell to maintain its structural integrity while responding to external and internal stimuli through shape alterations and movements.

Actin filaments maintain cell structural integrity while responding to external and internal stimuli

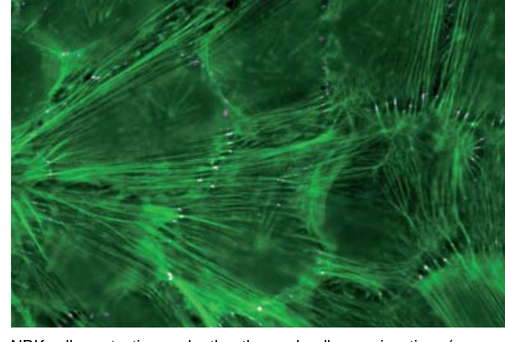
The behavior of actin is intimately linked to the activity of a group of related small GTPase proteins known as the Rho family, which is characterized by the actin-regulatory functions of its three primary members, Rho, Rac and Cdc42. The spatial and temporal localization of Rho-family proteins has been shown to be important in the structural organization of cells in culture. Shigenobu Yonemura is working to develop technologies to allow the precise determination of the localization of these proteins in cultured cells and tissues within specific biological contexts.

Cleavage plane determination

The final stages of mitotic cell division involve a process known as cytokinesis, in which the parent cell cleaves in two, partitioning cytoplasmic elements and genetic information into each of the daughter cells. Cytokinesis begins with the appearance of a cleavage furrow at the cell surface, a process which is known to be specified by micro-



NRK cells at cytokinesis. Rho (magenta) accumulates at the equatorial cell cortex depending on microtubule organizations (green).



NRK cells contacting each other thorough adherens junctions (magenta) where actin filament bundles (green) are tightly connected.

tubules in the mitotic apparatus, and which is essential to ensuring that each progeny cell receives one and only one set of chromosomes. However, the question of which complement of mitotic apparatus microtubules — astral or central — spatially determines this cleavage remains unresolved. Yonemura studied Rho accumulation at the putative furrow site, taking this as an index of cleavage plane determination signaling, and analyzed the roles of both microtubule organizations by selectively disrupting each. The study showed that both astral and central microtubules are able to transmit determinant signals to the cell cortex, a finding that helps explain how astral microtubules that extend to the cell cortex can play an important role in cleavage plane determination in larger cells, such as oocytes, in which central microtubules are located more remotely from the cortex, while being dispensable in the same process in smaller cells in which central microtubules are located more proximally to the cell's periphery.

Actin fibers and the adherens junction

The main zone of contact between a cell and its neighbors is called the adherens junction, which is defined by the selective binding of transmembrane adhesion molecules that are themselves anchored to molecular complexes located within each cell's interior. In fibroblastic cells in culture, bundles of actin filaments that run perpendicular to the plasma membrane are connected to the components of the adherens junction, a phenomenon that is also observed in epithelial cell adhesion. In many cells, the mature adherens junction forms a contiguous belt, although during the process of its formation it passes through a stage characterized by discrete, spot-like adhesions. The Yonemura lab used NRK cells, a type of non-polarized fibroblast which forms spot-like junction zones, to study the role of actin fibers in adherens junction formation. In this cell line, the adherens junction and its associated actin bundles can be disrupted and reconstituted by removing or adding serum to the culture medium. Using this system, he revealed that the activity of the small GTPase, Rho, is required both for the formation of the adherens junction and of actin bundles. The study showed that disruption of actin bundles affects the integrity of the adherens junction, indicating the importance of Rho-actin signaling in this form of cell-cell adhesion.

Mouse neutrophils migrating on a substratum. Phase contrast (blue) and fluorescence image of ERM proteins (magenta) are merged.

ERM protein recognition-site structure

ERM proteins, named for three representative members of the family, ezrin, radixin and moesin, act as links between actin filaments and the plasma membrane. Yonemura and colleagues studied the crystal structure of a functional domain of radixin to identify the structural basis for adhesion molecule recognition by one ERM protein, radixin. Radixin plays a role in the formation of microvilli, cellular extensions in which actin filaments are closely associated with the plasma membrane. The importance of radixin in sites of bilirubin secretion is suggested by the finding that mice lacking radixin, which is the dominant ERM protein in the liver, show severely impaired hepatic microvilli formation, resulting in a phenotype similar to human conjugated hyperbilirubinemia, a rare autosomal recessive liver disorder. This work provides the first insights into the structural bases for the recognition between an ERM protein and an adhesion molecule, and may further serve as an experimental model for the study of molecular processes involved in bile secretion.

In addition to its full-time research efforts, the Yonemura lab offers site-wide support for electron microscopy, services available to all research staff at the CDB. By providing fluorescence and electron microscopic visualizations as well as instruction in techniques, the team hopes to help ensure the quality, fidelity and ease-of-interpretation of visualization data generated at the Center.

The tip of a villus of the small intestine of the rat. Aged cells that are exfoliated highly express Rho protein (green).

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Publications

Hamada K, Shimizu T, Yonemura S, Tsukita S and Hakoshima T. Structural basis of adhesion-molecule recognition by ERM proteins revealed by the crystal structure of the radixin-ICAM-2 complex. *Embo J* 22:502-14 (2003).

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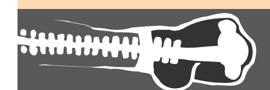

Supporting Laboratories

animal resources and genetic engineering

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Hirotake Nishino
Assistant
Kaori Nasu

The study of model organisms and systems is integral to biological research. Such systems provide scientists with the means to search for broadly shared mechanisms underlying developmental and regenerative processes across species, and conversely to identify those traits that earn each species its unique branch on the phylogenetic tree. The mouse is one of the most important and widely used model organisms in science today, prized for its amenability to genetic manipulation, its high level of homology with humans, and the trove of data regarding its physiology, genetics and development that has accumulated over nearly one century of intensive scientific research.

The Laboratory for Animal Resources and Genetic Engineering (LARGE) provides an important suite of services related to the generation of experimental mice to labs within the CDB and around Japan. In its role as a CDB support laboratory, the LARGE team produces transgenic and knockout mouse models to the specifications of scientists working in a wide range of genetic, embryological and biomedical research projects, maintaining the highest quality standards and rapid turnaround to ensure fast and easy access to researchers working within the Center and throughout the country. In addition to these core functions, the LARGE staff provides a number of other services, such as cloning by nuclear transfer and cryopreservation of mouse zygotes and sperm. The lab also performs a number of maintenance and logistical functions, such as the specific pathogen free (SPF) housing, cleaning, processing and distribution of animals.

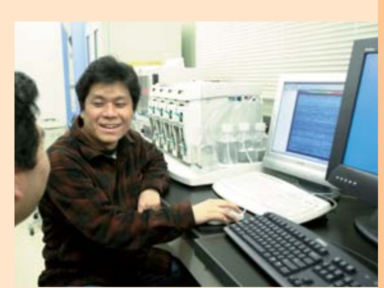
Chimera mice

sequencing

Supporting Laboratories

The CDB Sequencing Laboratory provides DNA sequencing and analysis services for use by all laboratories at the Center. Equipment is available for small, medium and large-scale requests, with turnaround times ranging from under one week for medium-scale projects to one month for larger jobs, at rates of up to 1,920 samples per day. Smaller request are fulfilled especially rapidly, and results can be delivered as soon as the following day when sequence-ready samples are submitted. Analysis services for editing, homology search, and assembling are also available.

In addition to DNA sequencing, the lab also offers a full range of DNA microarray services, from the amplification of target DNA and the preparation of probe cDNA, to microarray image data analysis. All work requests can be submitted and tracked online, and results are returned to the applicant's personal folder on the CDB intranet file server, making it possible for CDB research staff to sequence samples of interest without leaving their desktop computers. The lab also stores and distributes DNA resources, making clones available to all members of the CDB staff.



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Publications

K Chida, T Hara, T Hirai, C Konishi, K Nakamura, K Nakao, A Aiba, M Katsuki and T Kuroki. Disruption of Protein Kinase Ceta Results in Impairment of Wound Healing and Enhancement of Tumor Formation in Mouse Skin Carcinogenesis. *Cancer Res* 63:2404-8 (2003).

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In 2003, the LARGE team started to expand its services and initiate new programs, notably the generation of target vectors from sequence information alone, and the independent production of novel genetically-modified constructs, a drive that generates on the order of 40-50 new mutant strains per year. Such strains serve as research platforms with the potential to provide new insights into a range of important research problems, from the developmental mechanisms of organ development to the genetic bases of human disease. The lab will also function as part of Japan's system of Mouse Embryo Banks, with a special emphasis on producing, storing and cataloging embryos for use in developmental biology and regenerative medical research.

Animal Facility

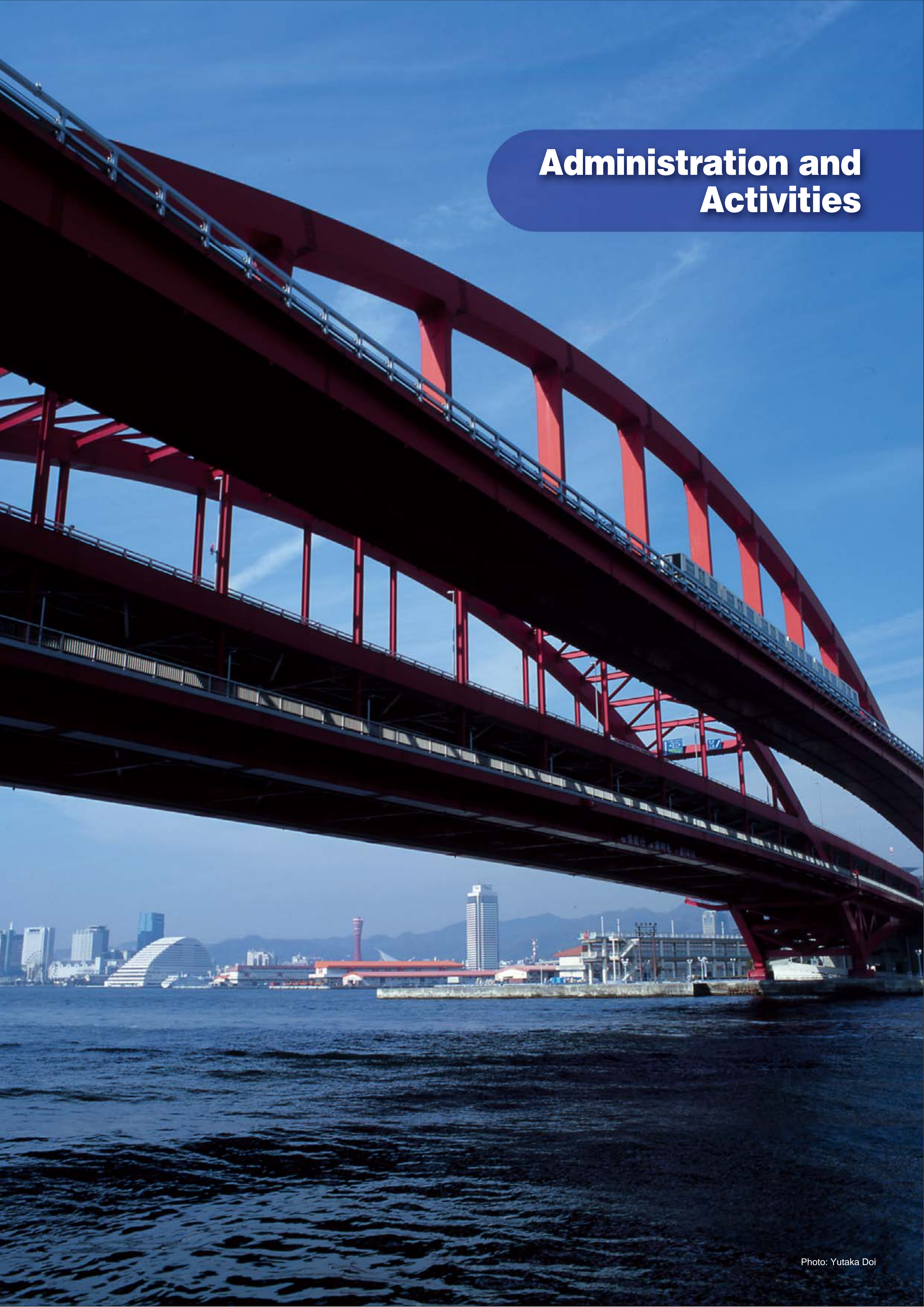
Mouse biology provides a valuable platform for investigations into many of the fundamental processes and mechanisms of mammalian physiology and metabolism, and is widely used as a model organism in developmental biology research. With the sequencing of the mouse genome, mice can now be modified for use in research into the developmental consequences of genetic alterations, and as models of human disease.

The CDB animal facility provides the technology, equipment and staff to enable the humane, hygienic, efficient and economical handling of large numbers of experimental mice. This 24,000 cage SPF facility has the capacity to handle and care for up to 100,000 mice. Cutting edge technology, such as semi-automated cage cleaning, makes it possible to maintain high standards of hygiene and sterility, minimizing the risk of outbreaks of contagion among the mouse populations. The facility also provides services for the production of transgenic and knockout mice to specifications and on demand.

Research Aquarium

The CDB research aquarium serves as home to a number of water-dwelling species used in research into development and regeneration, including the zebrafish (*Danio rerio*) and the African clawed frog (*Xenopus laevis*). These, and other aquatic species, provide useful models for studies ranging from developmental genomics to classical embryology.

The aquarium includes seven climate-controlled rooms geared to providing optimal environments for the handling and breeding of freshwater and marine species, and utilizes reverse osmotic technology to maintain consistent tank-water purity. In addition to the commonly used zebrafish and African clawed frog, the facility also houses specimens from more novel models used in evolutionary development research, such as the lamprey, *Lampetra japonica*.

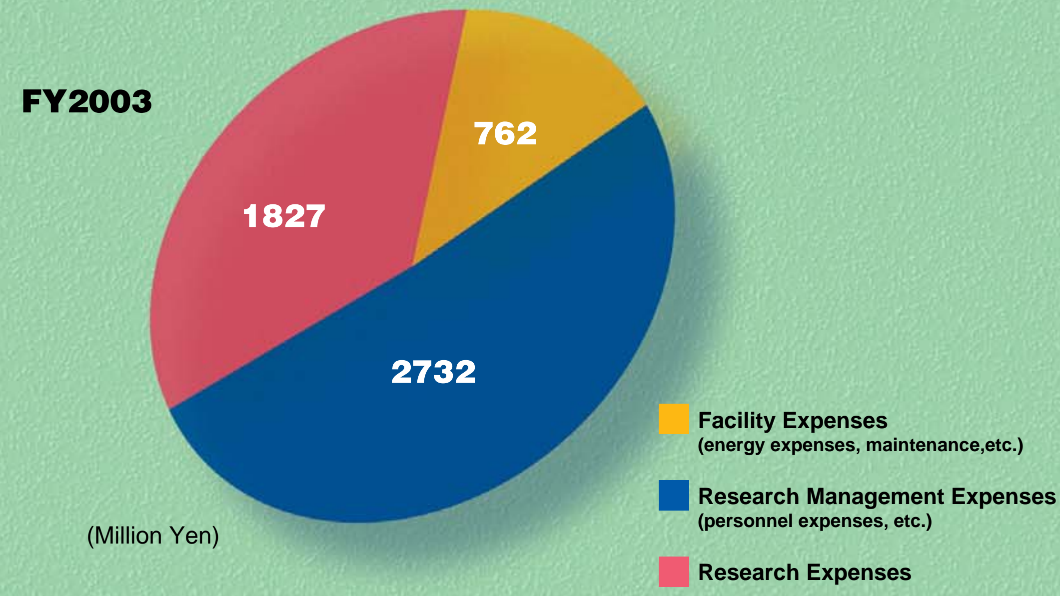


Administration and Activities

Photo: Yutaka Doi

2003 CDB Budget

The Center for Developmental Biology was launched in April 2000 under the auspices of the Millennium Project research initiative launched by former Prime Minister Keizo Obuchi. The Millennium Projects were established to drive research in the fields of information technology, environmental science and the study of aging, areas of vital importance to both Japan and the world in the 21st century. The organization and operations of the CDB were consigned to RIKEN, which coordinates the construction management and administration of the Center.



CDB Staff (December 2002)

Category	Number
GD/TL/UL	30
Research scientists	101
Research associates	3
Technical staff	105
Assistants	25
Visiting researchers	9
Collaborative scientists	30
Student trainees	47
Part time staff	53
Research promotion division	43
Other	65
Total	511

Advisory Council

The CDB Advisory Council, chaired by Dr Igor Dawid (National Institute of Child Health and Human Development, NIH) submits regular external reports regarding the scientific administration and the state of research progress at the Center. The ten-member Council includes top scientists in related fields from Japan and around the world and serves as an unbiased review board for CDB research activities.

Institutional Review Board

The Institutional Review Board includes representatives from local academic, research and lay organizations as well as CDB research staff, and meets regularly to review and discuss the ethical and social implications of programs and investigations being conducted at the CDB. The results of the Board's discussions are submitted to the Center Director and taken into consideration when planning research activities.

Graduate School Affiliates

Encouraging young scientists to participate in advanced research projects as they prepare for careers in the life sciences is essential to ensuring the development of future generations of researchers. The CDB has collaborative educational programs with a number of graduate schools in Western Japan, providing students with opportunities for hands-on laboratory benchwork and lectures on topics in development and regeneration. The center also has also accepted 50 students from other universities as research associates or trainees. In addition to regularly scheduled scientific seminars, students can attend events such as the CDB Forum research progress series and the Center's annual retreat. The Junior Research Associate program established by RIKEN also provides financial support to 8 graduate students working in CDB labs.

Graduate School of Biostudies, Kyoto University

Graduate School of Medicine, Kyoto University
Department of Developmental and Regenerative medicine

Kobe University, Graduate School of Science and Technology
Department of Life Science

Nara Advanced institute of Science and Technology
Graduate School of Biological Sciences

Kwansei Gakuin University
Bioscience Department

University of Texas Graduate School of Biomedical Sciences at Houston

Temasek Life Sciences Laboratory

Scientific Exchange Programs

The CDB is also engaged in programs of cooperation with research organizations in other parts of Japan and around the world. This activity is based primarily on cooperation between individual research laboratories. These scientific exchange agreements facilitate processes such as the hosting and dispatch of research staff for international collaborative research, the joint sponsorship of scientific meetings and the sharing of intellectual property produced in collaborations.

Leading Project in

In 2003, the Japanese Ministry of Education, Culture, Sports, Science and Technology (MEXT) inaugurated a number of Leading Projects to drive research into areas identified as having great potential to make contributions to the nation's economy, environment, social climate or health and welfare. One such project was introduced to promote research leading to applications for stem cells in regenerative medicine.

The implementation of the Leading Project in Regenerative Medicine has been consigned to universities and research organizations across Japan, and a number of CDB labs have received funding to conduct research into key areas including the experimental manipulation and maintenance of stem cells, the reprogramming and targeted differentiation of somatic stem cells, and the ex vivo reconstitution of physiological structures with potential clinical uses in regenerative medicine. Shin-ichi Nishikawa, Group Director of the CDB Laboratory for Stem Cell Biology, was appointed to head this Leading Project.

Mitsuko Kosaka
Ph. D.

Unit Leader
Mitsuko Kosaka

Research Scientist
Maki Asami
Junji Tsuchida

Visiting Scientist
Guangwei Sun

Cell Plasticity

Mitsuko Kosaka heads the Research Unit for Cell Plasticity, which seeks to determine the limits and possibilities in the differentiative potential of somatic stem cells. Their work focuses on differentiation in the iris of the eye, a population of pigmented cells that contains stem cells that show an intriguing plasticity, or ability to assume more than one cellular fate. The Kosaka lab will investigate the mechanisms by which stem cells in the iris can be induced to give rise to cells of other types, such as photoreceptive neural cells. This work is of great clinical promise in the treatment of vision loss due to nerve damage, as cells from the iris are readily obtainable and may one day be used as an autologous source of retinal replacement cells if they can be steered reliably to take up a neural fate. Recent findings from the lab suggesting that iris stem cells express genes characteristic of highly undifferentiated pluripotent stem cells also offer new avenues for exploration for researchers studying the genetic regulation of differentiative potency.

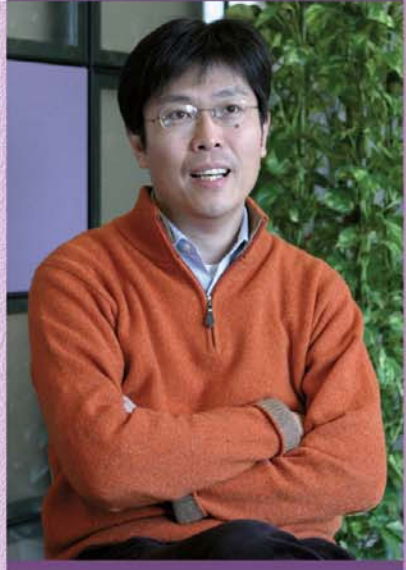
Regenerative Medicine

Two new Research Unit laboratories have been recruited to conduct goal-oriented, fixed-term research projects as part of this program. These Research Units are funded by Leading Project grants and are financially independent of RIKEN, but operate, in whole or in part, in laboratories located within the RIKEN CDB campus, enabling them to capitalize on the extensive shared-use equipment and facilities at the Center.

Organ Regeneration

The Research Unit for Organ Regeneration, headed by Hideki Taniguchi, focuses on studies of the differentiative and regulatory mechanisms underlying endodermal tissues and organs in the digestive system, which include liver, pancreas, intestine and salivary glands. By analyzing stem cells isolated from each of these tissue types, the Taniguchi lab seeks to confirm the existence of "endodermal stem cells" that enable the high levels of cellular plasticity seen in digestive organs. Using cell sorting technologies, Taniguchi is working to characterize these putative endodermal stem cells by sorting and analyzing cells from the livers and other digestive organs of fetal mice. The identification of molecular markers specific to such cells will make it possible to isolate them by flow cytometry and to pursue further investigations into their functional roles in the development of the endoderm-derived digestive system. The ability to identify and isolate stem cells capable of giving rise to endodermal lineage tissue types would also be of enormous potential value in the development of regenerative medical applications for the treatment of diabetes.

In a concurrent project, Taniguchi is working to develop a system for isolating and guiding the differentiation of stem cells from the pancreases of adult mice, which is aimed at establishing the foundations for cell replacement therapies using insulin-producing pancreatic beta cells grown in vitro.



Hideki Taniguchi
M. D. , Ph. D.

Unit Leader
Hideki Taniguchi

Collaborative Scientist
Takeshi Sugaya

Technical Staff
Midori Yokura
Naoyo Kajitani (2004)

Student Trainee
Masato Sugano (2004)

2003 Seminars

The CDB hosts regular seminars by distinguished speakers from within Japan and abroad as part of its committed effort to promote the borderless exchange of scientific information. All CDB seminars are held in English. In addition to these seminars, the CDB also holds monthly Forums, in which CDB researchers share findings with their colleagues, and a range of informal lecture offerings in English and Japanese.

Date	Title	Speaker
2003.1.15	Hox genes and brainstem patterning	Filippo M Rijli
2003.1.16	Neural patterning in vertebrate embryogenesis	Nobue Itasaki
2003.1.17	Left-right asymmetry in the zebrafish forebrain	Marnie Halpern
2003.1.27	Patterning formation in neocortical area by FGF8	Tomomi Fukuchi-Shimogori
2003.1.29	To branch or not to branch: HAMLET controls dendritic arbor branching and lineage restriction in the *Drosophila* peripheral nervous system	Adrian Moore
2003.2.21	Wnt signaling in development and disease	Randall T. Moon
2003.3.11	The new revolution in developmental biology: Expansion and reconciliation	Scott Gilbert
2003.3.27	Imaging cell movements that pattern the vertebrate embryo	Scott Fraser
2003.3.27	Induction and evolution of the neural crest	Marianne Bronner Fraser
2003.3.27	Functional expression cloning of *Nanog*, a gene conferring cytokine independent ES cell self-renewal	Ian Chambers
2003.4.22	Differentiation of embryonic stem cells into hepatocytes: biological functions and therapeutic applications	Takahiro Ochiya
2003.4.22	ES cells as a model system for pancreatic differentiation	Shoen Kume
2003.5.13	Dynamics of cellular interactions during T cell recognition in 3-dimensional tissue visualized by two-photon microscopy	Ellen Robey
2003.5.16	BMP-3b and BMP-3 function as different dorsalizing factors in *Xenopus* embryos	Shin-ichiro Nishimatsu
2003.5.21	Building brains from embryonic stem cells: Naive cultures offer greatest potential	Hidemasa Kato
2003.5.22	Considerations in microarray experimental design and data analysis	Ali R Zareh
2003.6.18	The role of myosin VI in the spermatogenesis of *Drosophila*	Noguchi Tatsuhiko
2003.6.23	Cellular functions and molecular mechanisms of signaling pathways mediated by target of rapamycin	Kazuyoshi Yonezawa
2003.7.3	Diversity of CNR/protocadherin family in the brain	Takeshi Yagi
2003.7.4	Membrane traffic: Molecular mechanisms and real-time imaging	Akihiko Nakano
2003.7.8	Ontogeny of the hematopoietic system	M Bruno Peault
2003.7.17	*PAX6* and *SOX2* in disease, development and evolution	Veronica van Heyningen
2003.7.18	Quiescent hematopoietic stem cells in the niche	Toshio Suda
2003.8.4	Manipulating mouse genome from single gene knockout to chromosome engineering	Ichiko Nishijima
2003.8.4	Genetic regulation of retinal interneuron differentiation	Akihira Otoshi
2003.8.6	Self-renewal of neural stem cells and CNS-repair	Hideyuki Okano
2003.8.7	Regulation of spindle orientation by centrosome and APC tumor suppressor	Yukiko M Yamashita
2003.8.8	A protein interaction matrix for an integrin adhesion complex within *C. elegans* muscle	Hiroshi Qadota

Date	Title	Speaker
2003.8.26	Impaired spermatogenesis in mice lacking Caf1	Tadashi Yamamoto
2003.9.11	Mechanisms controlling heart and blood vessel growth	Kenneth Walsh
2003.9.12	Genomic approaches for functional annotation of the mammalian genome	John Hogenesch
2003.9.19	Genetic analysis of mouse forebrain and midbrain development	Siew-Lan Ang
2003.9.29	Fibronectin requirement in branching morphogenesis	Takayoshi Sakai
2003.10.3	Chemical modulation of embryonic development and oncogenesis	James K Chen
2003.10.10	*Hox* in leukemia and as avenues to hematopoietic stem cell expansion	R Keith Humphries
2003.10.14	Characterisation of two modulators of the FGF signal transduction pathway,and their role in axial polarity	Michael Tsang
2003.10.14	Patterning neurogenesis in the zebrafish embryo	Motoyuki Itoh
2003.10.16	Endoderm as a source of patterning information for early chick embryogenesis	Gary Schoenwolf
2003.10.23	Structure and function of Spen proteins: A conserved repressive function in developmental signalling pathways	Mariko Ariyoshi
2003.10.24	Regulation of temporal and spatial patterns of gene expression in *Dictyostelium*	William Loomis
2003.10.27	Phylogenetic conservation of a cis-acting regulator that controls polarized expression of Sonic hedgehog (Shh) in limb buds	Toshihiko Shiroishi
2003.10.29	Sniffing out smell's code and map	Naoshige Uchida
2003.11.14	Molecular mechanisms specifying muscle-identity and myotube number in the *Drosophila* adult	K.VijayRaghavan
2003.11.18	Genesis of the neural primordia and their regional diversities through regulation of *Sox2*	Hisato Kondoh
2003.11.20	Nuclear interpretation of the Dpp morphogen in *Drosophila melanogaster*	Markus Affolter
2003.11.26	Genes that regulate the formation and regeneration of skeletal muscle	Margaret Buckingham
2003.11.27	Patterning and growth in *Drosophila* limb development	Konrad Basler
2003.12.2	Secondary rearrangement of TcR alpha chain in vivo: shaping and reshaping T cell repertoire	Osami Kanagawa
2003.12.3	Proteolytic cleavage of meiotic cohesin REC8 by separase is required for murine spermatogenesis	Nobuaki Kubo
2003.12.5	Function and regulation of centralspindlin, a kinesin/RhoGAP complex, in cytokinesis	Masanori Mishima
2003.12.9	Eph receptors and ephrins in neural map development	Masaru Nakamoto
2003.12.15	Establishment of the vertebrate body plan in relation to limb formation	Mikiko Tanaka
2003.12.15	Genetic cascades that regulate gastrulation cell movements in zebrafish	Masazimi Tada
2003.12.16	Wnt-mediated axon guidance at the midline of *Drosophila*	Shingo Yoshikawa
2003.12.26	*Msx* genes function in neural crest development	Mamoru Ishii

The First Annual CDB Symposium

The Origin and Formation of Multicellular Systems

The CDB held its first annual symposium at the nearby Portopia Hotel. The meeting drew more than 200 speakers and attendees from around the world, with lectures, poster presentations and lively discussion on the origins of metazoan biology, the fundamental processes of development and regeneration and the formation of higher-order structures, such as neural networks.

The Symposium was organized into five sessions over three days, with full and short talks by 28 distinguished speakers.

Monday, March 24

Session 1
The Origin of Multicellular Systems

9:00 am	9:40 am	Peter W Holland, Oxford University
9:40 am	10:00 am	Hitoshi Suga, Kyoto University
10:00 am	10:40 am	Nicole King, University of Wisconsin
10:50 am	11:30 am	Yoko Watanabe, National Science Museum, Japan
11:30 am	12:10 pm	Peter Devreotes, Johns Hopkins University

Session 2
Germ Cells, Stem Cells and Regeneration

1:30 pm	2:10 pm	Judith C Kimble, University of Wisconsin-Madison
2:10 pm	2:50 pm	Allan Spradling, Carnegie Institution of Washington
3:10 pm	3:30 pm	Yuzo Niki, Ibaraki University
3:30 pm	4:10 pm	Christopher Wylie, Cincinnati Children's Hospital
8:00 pm	8:40 pm	Shin-ichi Nishikawa, RIKEN CDB
8:40 pm	9:00 pm	Ian Chambers, University of Edinburgh
9:00 pm	9:40 pm	Kiyokazu Agata, RIKEN CDB

Tuesday, March 25

Session 3
Cellular Polarization and Asymmetric Division

9:00 am	9:40 am	David Ish-Horowicz, Cancer Research UK
9:40 am	10:20 am	Anthony A Hyman, Max Planck Institute
10:40 am	11:20 am	Yuh-Nung Jan, UCSF
11:00 am	11:40 am	Fumio Matsuzaki, RIKEN CDB
11:40 am	12:00 pm	Tadashi Uemura, Kyoto University

Session 4
Intercellular Signaling and Morphogenetic Cell Behavior

1:30 pm	2:10 pm	Hiroshi Hamada, Osaka University
2:10 pm	2:50 pm	Edward M DeRobertis, UCLA
2:50 pm	3:30 pm	Olivier Pourquie, Stowers Institute for Medical Research
3:50 pm	4:10 pm	Yasumasa Bessho, Kyoto University
4:10 pm	4:30 pm	Yukiko Gotoh, University of Tokyo
4:30 pm	5:10 pm	Elaine Fuchs,The Rockefeller University
5:10 pm	5:50 pm	Tom Curran, St Jude Childrenís Research Hospital

Wednesday, March 26

Session 5
Cellular Basis for Neuronal Circuit Formation

9:00 am	9:40 am	Hajime Fujisawa, Nagoya University
9:40 am	10:20 am	Peter Mombaerts, The Rockefeller University
10:40 am	11:20 am	Christine E Holt, Cambridge University
11:20 am	12:00 pm	Mu-ming Poo, UC Berkeley

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RIKEN is a publicly funded research organization established to conduct comprehensive research in science and technology and to disseminate the results of its scientific research and technological developments. RIKEN carries out basic and applied research in a wide range of fields, including physics, chemistry, medical science, biology, and engineering.

RIKEN was first founded in 1917 as a private research foundation, Rikagaku Kenkyuusho (The Institute of Physical and Chemical Research). In 2003, the Institute was reorganized as an Independent Administrative Institution under the Ministry of Education, Culture, Sports, Science and Technology (MEXT), since which time it has engaged in wide-ranging research activities spanning the basic and applied sciences.

ISSN 1349-0494

RIKEN Center for Developmental Biology 2003 Annual Report

http://www.cdb.riken.jp

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On The Cover
Terminal branch of the *Drosophila* trachea. A single terminal cell labeled with GFP (green) with a large polyploid nucleus (purple) spreads numerous cytoplasmic tubules with intracellular lumen (tracheole) over the surface of the larval gut.
PHOTO: Shigeo Hayashi

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Printed in Japan

R100 Printed on 100% Recycled Paper