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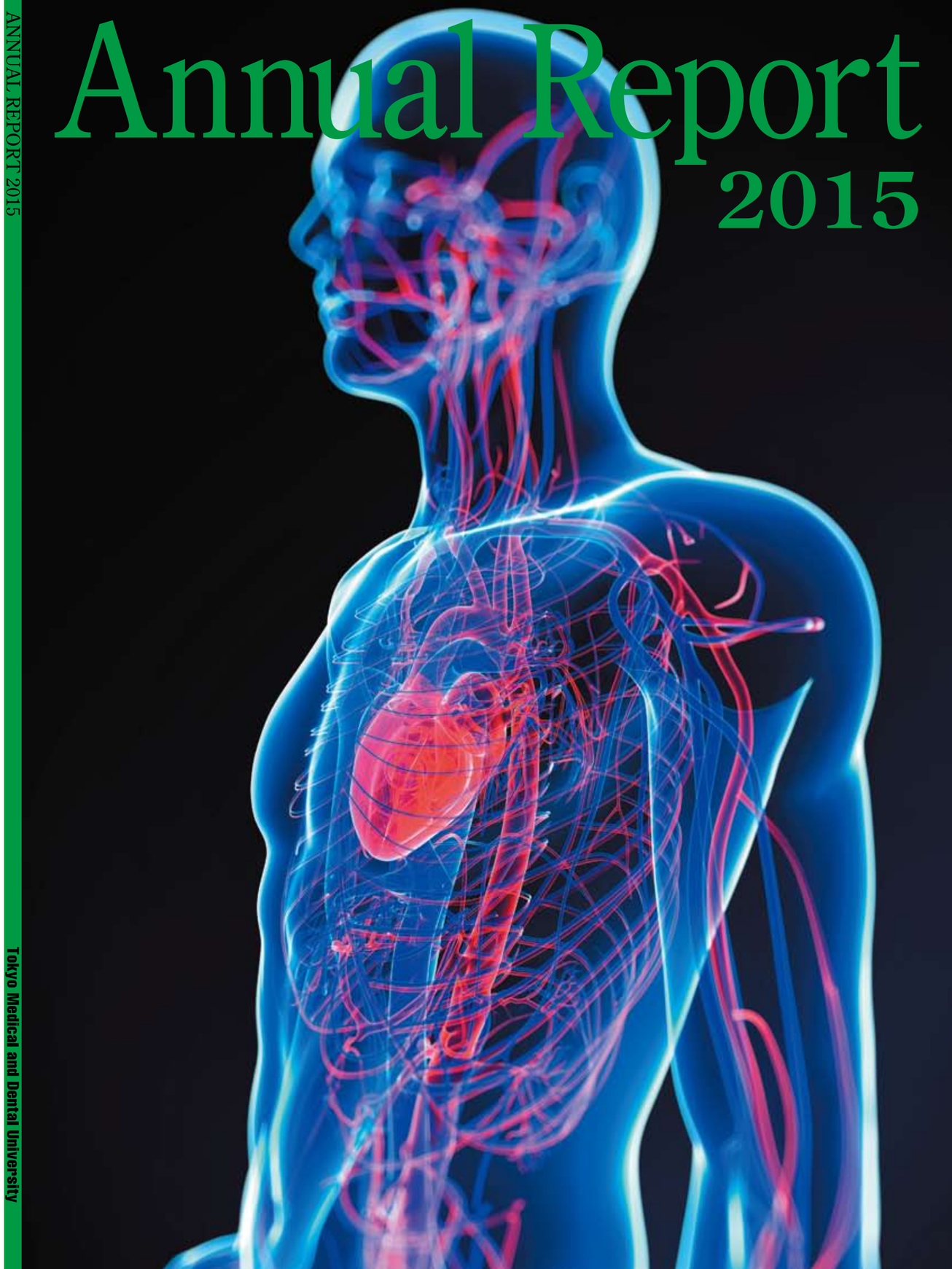
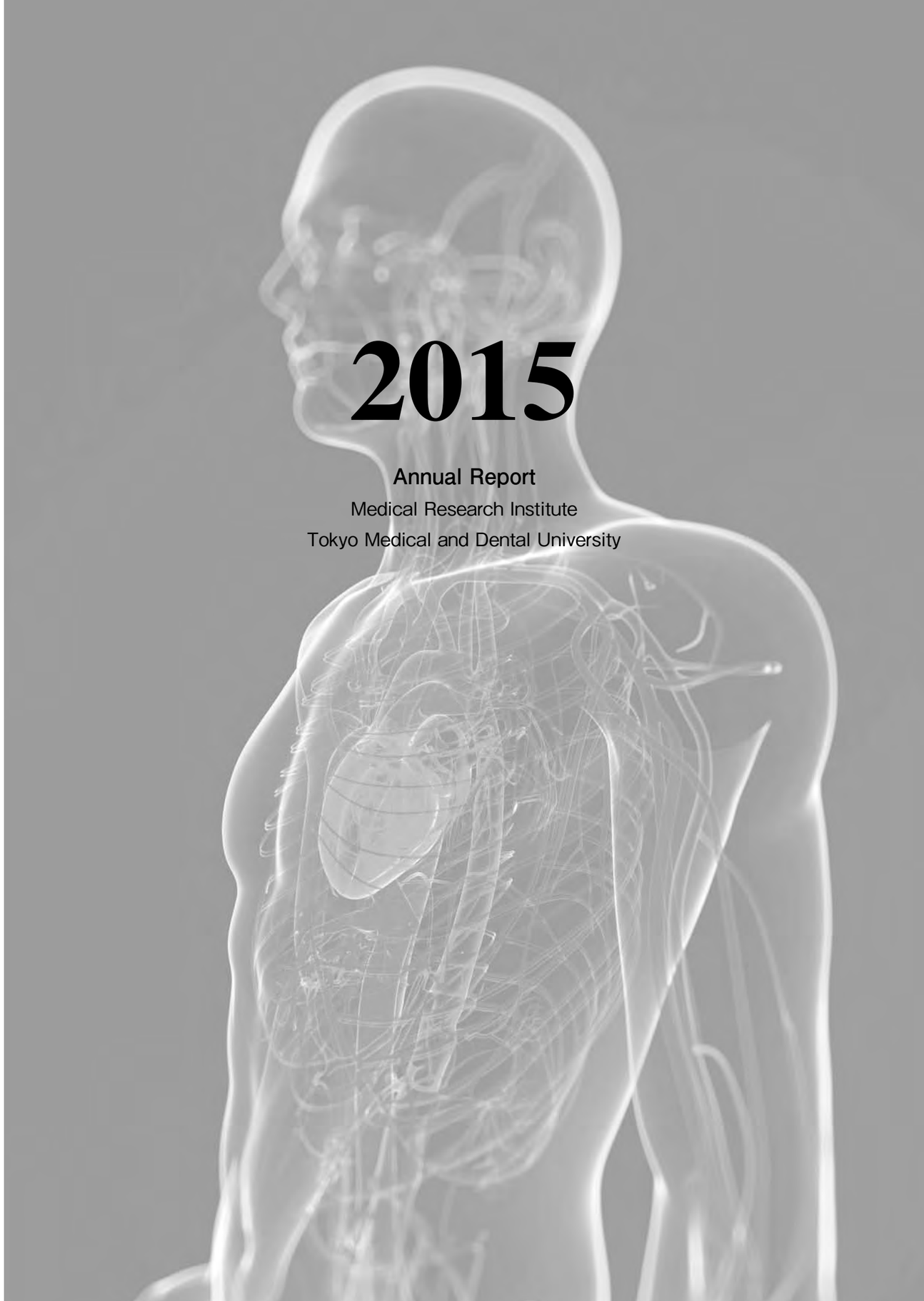
ANNUAL REPORT 2015

Tokyo Medical and Dental University

Medical Research Institute / Tokyo Medical and Dental University

Annual Report 2015





2015

Annual Report

Medical Research Institute

Tokyo Medical and Dental University

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Medical Research Institute

Yushima Area

1-5-45 Yushima Bunkyo-ku Tokyo 113-8510 Japan

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Surugadai Area

2-3-10 Kanda-surugadai Chiyoda-ku Tokyo 101-0062 Japan

Medical Research Institute
Department of Molecular Epidemiology, Frontier Research Unit Virus Research Unit, Frontier Research Unit Laboratory of Gene Expression, Project Research Unit

Medical Research Institute

Director
- Fumitoshi Ishino
- Faculty Meeting

Research Divisions

Division of Advanced Molecular Medicine	Department of Molecular Pharmacology	Professor	Masaki Noda
	Department of Molecular Cell Biology	Professor	Hiroshi Shibuya
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	Frontier Research Unit Laboratory of Oxygen Biology		
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	Department of Pathological Biochemistry	(under consideration)	
	Department of Pathological Cell Biology	Professor	Shigeomi Shimizu
	Department of Developmental and Regenerative Biology	Professor	Hiroshi Nishina
	Department of Stem Cell Biology	Professor	Emi K. Nishimura
	Department of Immunology	Professor	Takeshi Tsubata
	Department of Molecular Pathogenesis	Professoror	Akinori Kimura
	Project Research Unit		
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	Department of Molecular Genetics	Professor	Yoshio Miki
	Department of Molecular Epidemiology	Professor	Masaaki Muramatsu
	Department of Biochemical Genetics	Professor	Shigetaka Kitajima
	Department of Genomic Pathology	Professor	Shumpei Ishikawa
	Department of Epigenetics	Professor	Fumitoshi Ishino
	Department of Bioinfomatics	(under consideration)	
	Frontier Research Unit Laboratory of Gene Expression		
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Division of Integrative Research	System Biology for Intractable Diseases	Visiting Professor	Satoru Miyano
Laboratory for Integrated Research Projects on Intractable Diseases			
Advanced Technology Laboratory	Genome Laboratory		
	Laboratory of Cytometry and Proteome Research		
	Laboratory of Recombinant Animals		
	Laboratory of Anatomy and Cell Function		
	Bioresource Laboratory		
	Laboratory of Structural Biology		
	Stem Cell Laboratory		
Administrative Office	General Affairs Section		

Highlight

Identification of the driver gene in diffuse gastric cancer: RHOA mutation (Nat Genet. 2014 Jun; 46(6):583-7.)

Gastric cancer is one of the major causes of cancer death in Japan, killing 50,000 people a year. In particular, diffuse gastric cancer (also known as scirrhous stomach cancer) is one of the most highly malignant among gastric cancers, and since there are no effective molecular target drugs, this poses as a major health problem. Therefore, there is a need for the identification of causative genes and effective targets in scirrhous stomach cancer.

With the use of high depth whole exome sequencing, we determined the genomic sequence of almost all genes in surgically resected scirrhous stomach cancer. As a result, we identified RHOA as a gene that harbors somatic mutations with a high frequency, being found in more than 20% of clinical samples (Fig.1, arrow head). Among the identified mutations, amino acid substitutions were intensively concentrated in Arg5, Gly17 and Tyr42. In particular, Tyr42 was revealed to be located in the core effector region, which is important for RHOA to interact with its effector target proteins (Fig 2. a, b). Furthermore, to elucidate the function of mutated RHOA, we performed verification experiments by using culture cell lines. Knockdown of RHOA by siRNA suppressed only those cell lines with a mutated RHOA gene, and not with wild type RHOA. This siRNA-mediated suppression of proliferation was canceled by the overexpression of a siRNA-unresponsive RHOA gene. In conclusion, the results indicated that these mutations in the RHOA gene in scirrhous stomach cancer are important driver mutations that play a causative role in cancer progression.

This research finding is important as it involves the identification of mutated genes as the potential therapeutic target in scirrhous stomach, a cancer that does not yet have effective therapeutic targets.

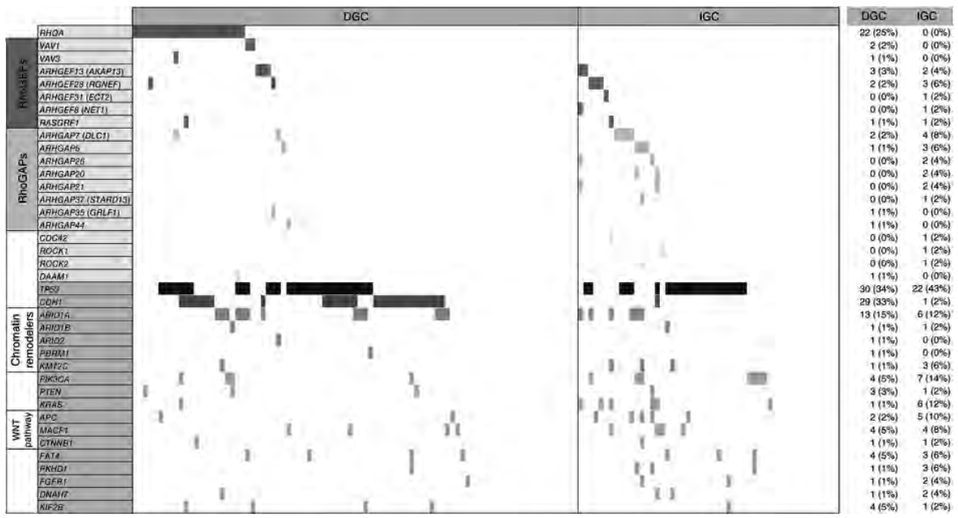


Fig 1. DGC : Diffuse-type gastric cancer IGC : intestinal-type gastric cancers

Genes closely linked to *RHOA* or frequently mutated in the discovery screen and in previous reports are shown. Cells represent somatically mutated genes. The total numbers and percentages of the mutant cases in each subtype are shown to the right.

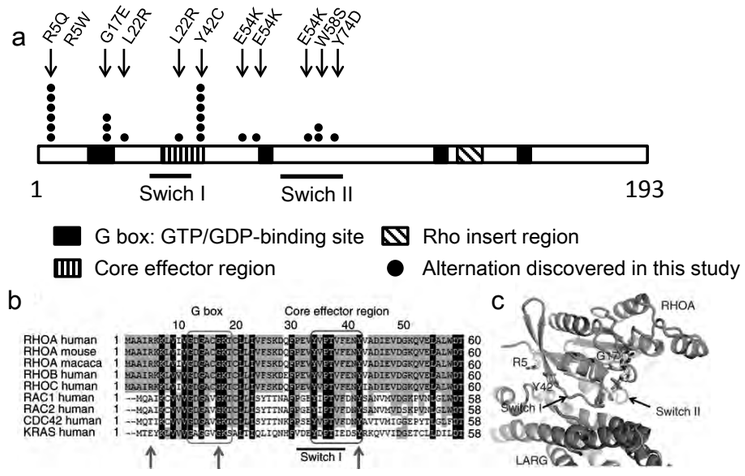


Fig 2. Distribution of RHOA alterations in DGC

(a) Map of RHOA functional regions and the sites of the amino acid substitutions discovered in this study. Recurrent alterations are indicated by multiple circles. (b) Amino acid alignment of RHOA and RHO family proteins (amino acids 1–60). Arrows indicate the most frequently mutated positions—Arg5, Gly17 and Tyr42. (c) Structure of RHOA and one of its representative RhoGEF proteins, LARG, in its GDP-bound form. Tyr42, one of the most frequently mutated residues in this study, is located on an interaction surface of RHOA with RhoGEFs.

Division of Advanced Molecular Medicine

Aim and Scope

The mission of Division of Advanced Molecular Medicine is to conduct basic and applied research on the cause, prevention and treatment of intractable diseases including life-style related diseases, bone diseases, immune diseases, neurological diseases, cardiovascular diseases and cancer. For this purpose, we are actively undertaking a broad spectrum of medical research with an emphasis on cross-disciplinary approaches. Topics of research projects in each Department are as follows:

【Molecular Pharmacology】

- FUCCI system was applied to visualize the link between PTH and mechanical stress signaling in osteoblasts in a real time manner.
- Cross talk between sympathetic tone and PTH was identified and analyses on the sympathetic tone action on BMP signaling was conducted.
- RANKL regulation in osteoporotic aged animal was discovered to be under the control of Cnot3 via its binding to 3' UTR to control posttranscriptionally.

【Molecular Cell Biology】

- WNK signaling pathway is involved in neural development via Lhx8 gene expression.
- IQGAP regulates the nuclear localization of β -catenin in Wnt signaling.

【Molecular Neuroscience】

- Glial dysfunction in the mouse habenula causes depression-like behaviors.
- Astroglial glutamate transporter deficiency leads to pathological repetitive behaviors in mice.
- Arundic acid attenuates retinal degeneration by increasing GLAST expression in a model of normal tension glaucoma.
- Valproic acid prevents retinal degeneration in a model of normal tension glaucoma.

【Biodefense Research】

- Discovery of commensal bacteria causing inflammatory bowel diseases (IBD).
  – A new point of view on treatment of IBD –
- Elucidation of a novel function of retinoic acid in the maintenance of intestinal homeostasis.
  – A new avenue of treatment for allergic responses to oral antigens –

【Bio-informational Pharmacology】

- Subthreshold analysis of GWAS for atrial fibrillation (AF)-related genes identified additional 6 AF-sensitive SNPs, resulting in identification of total 16 AF-sensitive SNPs.
- The system to simultaneously assay electrical and mechanical effects of drugs to iPS cell-derived cardiomyocytes has been established.
- By genetically modifying human iPS-derived cardiomyocytes, the system to assay rate-dependent effects of drugs has been established.

【Stem Cell Regulation】

- Sox17 was found to contribute to the maintenance of hematopoietic cell clusters containing HSCs in the midgestation AGM region.
- Abnormal number and functions of synapses were detected in histone demethylase gene mutant mice.
- A synthetic polymer-based approach revealed ECMs and iron as the components of cancer stem cell niche.

【Structural Biology】

- The crystal structure of an extracellular domain of the B-cell coreceptor CD72 was determined.
- The complex structures of a T-cell signaling protein with various phosphopeptides were determined.
- Interactions between the Alzheimer's disease-related tau protein and PPIase were investigated.

Department of Molecular Pharmacology

Professor	Masaki Noda, M.D., Ph.D.
Associate Professor	Yoichi Ezura, M.D., Ph.D.
Assistant Professor	Yayoi Izu, D.V.M., Ph.D.
Research Assistant Professor	Smriti Aryal A.C., DDS, Ph.D.

Research Summary

In order to contribute to the establishment of therapy and prevention for osteoporosis and the other calcium-related disorders, we are elucidating molecular mechanisms underlying regulation of calcium metabolism with emphases on bone formation and resorption. Skeletal system is the largest storage site for calcium in a living body and its metabolism is regulated by a complex cell society consisting of bone-forming osteoblasts and bone-resorbing osteoclasts as well as stromal cells and chondrocytes. In our department, we take molecular and cellular biological approaches to study the reguratory mechanisms of the development and differentiation as well as the function of each group of these cells.

Research Projects

1. Stability of mRNA influences osteoporotic bone mass via CNOT3 (Watanabe C, Hayata T, Notomi T, Ezura Y, Moriyama K, Noda M).

CNOT3 is a molecule involved in mRNA stability in yeast to mammalian cells, but its role in bone regulation is not known. We discovered that Cnot3 deficiency specifically enhances receptor activator of NF-κB (RANK) mRNA stability and leads to osteopenia in healthy young adult animals. Moreover, Cnot3 levels are reduced in ageing-induced osteoporosis, and Cnot3 deficiency further exacerbates such osteoporosis significantly. As a mechanism, Cnot3 binds to RANK mRNA and its 3'-UTR renders Cnot3-dependent instability to the reporter gene. Our results reveal Cnot3 regulation in aging-induced osteoporosis. (Proc Natl Acad Sci USA, 2014).

2. PTH regulates β2-adrenergic receptor expression in osteoblast-like MC3T3-E1 cells (Moriya S, Hayata T, Izu Y, Ezura Y, Kaneko K, Noda M).

As the aged population is soaring, prevalence of osteoporosis is increasing. However, the molecular basis underlying the regulation of bone mass is still incompletely understood. Sympathetic tone acts via beta2 adrenergic receptors in bone and regulates the mass of bone which is the target organ of parathyroid hormone (PTH). Here we investigated the effects of PTH on beta2 adrenergic receptor (*Adrb2*) gene expression in osteoblast-like MC3T3-E1 cells. PTH treatment immediately suppressed the expression levels of *Adrb2* mRNA. The effect was dose-dependent manner. PTH action on *Adrb2* expression was inhibited by a transcriptional inhibitor, DRB, but not by a protein synthesis inhibitor, cycloheximide suggesting direct transcription control. Knockdown of *Adrb2* promoted PTH-induced expression of *c-fos*, an immediate early response gene. With respect to molecular basis for this phenomenon, knockdown of *Adrb2* enhanced PTH-induced transcriptional activity of cyclic AMP response element-luciferase construct in osteoblasts. Knockdown of *Adrb2* also enhanced forskolin-induced luciferase expression, revealing that adenylate cyclase activity is influenced by *Adrb2*. As for phosphorylation of transcription factor, knockdown of *Adrb2* enhanced PTH-induced phosphorylation of cyclic AMP response element-binding protein (CREB). These data reveal that beta2 adrenergic receptor is one of the targets of PTH and acts as a suppressor of PTH action in osteoblasts. (J Cell Biochem, 2014).

3. b2 Adrenergic receptor activation suppresses BMP-induced alkaline phosphatase expression in osteoblast-like MC3T3E1 cells (Yamada T, Hayata T, Izu Y, Ezura Y, Harada K, Noda M).

Beta adrenergic stimulation suppresses bone formation in vivo while its actions in osteoblastic differentiation are still incompletely understood. We therefore examined the effects of beta 2 adrenergic stimulation on osteoblast-like MC3T3-E1 cells focusing on BMP-induced alkaline phosphatase expression. Morphologically, isoproterenol treatment suppresses BMP-induced increase in the numbers of alkaline phosphatase-positive small foci in MC3T3-E1 cells. Biochemically, isoproterenol treatment suppresses BMP-induced alkaline phosphatase activity in a dose dependent manner. W also found that continuous isoproterenol treatment is more suppressive against BMP-induced increase in alkaline phosphatase expression than cyclic regimen. At molecular level, isoproterenol treatment suppresses BMP-induced enhancement of alkaline phosphatase mRNA expression and BRE-luciferase activity. These data indicate that isoproterenol regulates BMP-induced alkaline phosphatase expression in osteoblast-like MC3T3E1 cells. (J Cell Biochem, 2014).

Highlight

Migration linked to FUCCI indicated cell cycle is controlled by PTH and mechanical stress (Shirakawa J, Hayata T, Ezura Y, Omura K. Noda M).

Bone metabolism is maintained via balanced repetition of bone resorption by osteoclasts and bone formation by osteoblasts. Osteoblastic cells are capable of conducting self-renewal and differentiation that are basically associated with cell-cycle transition to enable cell specification and bone formation. Osteoblasts are also migrating to fill the resorption cavity curved by osteoclasts during bone remodeling to maintain homeostasis of bone mass whose imbalance leads to osteoporosis. However, technical difficulties have hampered the research on the dynamic relationship between cell cycle and migration in osteoblasts. In this report, we overcome these problems by introducing fluorescent ubiquitination-based cell cycle indicator (FUCCI) reporter system in calvarial osteoblastic cells and reveal that the cells in G_1 as well as $S/G_2/M$ phase are migrating. Furthermore, the osteoblastic cells in $S/G_2/M$ phase migrate faster than those in G_1 phase. Interestingly, parathyroid hormone (PTH) as an anabolic agent enhances migration velocity of the cells. Mechanical stress, another anabolic signal, also enhances migration velocity. In contrast, in the presence of both PTH and mechanical stress, the migration velocity returns to the base line levels revealing the interaction between the two anabolic stimuli in the regulation of cell migration. Importantly, PTH and mechanical stress also interact when they regulate the transition of cell cycle. These data demonstrate that osteoblastic migration is linked to cell cycle and it is under the control of mechanical and chemical stimuli that coordinate to regulate bone mass. (J Cell Physiol, 2014).

Significance

Osteoporosis is a highly prevalent disease affecting nearly 20 million people in the United States and is life-threatening in elderly patients. The regulation of cell cycle and cell migration is required for bone formation at appropriate sites. Here we first successfully demonstrate the real time relationship between cell cycle and migration by using primary osteoboasts obtained from calvarias of the transgenic mice expressing fluorescent ubiquitination-based cell cycle indicator (FUCCI) reporter. This system revealed that the osteoblastic cells in $S/G_2/M$ phase migrate faster than those in G_1 phase. We also found that parathyroid hormone (PTH) and mechacnical stress that are the major anabolic potency enhance migration velocity of the cells. Interestingly, the presence of both PTH and mechanical stress keep the base line levels of cell velocity, revealing the interaction between the two anabolic stimuli in the regulation of cell migration. These data demonstrate that the linkage of osteoblast migration and cell cycle and it is under the coordinate control of mechanical and chemical stimuli that regulates bone mass.

Publications

[Original articles]

1. Watanabe C, Morita M, Hayata T, Nakamoto T, Kikuguchi C, Li X, Kobayashi Y, Takahashi N, Notomi T, Moriyama K, Yamamoto T, Ezura Y, Noda M. Stability of mRNA influences osteoporotic vone mass via CNOT3. ***Proc Natl Acad Sci U S A.*** 2014 Feb 18;111(7):2692-7.
2. Yamada T, Ezura Y, Hayata T, Moriya S, Shirakawa J, Notomi T, Arayal S, Kawasaki M, Izu Y, Harada K, Noda M. β2 Adrenergic receptor activation suppresses BMP-induced alkaline phosphatase expression in osteoblast-like MC3T3E1 cells. ***J Cell Biochem.*** 2014 *In Press*
3. Moriya S, Hayata T, Notomi T, Aryal S, Nakamaoto T, Izu Y, Kawasaki M, Yamada T, Shirakawa J, Kaneko K, Ezura Y, Noda M. PTH regulates β2-adrenergic receptor expression in osteoblast-like MC3T3-E1 cells. J Cell Biochem. 2015 Jan;116(1):142-8.
4. Hayata T, Yoichi, Ezura, Asashima M, Nishinakamura R, Noda M. Dullard/Ctdnep1 regulates endochondral ossification via suppression of TGF-beta signaling. ***J Bone Miner Res.*** 2015 Feb;30(2):318-29.
5. Ezura Y, Nagata J, Nagao M, Hemmi H, Hayata T, Rittling S, Denhardt DT, Noda M. Hindlimb-unloading suppresses B cell population in the bone marrow and peripheral circulation associated with OPN expression in circulating blood cells. ***J Bone Miner Metab.*** 2015 Jan;33(1):48-54.
6. Shirakawa J, Ezura Y, Moriya S, Kawasaki M, Yamada T, Notomi T, Nakamoto T, Hayata T, Miyawaki A, Omura K, Noda M. Migration linked to FUCCI-indicated cell cycle is controlled by PTH and mechanical stress. ***J Cell Physiol.*** 2014 Oct;229(10):1353-8.

Department of Molecular Cell Biology

Professor **Hiroshi Shibuya**
Associate Professor **Toshiyasu Goto**
Assistant Professor **Atsushi Sato**

Various signaling molecules inducing the cell-growth and differentiation regulate morphogenesis and organogenesis of the vertebrate. The failure of these signal molecules has also been caused with induction of the diseases. Therefore, the elucidation of signal transduction network regulating generation and differentiation is important upon clarifying the mechanism of morphogenesis, organogenesis and diseases. We focus on the signal transduction network regulating the mechanisms of morphogenesis and organogenesis in developmental process.

WNK protein kinases, the causative genes of pseudohypoaldosteronism type II (PHAII) disease

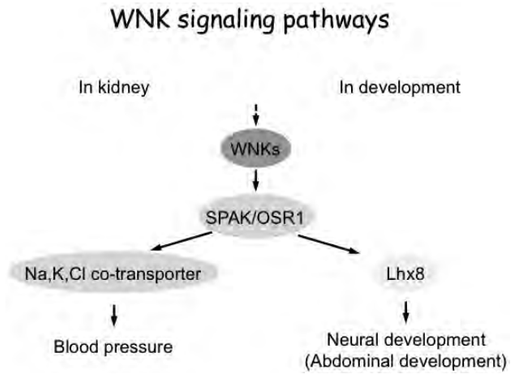
WNK (with no lysine (K)) kinase family that has been recently identified serine/threonin protein kinase family conserved among many species. There are four mammalian WNK family members, and mutations in two of them, WNK1 and WNK4 have been linked to a hereditary form of human hypertension known as Pseudohypoaldosteronism type II (PHAII). Previously, we identified that WNKs phosphorylated and activated SPAK/OSR1 kinases, which in turn regulated various ion co-transporters, such as NKCC1, NKCC2 and NCC. We also presented that the malfunction of this regulation caused the similar phenotypes of PHAII in mouse. However, this misregulation cannot cause all of pathological conditions of PHAII, such an intellectual impairment, dental abnormalities and impaired growth. This suggests that WNK is involved in the other signaling cascade. We started to look for the other interacting factor(s) of WNK using model animals.

1. WNK signaling is involved in neural development

We identified Arrowhead (Awh) as a new downstream element of WNK signaling pathway in *Drosophila*. Awh is conserved in vertebrates as Lhx8. *Lhx8* expression was also regulated by WNK signaling pathway. These results suggest that WNK-Lhx8/Awh pathway is the new conserved pathway in many species. In mouse brain, Lhx8 is known to be involved in the specification of cholinergic neurons. When we knocked down both *WNK1* and *WNK4* in differentiated Neuro2A cells, the elongation of neurites was suppressed and the marker gene expression of cholinergic neurons was reduced. We also showed that the expression of constitutive active form of OSR1, the downstream kinase of WNK, could rescue these phenotypes caused by the knockdown of both *WNK1* and *WNK4*. These results suggest new findings that WNK signaling pathway is involved in the neural differentiation and the specification of cholinergic neurons. Since the pathological conditions of PHAII showed an intellectual impairment, these may suggest that WNK pathway is involved in the pathogenesis of PHAII via Lhx8.

2. WNK4 is an essential effector of anterior formation in FGF signaling

In *Xenopus* embryos, depletion of *WNK4* by antisense morpholino oligonucleotides (MOs) results in a severe defect in anterior development and impaired expression of endogenous anterior markers. Defects in head formation or expression of anterior marker genes caused by suppression of endogenous *WNK4* expression could be rescued by expression of wild-type WNK4, but not mutant WNK4 lacking its kinase activity. It is notable that morphants of Xenopus *WNK4* inhibited the expression of anterior marker genes and the target genes induced by FGF signaling. Moreover, knockdown of Wnk4 significantly reduced the phosphorylation level of OSR1 induced by FGF. These results provide the first evidence that FGF signaling regulates WNK4 function required for anterior formation in *Xenopus* development.



Publications

Shimizu, N., Ishitani, S., Sato, A., Shibuya, H. and Ishitani, T. (2014). Hipk2 and PP1c Cooperate to Maintain Dvl Protein Levels Required for Wnt Signal Transduction. **Cell Rep.** 8, 1391-1404.

Department of Molecular Neuroscience

Professor	Kohichi Tanaka
Associate Professor	Hidenori Aizawa
Assistant Professor	Tomomi Aida
Assistant Professor	Miho Soma
Assistant Professor	Yukiko Ito
Assistant Professor	Michiko Yanagisawa

The final goal of our research is to understand molecular, cellular, and neuronal ensemble mechanisms underlying higher order brain functions including learning and memory. For that purpose, we combine molecular genetics, physiological and behavioral methods. The laboratory also studies the mechanism that underlies neuronal cell death and regeneration.

1. Functions of glutamate transporters in the brain

Glutamate is a major excitatory neurotransmitter and plays an important role in neuronal plasticity and neurotoxicity in the central nervous system. Glutamate transport proteins provide the mechanism by which synaptically released glutamate is inactivated and kept below toxic levels in the extracellular space. By now, five subtypes of high-affinity glutamate transporters have been identified in the mammalian brain. Our lab studies the physiological and pathological roles of glutamate transporter subtypes using subtype-specific knockout mice.

We show that astrocyte-specific glutamate transporter GLT1 inducible knockout (iKO) mice exhibit pathological repetitive behaviors including excessive and injurious levels of self-grooming and tic-like head shakes. Electrophysiological studies reveal that excitatory transmission at corticostriatal synapse is normal in a basal state but is increased after repetitive stimulation. Furthermore, treatment with an N-methyl-D-aspartate receptor antagonist memantine ameliorated the pathological repetitive behaviors in iKO mice. These results suggest that astroglial GLT1 plays a critical role in controlling the synaptic efficacy at cortico-striatal synapses and its dysfunction causes pathological repetitive behaviors.

We found that arundic acid induces GLAST expression *in vitro* and *in vivo*. In addition, arundic acid treatment prevented RGC death by upregulating GLAST in heterozygous (*GLAST*$^{+/-}$) mice. Furthermore, arundic acid stimulates the human GLAST ortholog, EAAT1, expression in human neuroglioblastoma cells. Thus, discovering compounds that can enhance EAAT1 expression and activity may be a novel strategy for therapeutic treatment of glaucoma.

2. Glial dysfunction of the lateral habenula causes the depressive-like behaviors and sleep disturbance

Lateral habenula (LHb) has recently attracted a surge of interest in psychiatry because recent studies have reported the pathological activation of the habenula in patients with major depression and in animal models. However, how habenular neurons are activated to cause various depression symptoms, such as reduced motivation and sleep disturbance, remain unclear. Since astrocyte primarily regulates the extracellular level of excitatory neurotransmitter glutamate, we hypothesized that dysfunctional astrocytes may cause LHb hyperactivity due to the defective uptake activity of extracellular glutamate, which induces depressive-like behaviors. The habenula-specific inhibition of glial glutamate transporter GLT-1 increased the neuronal firing rate and the level of c-Fos expression in the LHb. Mice with reduced GLT-1 activity in the habenula exhibited a depressive-like phenotype in the tail suspension and novelty-suppressed feeding tests. These animals also displayed increased susceptibility to chronic stress, displaying more frequent avoidant behavior without affecting locomotor activity in the open-field test. Intriguingly, the mice showed disinhibition of rapid eye movement sleep, which is a characteristic sleep pattern in patients with depression. These results provide evidence that disrupting glutamate clearance in habenular astrocytes increases neuronal excitability and depressive-like phenotypes in behaviors and sleep.

3. Development of genome editing technologies

Knockout and knockin mice have drastically improved our understanding of the functions of genes *in vivo*. However, the generation of knockout and knockin mice relies on homologous recombination in ES cells, which is a time-consuming, laborious, and expensive process. Recent development of genome editing technologies has enabled direct manipulation of the genome in mouse zygotes (*in vivo* genome editing), thereby providing new avenues for simple, convenient, highly efficient, and ultra-rapid production of knockout and knockin mice. We developed highly efficient CRISPR/Cas (clustered regularly interspaced short palindromic repeat/CRISPR-associated)-mediated *in vivo* genome editing system. By harnessing these technologies, we can produce any kind of genetically modified mice including gene knockout, human mutation knockin, and gene cassette knockin with extreme high efficiencies. Taken together, our CRISPR/Cas system provides a fast, convenient, efficient, and cost-effective approach to the production of genetically modified mice and brings about drastic developments in the field of genome editing, leading to a boost in functional genomic research.

Publications

[Original papers]

1. Yanagisawa, M., Aida, T., Takeda, Namekata, K., Harada, T., Shinagawa, R., Tanaka, K. Arundic acid attenuates retinal ganglion cell death by increasing glutamate/aspartate transporter (GLAST) expression neural cell death in a model of normal tension glaucoma. *Cell Death Dis* (in press).
2. Aida, T., Yoshida, J., Nomura, M., Tanimura, A., Iino, Y., Soma, M., Bai, N., Ito, Y., Cui, W., Aizawa, H., Yanagisawa, M., Nagai, T., Takata, N., Tanaka, KF., Takayanagi, R., Kano, M., Gotz, M., Hirase, H., Tanaka, K. Astroglial glutamate transporter deficiency increases synaptic excitability and leads to pathological repetitive behaviors in mice. *Neuropsychopharmacology* (in press).
3. Kimura, A., Guo, X., Noro, T., Harada, C., Tanaka, K., Namekata, K., Harada, T Valproic acid prevents retinal degeneration in a murine model of normal tension glaucoma. *Neurosci Lett* 588. 108-113, 2015.
4. Nakamori, T., Sato, K., Kinoshita, M., Kanamatsu, T., Sakagami, H., Tanaka, K., Ohki-Hamazaki, H. Positive feedback of NR2B-containing NMDA receptor activity is the initial step toward visual imprinting: a model for juvenile learning. *J Neurochem* 132. 110-123, 2015.
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[Reviews]

1. Aida, T. Genome editing in mice using TALENs. *Targeted Genome Editing Using Site-Specific Nucleases* Chapter 11, 167-182, 2015 (Springer).

Department of Biodefense Research

Professor	Toshiaki Ohteki, Ph.D.
Junior Associate Professor	Nobuyuki Onai, Ph.D.
Assistant Professor	Hiroyuki Tezuka, Ph.D.
Adjunct Lecturer (JST PREST)	Taku Sato, Ph.D.
Assistant Professor	Yusuke Nakanishi, Ph.D.
Project Junior Assistant Professor	Jumpei Asano, Ph.D.
Research Technician	Shoko Kuroda, Kisho Shiseki, Rumiko Nakamura
Secretarial Assistant	Hisako Kamioka

Our research projects focus on understanding the dynamic maintenance and transfiguration of homeostasis in the living body. Our goal is to define the homeostasis mechanism under conditions of health and disease. To accomplish this goal, we are trying to clarify the molecular basis of induction and failure of homeostasis by focusing on immune cells in particular mononuclear phagocytes, tissue stem cells, and their functional interplay in the immunological and non-immunological organs, such as skin and intestine. On the basis of our findings, we will further pursue our research in the hope of developing new rational therapies for prevention and treatment of disease.

1. Research on mononuclear phagocytes

1) Discovery of a novel source of dendritic cells, the control tower of the immune system

Dendritic cells (DCs) maintain immune tolerance under steady-state conditions, and activate immune cells upon infection. In this context, it is important to identify the progeny cells committed solely to DC differentiation, i.e. DC progenitors from the points of view of DC development and clinical applications.

DCs consist of conventional DCs (cDCs) and plasmacytoid DCs (pDCs), both of which play critical regulatory roles in the immune system. cDCs exhibit prominent antigen-presenting ability, whereas pDCs are characterized by their capacity to produce large amounts of type I interferons (IFNs). We have discovered the DC progenitors in the mouse bone marrow, and named common DC progenitors (CDPs) (***Immunity*** 2013; ***Nat Immunol*** 2007). Interestingly, CDPs are divided into 2 subpopulations. One is M-CSF receptor (R)$^{+}$ CDPs mainly producing cDCs, and the other M-CSFR^{-}CDPs highly express E2-2, an essential transcription factor for pDC development, and produce a large number of pDCs. Together with the common monocyte/macrophage progenitors, cMoP, identified by other group, we proposed a current model for mononuclear phagocyte differentiation pathway (***Immunity*** 2014) (Fig. 1).

Based on these achievements, we are currently trying to identify human progenitors of mononuclear phagocytes. The discovery of human mononuclear phagocyte progenitors producing fresh DCs or monocytes/macrophages will be a milestone, leading to the development of prophylactic and therapeutic applications for infectious diseases, cancers, and autoimmune diseases.

2) Discovery of a new function of microbiota to attract monocyte/macrophage migration

Breakdown of the intestinal epithelial layer's barrier function results in the inflow of commensal flora and improper immune responses against the commensal flora, leading to inflammatory bowel disease (IBD) development. The microbiota in the large intestine is denser and more diverse than that in the small intestine, and mostly consists of Gram-positive Firmicutes or Gram-negative Bacteroidetes; which are conserved in humans and mice. Although the presence of Gram-negative bacteria, Bacteroides/Prevotella or Enterobacteriaceae in the colon is a risk factor for developing IBD, the role of Gram-positive bacteria in colitis is unknown.

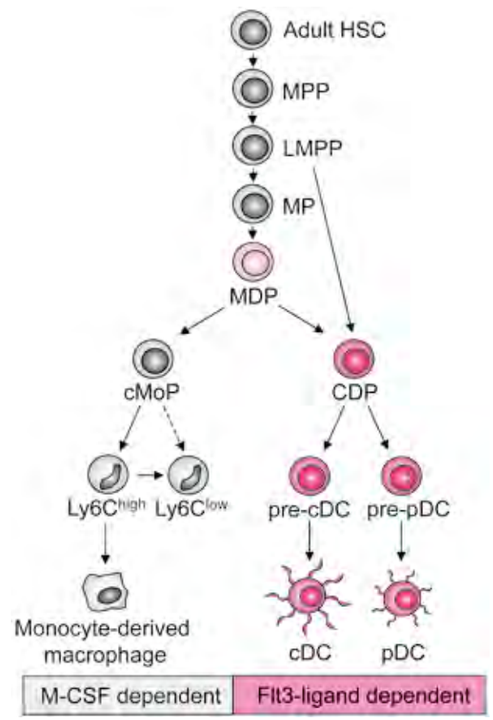


Fig.1 Development of mononuclear phagocytes

Using a mouse dextran sodium sulfate (DSS)–induced colitis model, we show here that commensal Gram-positive bacteria trigger the mobilization of inflammatory monocytes and macrophages into the colon (***Mucosal Immunol*** 2015). TNF-α is a representative cytokine that aggravates colitis, and predominantly produced by monocytes/macrophages. Interestingly, pretreating mice with vancomycin, which eliminated Gram-positive bacteria, particularly the Lachnospiraceae family, significantly reduced the severity of the colitis, evaluated by the body weight loss, colon length, pro-inflammatory cytokine level, massive leukocytic infiltrateon etc. Importantly, vancomycin treatment specifically downregulated the colonic epithelial cell (cEC) expression of CCR2 ligands, which are critical chemokines for monocyte/macrophage mobilization into the inflamed colon (Fig. 2). In addition, 16S rRNA analysis showed that vancomycin treatment dramatically reduced Lachnospiraceae, the most abundant order of Clostridiales in untreated control mice. As the sera from Crohn's disease patients and colitic mice react with *Lachnospiraceae bacterium A4* flagella, our findings provide a new environmental risk factor and new therapeutic approaches for IBD.

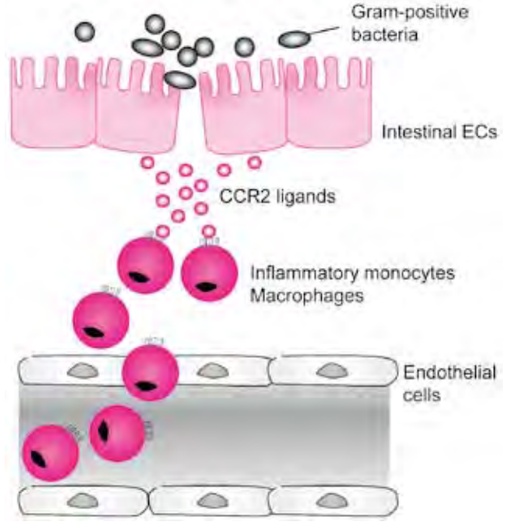


Fig.2 Gram-positive bacteria control migration of inflammtory monocytes/ macrophages in IBD

This paper has been selected by the Society for Mucosal Immunology as a Featured Paper of the Month.

2. Research on tissue stem cells

1) Understanding of tissue homeostasis on the basis of immune cell-tissue stem cell interplay

We recently found that type I IFNs induce proliferation and exhaustion in hematopoietic stem cells (HSCs), and that interferon regulatory factor-2 (IRF2), a transcriptional suppressor of type I IFN signaling, preserves the self-renewal and multi-lineage differentiation capacity of HSCs (***Nat Med*** 2009). Based on this finding, we show that type I IFN preconditioning, without irradiation or DNA alkylating agents, significantly enhanced the HSC engraftment efficiency in wild type (WT) recipient mice, which was applicable to the treatment of Sly syndrome, a congenital storage disorder with β-glucuronidase deficiency, in which it restored enzyme expression at the HSC level. Our findings suggest type I IFN-based preconditioning, combined with HSC transplantation, as a novel non-genotoxic treatment for some congenital diseases (***Blood*** 2013).

2) Maintenance machinery of intestinal stem cells

Intestinal stem cells (ISCs) are an exclusive source of intestinal epithelial cell regeneration. We have identified 2 genes that are essential for the maintenance of ISCs, and are currently studying the molecular basis of how these genes control ISC homeostasis.

Publications

[original papers]

1. Nakanishi , Sato T, and Ohteki T. Commensal Gram-positive bacteria initiates colitis by inducing monocyte/macrophage mobilization. ***Mucosal Immunol*** 8, 152-60, 2015.
2. Yokota-Nakatsuma A, Takeuchi H, Ohoka Y, Kato C, Song SY, Hoshino T, Yagita H, Ohteki T, and Iwata M Retinoic acid prevents mesenteric lymph node dendritic cells from inducing IL-13-producing inflammatory Th2 cells. ***Mucosal Immunol*** 7, 786-801, 2014.

[Awards]

Nobuyuki Onai, The best paper award of Medical Research Institute in 2014

Authors: Onai N, Kurabayashi K, Hosoi-Amaike M, Toyama-Sorimachi N, Matsushima K, Inaba, K, and Ohteki T.
Title of the paper: A clonogenic progenitor with prominent plasmacytoid dendritic cell developmental potential. ***Immunity*** 38, 943-57, 2013.

Department of Bio-informational Pharmacology

Professor Tetsushi Furukawa, M.D., Ph.D.
Associate Professor Junko Kurokawa, Ph.D.
Assistant Professor Yusuke Ebana, M.D., Ph.D.

This laboratory focuses on understanding pathogenesis of intractable and common cardiovascular diseases using multidisciplinary approach (patch-clamp, cell biology, optical recording, genetic analysis, and computational analysis). Our ultimate goal is to improve diagnosis and management of intractable and common cardiovascular diseases.

1. Pathogenesis of atrial fibrillation (AF)

Atrial fibrillation (AF) is the most frequent arrhythmias, reaching more than 1 million patients in Japan. Associated cerebral infarction due to cardiogenic thrombosis (250,000 patients /year in Japan) and higher incidence of cognitive impairment cause reduced QOL and are main causes of bedridden old people. Thus, establishment of therapeutic strategy for AF is an urgent requirement.

(1) GWAS for AF

We had carried out most extensive GWAS (genome-wide association study) in Japan to determine gene polymorphisms associated with AF. Since 2011, we have participated in the international Meta-analysis called as CHARGE study. Up to the last year, we identified totally 10 SNPs associated with AF (6 in Japanese population). This year, we performed sub-threshold study for SNPs that had low p-values but did not reach the genetically statistically significant level in the original GWAS. The sub-threshold analysis gave us 6 novel SNPs associated with AF (figure 1).

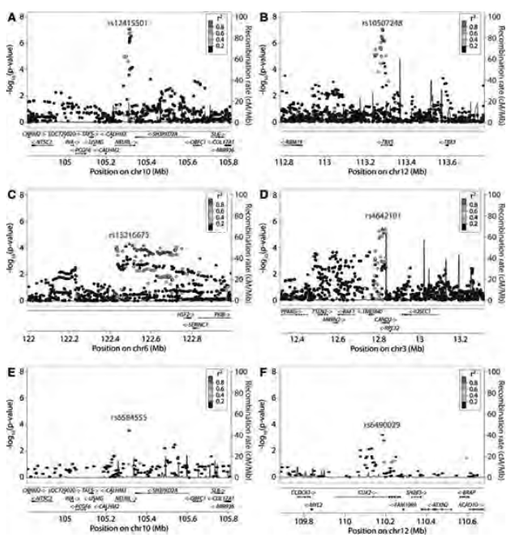


Fig.1 New 6 AF-sensitive SNPs identified in the sub-threshold study

(2) Functional analysis of AF associated genes

One of the sales-points of GWAS is the identification of novel pathogenic pathways and therapeutic targets due to its comprehensibility. We carried out functional analysis for 6 genes associated with Japanese AF patients. Up to the last year, we demonstrated a novel pathway generating abnormal automaticity in the pulmonary vein myocardium, which is the main triggering factor of atrial fibrillation. This year, we focused on another SNP that found to enhance actin depolymerization, and to cause myolysis frequently seen in fibrillated atrium.

2. Cardiovascular diseases and microRNA

The growing body of evidence indicates that microRNAs play important roles in development of diseases, and that circulating microRNAs can be biomarkers for development and progression of diseases. This year, we searched for microRNAs and circulating microRNAs that were up-regulated or down-regulated in various cardiovascular diseases in mouse and human. We found several microRNAs up-regulated in diabetic mouse hearts and in the hearts of mouse raised with high fat diet. We could identify the microRNA-target genes and the pathways involving in arrhythmia developments.

3. Safety cardiac pharmacology and toxicology using iPS cells and mathematical modeling

We aim to contribute to assessments for drug-induced lethal arrhythmias which have been a major reason for drug withdrawal from market. Novel multidisciplinary approaches using in silico mathematical models (collaborating with Dr. Takashi Ashihara at Shiga University of Medical Science) and human iPS cells-derived cardiomyocytes (collaborating with Dr. Yasunari Kanda at National Institute of Health Sciences) are developing in order to predict lethal drug-induced arrhythmias at pre-clinical safety pharmacological and toxicological tests.

4. Studies on molecular mechanisms for cardiac channelopathies

Mutations in *KCNQ1* which encodes the I_{Ks} channel α subunit associate with cardiac channelopathies including congenital long QT syndromes. In order to understand the molecular mechanisms, we analyze super-macromolecular complex of membrane transporters which bind to the KCNQ1 channel using biochemical and electrophysiological approaches and try to unravel physiological/pathophysiological roles of the KCNQ1-complex in the cardiac electrophysiological system (collaborating with Dr. Shushi Nagamori at Osaka Univesity Graduate School of Medicine).

5. Use of state-of-art technology for cardiovascular research

(1) Use of motion vector technology for in vitro analysis of cardiac contraction

Motion vector technology created by Sony Co. (Dr. Matsui E. et al.) is the in vitro system to assay non-invasively contraction and relaxation speed of cardiac myocytes. We have tried to broaden its application to screening of cardiac toxicity of medicines. This year, we performed two projects; one was to establish a system to monitor electrical and mechanical properties of iPS cell-derived cardiomyocytes using MEA system and the motion vector technology, and the other was to establish a system to monitor arrhythmias (spiral wave reentry) themselves.

(2) Use of 3-D cardiac simulator (UT-heart) for screening of cardiac toxicity of medicines

Prof. Hisada T. et al. in the University of Tokyo have developed a 3-D cardiac simulator (UT-heart). We have tried to broaden its application to screening of cardiac toxicity of medicines. This year, we examined 10 standard medicines (high risk, intermediate risk, and no risk), and found that the UT-heart predicted cardiac toxicity of medicines with a high accuracy. The UT-heart is a 3-D simulator and divides ventricular wall into 3 layers, endocardial, mid-myocardial, and epicardial layers. If we eliminated 3 layers structure from a simulation, then the accuracy to predict cardiac toxicity of medicines significantly dropped, suggesting the importance of a 3-D simulation for accurate prediction of cardiac toxicity of medicines.

Publications

[original articles]

1. Sinner MF, Tucker N, Lunetta K, Ozaki K, Smith G, Trompet S, Bis J, Lin H, Chung M, Nielsen JB, Lubitz S, Krijthe B, Magnani J, Ye J, Gollob M, Tsunoda T, Müller-Nurasyid M, Lichtner P, Peters A, Dolmatova E, Kubo M, Smith J, Psaty B, Smith N, Jukema JW, Chasman D, Ebana Y, Furukawa T, Macfarlane P, Harris T, Darbar D, Dorr M, Holst A, Hastrup J, Svensen H, Hofman A, Uitterlinden A, Gudnason V, Isobe M, Malik R, Dichgans M, Rosand J, Wagoner DV, Benjamin E, Milan D, Melander O, Heckbert SR, Ford I, Liu Y, Barnard J, Olesen M, Stricker B, Tanaka T, Kääb S, Ellinor P. (2014). Integrating genetic, transcriptional, and functional analyses to identify five novel genes for atrial fibrillation. ***Circulation*** 130, 1225-1235.
2. Hayakawa T, Kunihiro T, Ando T, Kobayashi S, Matsui E, Yada H, Kanda Y, Kurokawa J, Furukawa T. (2014). Image-based evaluation of contraction-relaxation kinetics of human-induced pluripotent stem cell-derived cardiomyocytes: Correlation and complementary with extracellular electrophysiology. ***J. Mol. Cell. Cardiol.*** 77, 178-191.
3. Tanaka A, Yuasa S, Mearini G, Egashira T, Seki T, Kodaira M, Kusumoto D, Kuroda Y, Okata S, Suzuki T, Inohara T, Arimura T, Makino S, Kimura K, Kimura A, Furukawa T, Carrier L, Node K, Fukuda K. (2014). ET-1 induces myofibrillar disarray and contractile vector variability in hypertrophic cardiomyopathy-iPS cell-derived cardiomyocytes. ***J. Amer. Heart Assoc.*** 13, e001263.

Department of Stem Cell Regulation

Professor	Tetsuya TAGA
Associate Professor	Tetsushi KAGAWA, Ikuo NOBUHISA
Project Assistant Professor	Kouichi TABU (-August 2014)
Part-time Lecturer	Toshio SUDA, Ryoichiro KAGEYAMA, Taichi KASHIWAGI
Administrative Assistant	Mako FUSHIMI
Technical Assistant	Kazuko INOUE
Graduate Student	Norihisa BIZEN, Maha ANANI, Genki SUDO, Yasuhiro KOKUBU, Wenqian WANG, Mayumi AMANO, Sachiko KANEKO, Kaho HARADA, Yoshitaka MUROTA, Tomoyo IKENOUE, Ryosuke KIMURA, Kiyoka SAITO, Aoi MINOWA, Shunki NOMOTO, Yuki YOKOI
Research Student	Kazuo TERASHIMA

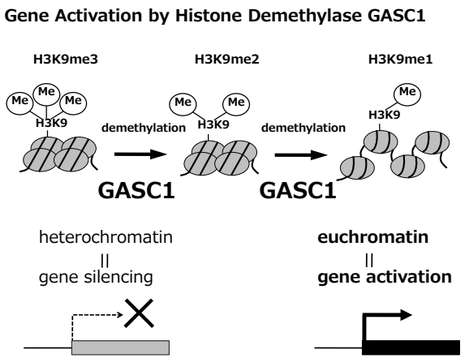
Research Outline

Our research has been conducted to elucidate the mechanisms by which stem cells are regulated. The major focus has been on neural stem cells, hematopoietic stem cells, and cancer stem cells. The study is aimed to understand development, maintenance, and regeneration of the central nervous system and the hematopoietic system, and to obtain a clue to tackle the problem of cancer recurrence. The projects performed in 2013 are categorized into three groups: 1. Understanding of molecular basis for self-renewal and fate determination of neural stem cells, 2. Characterization of fetal hematopoiesis, and 3. Characterization of cancer stem cells and their niche.

Research Projects

1. Epigenetic regulation of brain function

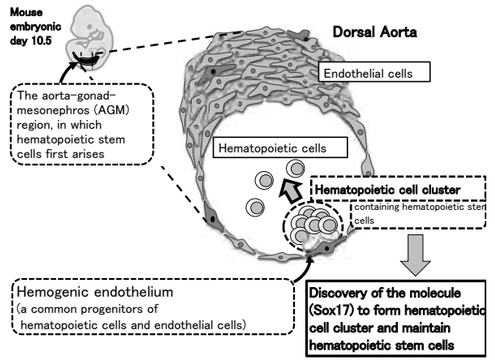
Accumulating evidence indicated importance of epigenetic modification in ES cells and normal tissue development. However, its physiological and pathological consequence in higher brain functions largely remained elusive. Gasc1 gene encodes a histone H3 lysine 9 (H3K9) demethylating enzyme, which is considered to serve to trigger a wide range of epigenetic activation of gene expression, and is strongly expressed in post-mitotic neurons in the embryonic and adult mouse forebrain. We started to analyze the mice with a hypomorphic mutation in Gasc1 gene by collaborating with Professor Inazawa at Medical Research Institute, Tokyo Medical and Dental University. Interestingly, Gasc1 homozygous mutants showed no obvious histological abnormalities in the brain, but exhibited abnormal behaviors, including hyperactivity, stereotyped behaviors and impaired learning and memory.



These symptoms appeared to be relevant to human psychiatric disorders. In this year, we detected increased dendritic spine densities of the mutant hippocampal neurons. Electrophysiological studies on hippocampal slices further demonstrated synaptic dysfunction in the mutants, including decreased paired-pulse facilitation and increased long-term potentiation. We are now in the process of detailed analysis of the mutant mice, which may enable us to discuss the cause of human neurological/psychiatric symptoms and future drug discoveries.

2. Analysis of the role of Sox17 in the maintenance of hematopoietic stem cells in the mouse embryo.

During mouse development, hematopoietic stem cells (HSCs) initially arise in hematopoietic cell clusters which are attached to the inside wall of the dorsal aorta at midgestation and are produced from the hemogenic endothelium. However, it is not known exactly how HSCs are maintained in the hematopoietic cell clusters. Forced expression of Sox17 in the major cells comprising the hematopoietic cell clusters, led to consistent formation of cell clusters and maintenance of multipotency in vitro during several passages of cocultures with stromal cells. Shutdown of the exogenously transduced Sox17 gene expression in the Sox17-transduced cell clusters resulted in the differentiation into hematopoietic cells in the cocultures with stromal cells. Moreover, intra-bone marrow transplantation of the Sox17-transduced cells into irradiated mice revealed that hematopoietic cells were repopulated over a relatively long period. These results indicated that Sox17-transduced cells maintained the HSCs. Our results suggest that Sox17 plays a pivotal role in controlling the HSC fate decision between indefinite self-renewal and differentiation during fetal hematopoiesis. In transplantation experiment, Sox17 transduction in the hematopoietic cluster cells increased the absolute number of common myeloid progenitors (CMPs) in the bone marrow of recipient mice to a greater extent. When Sox17-transduced cells were co-cultured with OP9 cells, CMPs and granulocyte/macrophage progenitors (GMPs) maintained their self-renewal capacity and the hematopoietic ability. Sox17 is suggested to regulate the maintenance and differentiation of hematopoietic progenitors, in particular, myeloid progenitors.

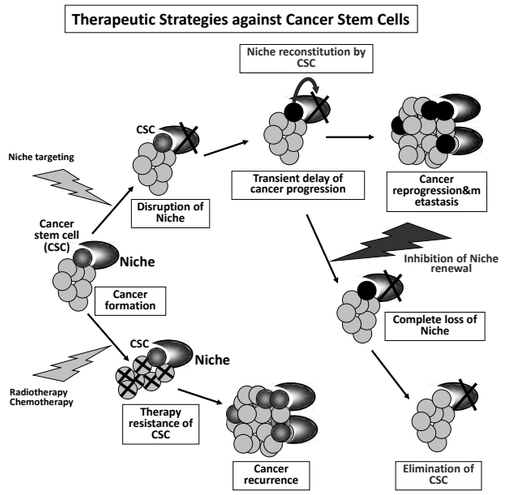


3. Characterization of cancer stem cells and their niche

"Cancer stem cells" (CSCs), a functional subset of tumor cells, are characterized by radio- and chemo-resistance and have been postulated as key drivers of tumor relapse and progression as shown in Figure. CSCs reside in a specialized microenvironment known as the niche composed of, for instance, various stromal cells. Elucidation of the CSC niche may help develop effective strategies of cancer therapy. However, to date, very little is known about the identity of niche components. As we have previously reported, C6 glioma cell line contains a sub-population of CSCs, which is enriched in the "side population (SP)" by Hoechst 33342 staining and FACS analysis.

As we published in 2004, SP cells in C6 are tumorigenic, but cells in the major population (main population, MP) are not. In the recent couple of years, we searched for CSC niche mimicries from hundreds of synthetic polymers in collaboration with Professor Mark Bradley (University of Edinburgh). Out of nearly 400 polymers arrayed on slides, one urethane polymer #10 (Pol10) was identified which enriches a higher tumorigenic cell fraction within SP when transplanted into the NOD/SCID mouse brain. TOF/MS analysis of the Pol10-binding proteins in collaboration with Professor Issay Kitabayashi (National Cancer Center Research Institute) further identified an iron-carrier transferrin as a niche candidate protein for CSCs. In mouse tumors formed by SP cells, iron was found to be stored in CD204(+) tumor-associated macrophages (TAMs), suggesting the pivotal contribution of TAMs to cancer progression.

Such a polymer-based approach will provide clues to understand the molecular basis for CSC niche and to develop effective therapeutic strategies against cancers.



Publications

[Original Article]

1. Nobuhisa I, Osawa M, Uemura M, Kishikawa Y, Anani M, Harada K, Takagi H, Saito K, Kanai-Azuma M, Kanai Y, Iwama A, and Taga T. Sox17-mediated maintenance of fetal intra-aortic hematopoietic cell clusters. Mol. Cell. Biol., 34:1975-1990, 2014
2. Bizen N, Inoue T, Shimizu T, Tabu K, Kagawa T, and Taga T. A growth-promoting signaling component cyclin D1 in neural stem cells has anti-astrogliogenic function to execute self-renewal. Stem Cells, 32:1602-1615, 2014
3. Anani M, Nobuhisa I, Osawa M, Iwama A, Harada K, Saito K, and Taga T. Sox17 as a candidate regulator of myeloid restricted differentiation potential. Dev. Growth Differ., 56, 469-479, 2014

Department of Structural Biology

Professor	Nobutoshi Ito
Associate Professor	Teikichi Ikura
Assistant Professor	Nobutaka Numoto
Technical Assistant	Michiko Hattori
Postgraduate Student	Kenrou Shinagawa

The advance of genome science and proteomic analysis has produced a large amount of information about the primary structure of proteins and their spatial and temporal distributions. On the other hand, most of the proteins only function when they take certain three dimensional structures. As obviously seen in so-called prion diseases, proteins which are chemically correct but structurally incorrect not only fail to function properly but also can harm cells. Our laboratory aims to understand the function of biological macromolecules at atomic level through structure analysis and other methods of physical chemistry, in the hope that accumulation of such knowledge will eventually lead to development of drugs. We are also involved in providing database of such structural data to scientists through the activities of Protein Data Bank Japan.

1. Crystallographic analysis of the B-cell inhibitory co-receptor CD72

B cells play a key role in the immune system by making antibodies. CD72 is an inhibitory co-receptor that regulates signaling through the B cell receptor (BCR). Activation of CD72 is thought to prevent overstimulation of the B cells. Thus, CD72 is necessary to avoid autoimmunity or allergy and make antibodies properly against antigens. CD72 is a type II membrane protein forming a homodimer, primarily expressed in B cells. The ligand binding region of CD72 is located at the C-type lectin-like domain in the C-terminal extracellular region. Some carbohydrate molecules are thought to be able to bind to CD72. However, the mechanism of the ligand recognition of CD72 is still unclear because no structure is available. To elucidate detailed model of the ligand binding site, and obtain structural bases to design novel ligands that regulate CD72 more efficiently, we have initiated crystallographic analysis of the C-type lectin-like domain of CD72 (CD72-CTLD).

We overexpressed CD72-CTLD in *Escherichia coli* but severe aggregation of CD72-CTLD resulted in the formation of inclusion bodies. To overcome the aggregation, we tried to some refolding techniques in which the misfolded and insoluble protein in the inclusion body is solubilized with a denaturing reagent, and then the protein is refolded to their native state. We also optimized the expression constructs and point mutants. Finally we found the optimal condition to obtain highly purified and large-scale production of the recombinant CD72-CTLD. We have obtained some crystals of CD72-CTLD after crystallization trials of hundreds conditions. In optimized condition, we have obtained rod-shaped crystals with a good reproducibility. These crystals diffracted X-ray up to 1.2 Å resolution at the synchrotron radiation facility. The structure of CD72-CTLD was determined using the molecular replacement method. In comparison to the previously reported structures of the C-type lectin-like domains, the structure of CD72-CTLD (Fig. 1) lacks the loop region which forms the ligand-binding site. These findings strongly suggest that CD72-CTLD would have novel ligand-binding manner. A search for the candidates of ligand-molecules and structural analysis of CD72-CTLD-lignad complexes are in progress. This work is performed in collaboration with Professor Tsubata at Medical Research Institute of TMDU.

Fig.1 Crystal structure of CD72-CTLD.

2. Molecular recognition mechanisms of T-cell activators and signal transduction proteins

T cells play a central role in immune system. Two sets of signals are needed to activate T-cells. One is the T-cell receptor (TCR) signal that is mediated by the major histocompatibility complex (MHC), and the other is the co-signal provided by CD28 family receptors. The intracellular domain of CD28 consists of about 40 amino acids and has no enzymatic activity. A tyrosine kinase is recruited to the intracellular domain of CD28, and then phosphorylated YMNM motif (pYMNM) is recognized by the SH2 domains of signal transduction properties such as Grb2, Grb2-related adaptor downstream of Shc (Gads), and PI3-kinase. Structural bases of the molecular recognition mechanisms between CD28 and these signaling factors will be useful for the development of novel drugs which prevent the autoimmune disease or the rejection of an organ transplant.

We have obtained the crystals of the complex of Gads SH2 domain with the synthesized 8 amino acids peptide including the pYMNM motif of CD28. The diffraction data was collected at 1.1 Å resolution. Moreover, we have crystallized the complexes of two kinds of the SH2 domains (nSH2 and cSH2) of PI3-kinase with the same peptide of CD28. These crystals diffracted X-ray up to 0.9 and 1.0 Å resolution, respectively. These three structures reveal a more straight conformation of CD28 (Fig. 2) than previously reported structure of Grb2-CD28 complex. Comparison among the structures of the three complexes shows variation of the environments around phosphorylated tyrosine of CD28. Thermodynamic parameters of the interactions between the SH2 domains and CD28 analyzed using isothermal titration calorimetry (ITC) would provide more comprehensive understanding for the molecular recognition mechanisms of the SH2 domains and CD28. This work is performed in collaboration with Associate Professor Oda at Kyoto Prefectural University.

3. Improvement in Protein Data Bank

The advance of structural biology with X-ray crystallography and NMR has generated a huge amount of data such as atomic coordinates. The increase is expected to continue with even more rapidly by the advent of structure genomics. The vast information is now a precious resource of wide range of science such as bioinformatics. Ever since 1970's, Protein Data Bank (PDB) has been acting as the main and single international archive of the structural data of biological macromolecules. Currently wwPDB, a consortium of Research Collaboratory of Structural Bioinformatics (RCSB), European Bioinformatics Institute (EBI) and Protein Data Bank Japan (PDBj) maintains it. We, as a member of PDBj, contribute to the continuous effort by wwPDB to improve PDB.

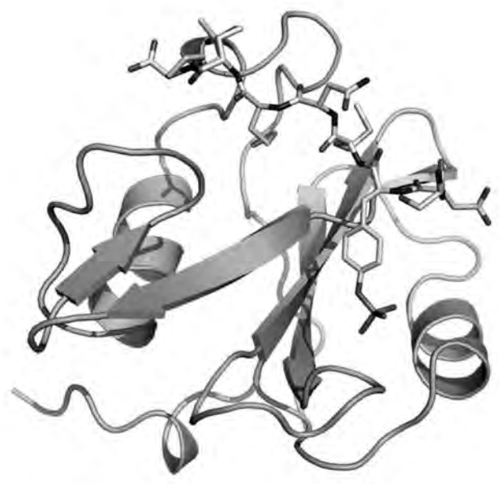


Fig.2 Crystal structure of the complex of PI3-kinase SH2 domain and the phosphorylated peptide of CD28.

Publications

1. Yamamoto M, Onogi H, Kii I, Yoshida S, Iida K, Sakai H, Abe M, Tsubota T, Ito N, Hosoya T, Hagiwara M: CDK9 inhibitor FIT-039 prevents replication of multiple DNA viruses. J Clin Invest, 124: 3479-3488, 2014.

2. Kudo T, Ishizawa M, Maekawa K, Nakabayashi M, Watarai Y, Uchida H, Tokiwa H, Ikura T, Ito N, Makishima M, Yamada S: Combination of triple bond and adamantane ring on the vitamin D side chain produced partial agonists for vitamin D receptor. J Med Chem, 57: 4073-4087, 2014.

3. Numoto N, Nakagawa T, Ohara R, Hasegawa T, Kita A, Yoshida T, Maruyama T, Imai K, Fukumori Y, Miki K.: The structure of a deoxygenated 400 kDa haemoglobin reveals ternary- and quaternary-structural changes of giant haemoglobins. Acta Crystallogr D Biol Crystallogr, 70: 1823-1831, 2014.

Frontier Research Unit
Laboratory of Oxygen Biology

Associate Professor　Koh Nakayama, Ph.D.
Assistant Professor　Kouichi Tabu, Ph.D.

Aim of the Research

Study in our laboratory aims to understand how changes in the ambient oxygen concentration affect our body function. It is well known that oxygen is required for the efficient energy production in mitochondria. Furthermore, recent studies revealed the role of oxygen, particularly a hypoxic condition, involved in developmental processes, tumorigenesis, and stem cell proliferation. Our goal is to understand the molecular mechanism of hypoxic response and establish new tools for cancer therapy and regenerative medicine.

Research Projects

1. Signal transduction of hypoxic response

Hypoxia-Inducible Factor (HIF)-α is a transcription factor which plays a central role during hypoxic response by regulating multiple cellular functions including metabolism, respiration, and cell growth. PHD is a HIF-prolyl hydroxylase which hydroxylates and regulates the expression of HIF-α. There are three PHDs identified, which are named PHD1, 2, and 3. These proteins hydroxylate HIF-α to negatively regulate its expression. Moreover, it is suggested to have substrates besides HIF-α. We have been focusing on PHD3, and studying hypoxic cell signaling pathways which are connected to the HIF-dependent and -independent pathways.

2. Identification and characterization of *in vivo* oxygen senor

Our recent study demonstrated the formation of 'hypoxia complex' under hypoxic condition which consists of PHD3 and other unidentified proteins (Figure). We hypothesized that the hypoxia complex contains an oxygen sensing molecule(s) which regulates the complex formation. We have been working on identifying the components of complex by multiple proteomics approaches. Up to date, we identified proteins involved in metabolism, cell structural organization, and splicing. We now work on functional characterization of the proteins to understand their role in hypoxic response, and as an oxygen sensor. Ultimately, we challenge to invent means to suppress the progression of hypoxic tumor by modifying the oxygen sensor.

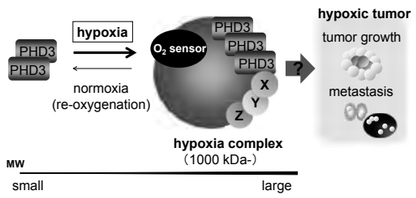


Figure Characterization of Hypoxia Complex

3. Metabolic Regulation under hypoxic condition

Cancer cells are known to exhibit glycolytic metabolism. The molecular mechanism of how cancer cells utilize glycolysis as a main source of energy production has been a central focus in the cancer research field. We recently identified pyruvate dehydrogenase (PDH) as a component included in the hypoxia complex (Figure). PDH is an enzyme which converts pyruvate into acetyl CoA, and connect glycolysis and TCA cycle. We have demonstrated that PDH interacts with PHD3 under hypoxic condition. In *PHD3*$^{-/-}$ cells, PDH activity is significantly decreased, suggesting that PHD3 positively regulates PDH activity by directly interacting with it. Thus, PHD3 possibly fine-tunes the PDH activity under hypoxic condition to balance the glycolysis and TCA cycle to carry out the most efficient energy production state. We now challenge to alter the cancer metabolism by changing the PHD3 expression and increasing the PDH activity, which would break up the glycolytic metabolism in tumors.

Publications

1. Kikuchi D., Minamishima YA., and Nakayama K.* Prolyl-hydroxylase PHD3 interacts with pyruvate dehydrogenase (PDH)-E1beta and regulates the cellular PDH activity. ***Biochem. Biophys. Res. Commun.,*** 451, 288-294, (2014).

Tenure Track Research Unit
Department of Cellular and Molecular Medicine

Associate professor　Yumiko Oishi MD, PhD
Assistant professor　Daiki Taneichi PhD, Shinichiro Hayashi, PhD
Project Assistant professor　Sumio Hayakawa, PhD

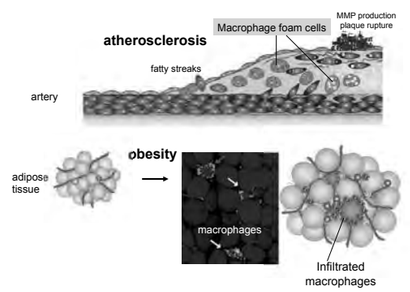
Research outline

Cardiovascular disease, as a consequent of the obesity related metabolic syndrome, remains a significant cause of morbidity and mortality in industrialized societies despite major advantages in treatment of hypercholesterolemia and hypertension. A major effort of our laboratory has been to investigate the molecular mechanism of an initiation and progression of metabolic syndrome which leads to the life-threatening cardiovascular diseases from the viewpoint of transcriptional regulation. Because macrophages contribute to all phages of the pathogenesis of atherosclerosis, we have extensively studied the macrophage diversity which respond to various stress within tissue environment. The long term goals of our current study are to elucidate: 1) the mechanism by which cellular homeostasis is regulated through inflammatory response, and 2) mechanism responsible for pathogenesis of metabolic syndrome by disruption of macrophage homeodynamics.

Research Projects

Mechanisms of Coordinated regulation of inflammatory response and lipid homeostasis in macrophage

Chronic low-grade inflammation has been recognized as a key contributing factor in the onset and progression of metabolic syndrome and atherosclerosis. As a multifunctional effector cell, macrophage play pivotal roles in both the enhancement and resolution of this inflammatory process. Recent lipidomic analysis based on the mass spectrometry revealed that macrophages synthesize variety of fatty acids and sterols by responding various signals. Activated macrophages rapidly activate arachidonate cascade to produce inflammatory mediators such as leukotrienes and prostagrandins. On the other hand, the production of the anti-inflammatory omega-3 unsaturated fatty acids (ω-3 PUFAs) was significantly increased in the chronic phase of inflammation. By utilizing both molecular biology technique and bioinformatics, we found that the inflammatory activated NF-κB kicks off both pro-inflammatory and anti-inflammatory signaling pathway. TLR4 activation rapidly, and transiently inhibits Liver X receptor (LXR) signaling through NF-κB, and subsequently activates Sterol regulatory element-binding protein (SREBP) by processing from the ER membrane. In the chronic phase of inflammation, LXR and SREBP work together to increase production of anti-inflammatory fatty acids, then actively resolve inflammation. Thus, transcriptional/signaling network between LXR and SREBP play an important role in the regulation of the fatty acid synthesis to regulate homeostasis. By elucidating the cross-talk between cellar function and metabolism, we would be able to accumulate beneficial knowledge to develop novel therapeutic strategy targeting macrophages for the prevention and treatment of metabolic syndrome. We also pay marked attention to the interplay between macrophages and skeletal muscle cells, since recent studies suggest that macrophages are indispensable for the normal skeletal muscle degeneration and development.



Macrophage is important for a chronic inflammation.

Publications

1. Zalc A[1], Hayashi S[1], Auradé F, Bröhl D, Chang T, Mademtzoglou D, Mourikis P, Yao Z, Cao Y, Birchmeier C, Relaix F. Antagonistic regulation of p57kip2 by Hes/Hey downstream of Notch signaling and muscle regulatory factors regulates skeletal muscle growth arrest. Development. 2014 Jul;141(14):2780-90. [1]: These authors contributed equally to this work

Division of Pathophysiology

【Aim and Scope】

Research purpose of the Division of Pathophysiology is to understand the fundamental mechanisms underlying biological phenomena and their abnormalities in the disease conditions including intractable diseases and to develop new diagnostic or therapeutic tools for various diseases of which curative treatment is difficult. Topics of research projects in each Department are as follows;

【Neuropathology】

- Discovery of the earliest pathology of Alzheimer's disease
- Development of gene therapies against spinocerebellar ataxia and microcephaly at the basic research level

【Pathological Cell Biology】

- Discovery of a novel role of Atg5-independent macroautophagy in the elimination of mitochondria from reticulocytes
- Discovery of a biological role of autophagic cell death: compensation of apoptosis during development

【Developmental and Regenerative Biology】

- Discovery of the dual function of Hippo-Yap signaling pathway during retinal progenitor cell proliferation versus photoreceptor cell differentiation
- Elucidation of the PDZ-binding domain of YAP in cellular transformation

【Stem Cell Biology】

- Identification of melanocyte stem cells in sweat glands of volar skins
- Melanocyte stem cells are sensitive to ionizing irradiation during their dormancy

【Immunology】

- Development of synthetic sialosides that bind to the inhibitory B cell receptor CD22 with high affinity and carry an immune activation activity
- Elucidation of a crucial role of reactive oxygen species in B lymphocyte activation (ALPS)

【Molecular Pathogenesis】

- Identification of de novo mutations in CALM2 as novel mechanisms for congenital long QT syndrome via decreasing calcium binding to calmodulin 2 molecule
- Clarification of evolutionary aspects of ULBP gene family in higher primates; diversity and divergence of ULBP molecules as ligands for a NK receptor NKG2D

【Virus Research Unit】

- Establishment of a chronic active EBV infection model using NOG mice
- Development of an exhaustive and quantitative pathogen microbes screening system capable of screening dozens of virus, bacteria, fungous and protozoa simultaneously

Department of Neuropathology

Professor	Hitoshi Okazawa
Practical professor	Kazuhiko Tagawa
Project Lecturer/Part-time Lecturer	Nobuyuki Nukina, Toshiki Uchihara, Masaki Sone
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Secretary	Rumi Innami, Ayako Seki
Graduate Student	Kanoh Kondo, Shigenori Uchida, Juliana Bosso Taniguchi, Mao Ying, Eriko Hoshino
Research Student	Zhang Xuemei

Research contents

Following studies have been intensively carried out in our laboratory with various techniques including molecular biology, cell biology, biochemistry, Drosophila models, and mouse models.

1) Investigation of molecular pathologies of neurodegenerative diseases.
2) Studies on impairment of DNA-repair in polyglutamine diseases.
3) Development of new seed drugs for neurdegeneration.
4) Development of new seed drug for mental retardation.
5) Investigation of molecular functions of Oct-3/4

This year's progress.

1) The research group elucidated molecular basis for the earliest synapse pathology in preclinical Alzheimer' sdisease (AD) brain. In this study, the research group performed comprehensive phosphoprotein analysis with brain samples from AD patients and four mouse models by using high-end mass spectrometry, and analyzed the data by methods of systems biology using a super computer. They found that 17 phosphoproteins related to synapse functions are changed in the brains of mouse AD models and human AD patients. Especially, the change of MARCKS started at a preclinical stage even before histological A β deposition. Two-photon microscopic observation revealed recovery of abnormal spine formation in the AD model mice by targeting MARCKS or by inhibiting its candidate kinases. This study proposed a novel strategy of AD treatment which targets the earliest pathology.

2) The research group elucidated a molecular pathomechanism of microcephaly by mutations of PQBP1 (polyglutamine binding protein-1) gene, which is known as a major causative gene for microcephaly. In this study the research group made a conditional KO mouse which does not express PQBP1 in neural stem progenitor cells (NSPCs). The mouse model showed microcephaly without structural change (primary microcephaly) and a cell cycle time elongation in NSPCs, which is basically mediated by transcription/splicing abnormalities including a number of genes related to the cell cycle regulation such as APC2 and APC4. The mice did not showed accelerated production of neurons, increased cell death of NSPCs, or abnormal migration. They confirmed supplementation of APC4 recovered the cell cycle time elongation and NSPCs expansion. Moreover, the research group performed peritoneal injection of adeno-associated virus (AAV) vector into pregnant mice to express

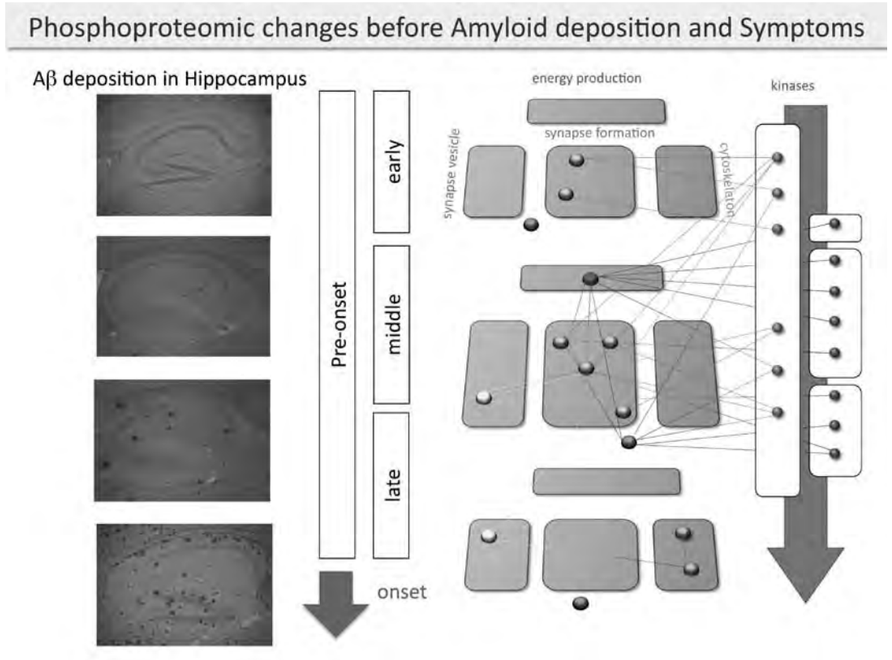
PQBP1 in embryos, and confirmed recovery of the microcephaly and behavioral abnormalities of offsprings. This study proposed a new mechanism of primary microcephaly and a treatment strategy.

3) The research group succeeded a gene therapy of a model mouse of spinocerebellar ataxia type 1 (SCA1). Previously, the research group found a candidate therapeutic molecule, HMGB1. This study extended the finding and applied HMGB1 for a SCA1 model mice therapy. We succeeded in remarkable elongation of lifespan and a rotarod test. Virus injection of AAV-HMGB1 also recovered the phenotypes of SCA1 model mice. This study proposed novel approach for therapy strategy of SCA1.

Highlight

"Comprehensive phosphoproteome analysis unravels the core signaling network that initiates the earliest synapse pathology in preclinical Alzheimer's disease brain"

Using a high-end mass spectrometry, we screened phosphoproteins and phosphopeptides in four types of Alzheimer's disease (AD) mouse models and human AD postmortem brains. Surprisingly, most of the core phosphoproteins were directly connected, and they formed a functional network linked to synaptic spine formation. The change of the core network started at a preclinical stage even before histological Ab deposition. Systems biology analyses suggested that phosphorylation of myristoylated alanine-rich C-kinase substrate (MARCKS) by overactivated kinases including PKC initiates synapse pathology. Two-photon microscopic observation revealed recovery of reduced spine density in the AD model mice by targeting a core protein MARCKS or by inhibiting candidate kinases, supporting our hypothesis formulated based on phosphoproteome analysis.



Publications

[Original Articles]
1. Sam S Barclay, Takuya Tamura, Hikaru Ito, Kyota Fujita, Kazuhiko Tagawa, Teppei Shimamura, Asuka Katsuta, Hiroki Shiwaku, Masaki Sone, Seiya Imoto, Satoru Miyano, Hitoshi Okazawa. Systems biology analysis of Drosophila in vivo screen data elucidates core networks for DNA damage repair in SCA1. Hum. Mol. Genet.. 23, 1345-1364 (2014)
2. Mineyuki Mizuguchi, Takayuki Obita, Tomohito Serita, Rieko Kojima, Yuko Nabeshima, Hitoshi Okazawa. Mutations in the PQBP1 gene prevent its interaction with the spliceosomal protein U5-15 kD. Nat Commun. 5, 3822 (2014)
3. Ito H, Shiwaku H, Yoshida C, Homma H, Luo H, Chen X, Fujita K, Musante L, Fischer U, Frints S G M,Romano C, Ikeuchi Y, Shimamura T, Imoto S, Miyano S, Muramatsu S-I, Kawauchi T, Hoshino M, SudolM, Arumughan A, Wanker E E, Rich T, Schwartz C, Matsuzaki F, Bonni A, Kalscheuer V M, OkazawaH. In utero gene therapy rescues microcephaly caused by Pqbp1-hypofunction in neural stem progenitor cells. Mol Psychiatry. 2014.07; [Epub ahead of print] doi: 10.1038/mp.2014.69
4. Tagawa Kazuhiko, Homma Hidenori, Saito Ayumu, Fujita Kyota, Chen Xigui, Imoto Seiya, Oka Tsutomu, Ito Hikaru, Motoki Kazumi, Yoshida Chisato, Hatsuta Hiroyuki, Murayama Shigeo, Iwatsubo Takeshi, Miyano Satoru, Okazawa Hitoshi. Comprehensive phosphoproteome analysis unravels the core signaling network that initiates the earliest synapse pathology in preclinical Alzheimer's disease brain. Hum Mol Genet. 2015. 24 (2): 540-558.
5. Hikaru Ito, Kyota Fujita, Kazuhiko Tagawa, Xigui Chen, Hidenori Homma, Toshikazu Sasabe, Jun Shimizu, Shigeomi Shimizu, Takuya Tamura, Shin-Ichi Muramatsu, Hitoshi Okazawa. HMGB1 facilitates repair of mitochondrial DNA damage and extends the lifespan of mutant ataxin-1 knock-in mice. EMBO Mol Med. 7, 78-101. (2014)
6. Risa Shiraishi, Takuya Tamura, Masaki Sone, Hitoshi Okazawa. Systematic analysis of fly models with multiple drivers reveals different effects of ataxin-1 and huntingtin in neuron subtype-specific expression. PLoS ONE. 2014.12; 9(12); e116567
7. Yuko Nabeshima, Mineyuki Mizuguchi, Asagi Kajiyama, Hitoshi Okazawa. Segmental isotope-labeling of the intrinsically disordered protein PQBP1. FEBS Lett. 588, 4583-4589 (2014)

[Misc]
1. Takuya Tamura, Hitoshi Okazawa. Cell death in neuro-degenerative diseases and Hippo pathway. Journal of Clinical and Experimental Medicine. 251, 449-454 (2014)
2. Kyota Fujita, Hitoshi Okazawa. Annual Review 2015「A functional deficiency of TERA/VCP/p97 contributes to impaired DNA damage repair in multiple polyglutamine diseases」65-72 (2015)
3. Hiroki Shiwaku, Hitoshi Okazawa. Impaired DNA Damage Repair as a Common Feature of Neurodegenerative Diseases and Psychiatric Disorders. Curr Mol Med. 2015. [Epub ahead of print]

Department of Pathological Cell Biology

Professor	Shigeomi SHIMIZU
Tokunin Junior Associate Professor	Masatsune TSUJIOKA, Satoru TORII
Assistant professor	Satoko ARAKAWA, Shinya HONDA
Tokunin Assistant Professor	Michiko MUROHASHI, Min Kyong SHIN, Hirofumi YAMAGUCHI, Yasuyuki SUGIMURA

This laboratory mainly focuses on understanding the molecular mechanisms of autophagy, programmed cell death, and the mitochondria-related diseases. We take biochemical, genetical, cellular biological approaches to elucidate them. The aims of this laboratory are to develop new strategies to control autophagy and cell death and to apply them to various diseases.

〈**Research Projects**〉

1, Discovery of Atg5/Atg7-independent alternative macroautophagy.

Macroautophagy is a process that leads to the bulk degradation of subcellular constituents through the creation of autophagosomes/autolysosomes. This mechanism is involved in the turnover of cytoplasmic components. Accumulating studies have shown that certain Atg genes, including Atg5, Atg6 (also called Beclin-1), and Atg7, are essential for induction of macroautophagy. However, recently we found that cells lacking Atg5 or Atg7 can still form autophagosomes/autolysosomes when subjected to certain stresses. Although lipidation of LC3 (LC3-II formation) is generally considered to be a good indicator of macroautophagy, it did not occur during the Atg5/Atg7-independent alternate macroautophagy. We also found that this alternative macroautophagy was regulated by several autophagic proteins, including Ulk1 and Beclin-1. In vivo, Atg5-independent alternate macroautophagy was detected in several embryonic tissues. These results indicate that mammalian macroautophagy can occur via at least two different pathways, which are an Atg5/Atg7-dependent conventional pathway and an Atg5/Atg7-independent alternate pathway.

In this year, we discovered the involvement of alternative macroautophagy in mitophagy during erythrocyte maturation. In the terminal stage of erythrocyte maturation, the erythroblasts lose their nuclei to become reticulocytes and reticulocytes are transformed into erythrocytes by elimination of organelles including the mitochondria. Because ultrastructural studies have detected autophagic structures engulfing mitochondria, mitochondrial clearance occurs by mitophagy. In fact, we have observed that mitochondria were engulfed and digested by autophagic vacuoles in wild-type reticulocytes, and surprisingly, an equivalent amount of mitophagy was observed even in Atg5-deficinet reticulocytes. Consistently, the number of persisting mitochondria in Atg5-deficinet reticulocytes and erythrocytes was the same as in wild-type cells of each type. Unlike Atg5, Ulk1 is crucial for mitochondrial clearance in reticulocytes as judged from the failure of mitophagy in Ulk1-deficient mice. These data indicated that the Ulk1-dependent Atg5-independent alternative macroautophagy is the dominant force for mitophagy in reticulocytes.

2, Molecular mechanisms of programmed cell death

Cell death is the essential mechanisms for selective elimination of cells. It plays an integral part in a variety of biological events, including morphogenesis and removal of harmful cells. Previously, apoptosis is considered as the only form of programmed cell death. Recent studies, however, have provided evidence that there is another mechanism of programmed cell death called non-apoptotic cell death, including include apoptosis, autophagic cell death, and programmed necrosis. Therefore, in order to understand the role of cell death in multicellular organisms, it would be required to elucidate all the molecular mechanisms of cell death. Our final goal is to understand the role of programmed cell death in humans.

In this year, we discovered the biological role of autophagic cell death. To this aim, we generated Atg5/Bax/Bak triple-knockout (TKO) mice, in which autophagic cell death is greatly suppressed. The formation of interdigital web of TKO embryos occurred at embryonic day 16.0 (two days after that of wild type mice). Brain malformation is a remarkable feature of failure of cell death, and which was appeared in TKO embryos. All the data show that autophagic cell death contributes for embryonic development.

3, Analysis of mitochondrial diseases

Mitochondria play an essential role in both cell survival and cell death. In both cell function, mitochondrial membrane permeability is crucial. Therefore, we are focusing on channel proteins located on mitochondrial membrane. We are also focusing on mitochondrial diseases. The motor neuron degeneration 2 (mnd2) mouse is considered to be an animal model of Parkinson disease (PD). Mnd2 mice possess a non-functional missense mutation $^{Ser}276^{Cys}$ in the mitochondrial protease HtrA2/Omi. We are trying to prolong the life of these mice.

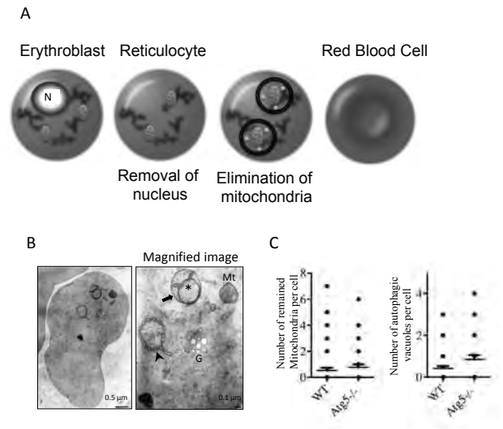


Fig.1 Involvement of alternative macroautophagy in mitophagy during erythrocyte maturation.
(A) Final stage of red blood cell maturation. During erythrocyte maturation, erythroblasts lose their nuclei to become reticulocytes and reticulocytes are transformed into erythrocytes by elimination of mitochondria. Macroautophagy is involved in the latter process. (B) Electron micrographs of Atg5-deficient erythrocytes. Mitophagy can be observed in the Atg5-deficient erythrocyte. Arrowhead indicates the isolation membrane-autophagosomal structure. Arrow indicates the autophagosome containing mitochondria (*). Mt: non-engulfed mitochondria. G: Golgi-deribed membranes. (C) The number of remained mitochondria per cell and autophagic vacuoles per cell in wild-type and Atg5-deficient erythrocytes are indicated. Lines indicate the mean and SD.

List of Publications

[Original paper]

1. Honda S, Arakawa S, Nishida Y, Yamaguchi H, Ishii E, Shimizu S.: Ulk1-mediated Atg5-independent macroautophagy mediates elimination of mitochondria from embryonic reticulocytes. ***Nature Commun*** 5 Article number:4004 (2014)
2. Shimizu S, Yoshida T, Tsujioka M, Arakawa S.: Autophagic Cell Death and Cancer. ***Int. J. Mol. Sci.*** 15(2):3145-53 (2014)
3. Shimizu S, Honda S, Arakawa S, Yamaguchi H.: Alternative Macroautophagy and Mitophagy. ***Int. J. Biochem. Cell Biol.*** 50:64-6 (2014)

[Review paper]

1. Shimizu S.: Autophagic Cell Death and Cancer Chemotherapeutics. "Innovative Medicine : Basic Research and Development" Springer *in press*

Department of Developmental and Regenerative Biology

Professor	Hiroshi Nishina, Ph.D.
Associate Professor	Jun Hirayama, Ph.D.
Assistant Professor	Yoichi Asaoka, Ph.D.
Project Assistant Professor	Norio Miyamura, Ph.D.
Project Assistant Professor	Yoshimi Uchida, Ph.D.

Our goal is to define the molecular basis for the mechanism of organ formation, regeneration, and maintenance using knockout mice and mutant fishes. To accomplish this goal, we have focused on defining signaling molecules and pathways that regulate liver and brain formation and maintenance. Our study will provide new insights into understanding the precise molecular mechanisms that underlie organ failures found in human disease and will lead to the development of new rational therapy for the diseases.

1. SAPK/JNK Signaling Pathway

Stress-activated protein kinase (SAPK)/c-Jun NH2-terminal kinase (JNK) is activated by many types of cellular stresses and extracellular signals. We showed that two JNK activators, MKK4/SEK1 and MKK7, are required for full JNK activation and fetal liver formation. MKK4-deficient embryos die between E10.5 and E12.5 with impaired liver formation and massive apoptosis. MKK7-deficient embryos die between E11.5 and E12.5 with similar defects in liver formation. These results indicate that MKK4 and MKK7 cannot substitute for one another *in vivo* and that both are important for hepatoblast proliferation and survival during mouse embryogenesis. Furthermore, we found that the Tyr and Thr residues of JNK are sequentially phosphorylated by MKK4 and MKK7, respectively. We propose the hypothesis that the strong or weak activation of SAPK/JNK is induced by MKK4 and MKK7 to regulate the cell fate.

2. Hippo Signaling Pathway

The Yes-associated protein (YAP) is a transcriptional co-activator that contributes to the regulation of multiple cellular processes by activating several transcription factors. Recently, YAP was shown to play an important role in organ size control and to be inhibited by the Hippo signaling pathway. Either transgenic overexpression of YAP or knockout of Hippo pathway genes in mouse liver results in enlargement of this organ and the eventual development of hepatic tumors. *In vitro*, YAP overexpression promotes cell proliferation and induces oncogenic transformation by activating TEAD family transcription factors. YAP also plays a critical role in maintaining stem cell pluripotency, as knockdown of YAP leads to loss of this property in ES cells, and YAP overexpression suppresses ES cell differentiation. In certain tumor-derived cell lines exposed to various genotoxic stimuli, YAP promotes apoptosis via the induction of pro-apoptotic genes controlled by the transcription factor p73.

The functions of YAP are regulated by multiple post-translational modifications. Cell-cell contact triggers YAP inactivation via phosphorylation mediated by the Hippo pathway. The Hippo pathway component Lats kinase phosphorylates serine 127 (S127) of human YAP (hYAP), promoting its recognition and cytoplasmic retention by 14-3-3 protein. In addition, phosphorylation of hYAP S381 by Lats primes subsequent phosphorylation of hYAP S384 by casein kinase 1, leading to hYAP ubiquitination and degradation. We are studying on the regulation and function of YAP.

3. Circadian Clock

Circadian clocks are intrinsic, time-tracking systems that endow organisms with a survival advantage. The molecular mechanism underlying the circadian clock is based on interconnected transcriptional–translational feedback loops in which specific clock-factors repress transcription of their own genes. In vertebrate CLOCK and BMAL1 heterodimerize to form an active transcription complex that binds to promoter E-box elements, which are present in *Per* and *Cry* genes. Once PER and CRY proteins have been translated, they form a complex that inhibits CLOCK–BMAL1-mediated transcription. It is important to note that the CLOCK–BMAL1 heterodimer also stimulates the transcription of many other clock-controlled genes, which, in turn, influence functions external to the oscillatory mechanism itself and mediate the output of the clock. This accounts for the circadian rhythmicity of many physiological processes, such as food intake, hormonal synthesis and release, body temperature maintenance and several aspects of metabolism. The principal cue that influences circadian rhythmicity is light. The molecular pathways that light uses to influence the clock mechanism are thereby of central importance. We have been studying the light signaling pathway for circadian transcription and clock entrainment using zebrafish as the model animal.

Highlight

In the vertebrate embryonic nervous system, neural progenitor cells proliferate and differentiate into diverse neuronal and glial cell types that finally build up functional neural circuits such as the retina. Recently, Hippo signaling has been shown to play an important role in promoting cell cycle exit and terminal differentiation during rodent retinogenesis. When Hippo signaling is activated, the Mst1/2 kinases activate the Lats1/2 kinases, which in turn phosphorylate and inhibit the transcriptional cofactor Yap. At present, the detailed molecular mechanism by which the Hippo-Yap pathway controls the differentiation of specific types of retinal neurons has remained unknown. In this study, we found that knockdown of zebrafish mst2 induced early embryonic defects, including altered retinal pigmentation and morphogenesis. Similar abnormal retinal phenotypes were observed in zebrafish embryos injected with a constitutively active form of yap [yap (5SA)]. Microarray analysis revealed that yap (5SA)-expressing embryos exhibited decreased expression of transcription factors such as Otx5 and Crx, which orchestrate photoreceptor cell differentiation by activating the expression of rhodopsin and other photoreceptor cell genes. Co-immunoprecipitation experiments identified the photoreceptor cell differentiation factor Rx1 as a novel interacting partner of Yap. Altogether, our results suggest that Yap is crucial for coordinating the timing of the terminal differentiation of photoreceptor neurons by suppressing Rx1-mediated transactivation of the otx, crx and rhodopsin genes during zebrafish retinogenesis (Fig. 1).

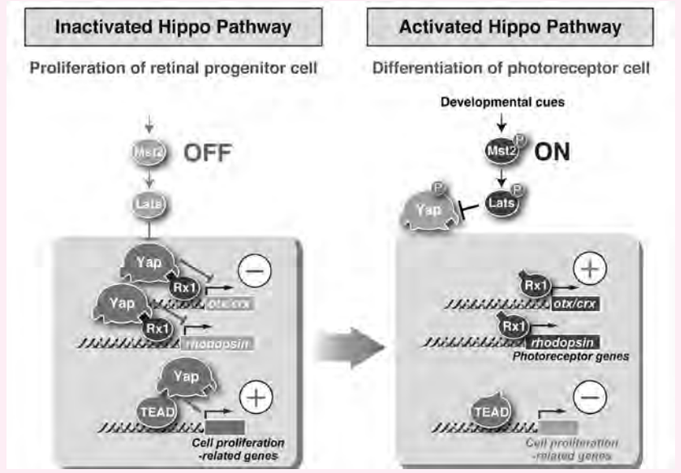


Fig.1. A proposed model for the dual function of Hippo-Yap signaling during retinal progenitor cell proliferation versus photoreceptor cell differentiation

Publications

1. Yoichi Asaoka, Shoji Hata, Misako Namae, Makoto Furutani-Seiki, and Hiroshi Nishina (2014) The Hippo pathway controls a switch between retinal progenitor cell proliferation and photoreceptor cell differentiation in zebrafish. ***PLoS ONE*** 9, e97365.
2. Tadanori Shimomura, Norio Miyamura, Shoji Hata, Ryota Miura, Jun Hirayama and Hiroshi Nishina (2014) The PDZ-binding motif of Yes-associated protein is required for its co-activation of TEAD-mediated *CTGF* transcription and oncogenic cell transforming activity. ***Biochem. Biophys. Res. Commun.*** 443, 917-923.
3. Keita Nakanaga, kotaro Hama, Kuniyuki Kano, Takanoa Sato, Hiroshi Yukiura, Asuka Inoue, Daisuke Saigusa, Hidetoshi Tokuyama, Yoshihisa Tomioka, Hiroshi Nishina, Atsuo Kawahara and Junken Aoki (2014) Overexpression of autotaxin, a lysophosphatidic acid-producing enzyme, enhances cardia bifida induced by hypo-sphingosine-1-phosphate signaling in zebrafish embryo. ***J. Biochem.*** 155, 235-241.
4. Zeyu Yang, Kentaro Nakagawa, Aradhan Sarkar, Junichi Maruyama, Hiroaki Iwasa, Yijun Bao, Mari Ishigami-Yuasa, Shigeru Ito, Hiroyuki Kagechika, Shoji Hata, Hiroshi Nishina, Shinya Abe, Masanobu Kitagawa, Yutaka Hata (2014) Screening with a novel cell-based assay for TAZ activators identifies a compound that enhances myogenesis in C2C12 cells and facilitates muscle repair in a muscle injury model. ***Mol. Cell. Biol.*** 34, 1607-1621.

Department of Stem Cell Biology

Professor Emi K. Nishimura, M.D., Ph. D.
Assistant Professor Hiroyuki Matsumura, Ph. D.
JSPS Postdoctoral Fellow (PD) Yasuaki Mohri, Ph. D., Hironobu Morinaga Ph. D.
Project Assistant Professor Go Yoshida MD.,Ph. D.

Stem cell systems play fundamental roles in tissue turnover and homeostasis. Our goal is to understand the mechanisms of tissue homeostasis driven by stem cell systems and to apply the knowledge to better understand the mechanisms underlying specific tissue decline, cancer development and other diseases associated with aging. We further aim to apply this knowledge to regenerative medicine using somatic stem cells and to the treatment of cancer as well as other age-associated diseases.

1) Identification of melanocyte stem cells in hair follicles and sweat glauds

The skin is the largest organ in the body. Hair follicles in the skin constantly renew themselves by alternate phases of growth, regression and rest. During this process, mature melanocytes (pigment cells) in hair follicles are replaced by a new melanocyte population every hair cycle. We previously identified the source of those melanocytes as “melanocyte stem cells” (McSC). They are located in the hair follicle bulge and supply mature melanocytes required for hair pigmentation to the hair matrix (Nishimura EK et al. Nature 2002) (Figure 1). We have searched for a similar stem cell population in non- hair-bearing skin areas and currently identified McSCs in eccrine sweat glands by using Dct-H2B・GFP transgenic mice in which melanocyte lineage cells can be stably visualized. We have characterized the H2B・GFP positive cell population in sweat glands and demonstrated that the population possess the features of somatic stem cells (Figure 2). As our analysis on human early acral melanoma have suggested the possibility that the population can be the early origin of melanoma, we are currently testing whether the population can be the major origin of melanoma in the acral volar skin on the palm and sole which contain abundant sweat glands instead of hair follicles.

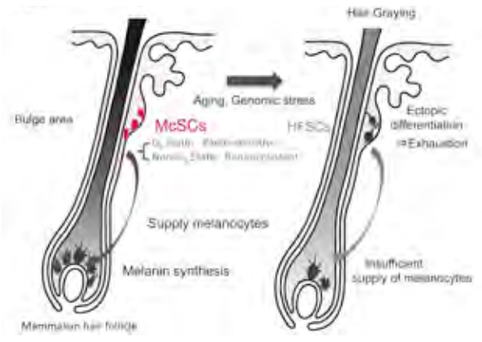


Figure 1 Mechanisms of hair graying in mammalian hair follicles.

2) Mechanisms of stem cell maintenance

The underlying mechanisms of stem cell maintenance and regulation are fundamental issues in stem cell biology and medicine. We previously demonstrated that the niche microenvironment plays dominant role in melanocyte stem cell fate determination (Nishimura EK et al. 2002) and also that the progressive hair graying phenotype, one of the most typical aging phenotype, is caused by incomplete maintenance of McSCs (Nishimura EK et al. Science 2005). The phenotype is characteristically seen in some coat color mutants such as *Bcl2* deficient mice and *Mitf-vit* mutant mice. We examined the roles of *Mitf* and *Bcl2* and found that these genes are intrinsic genes critically involved in McSC maintenance to prevent hair graying. Also we found that hair follicle stem cells (HFSC), which surround McSCs in the hair follicle bulge-subbulge area, play essential role in McSC maintenance as functional niche cells through transforming growth factor β (TGF-β) signaling to prevent premature hair graying (Nishimura EK et al. Cell Stem Cell, 2010) (Tanimura S et al. Cell Stem Cell, 2011).

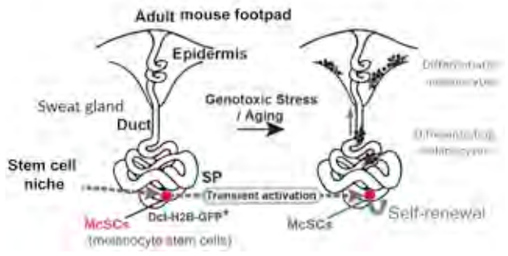


Figure 2 Localization of melanocyte stem cells and their niche in murine sweat glands.

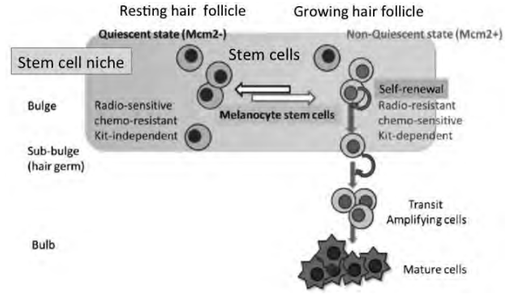


Figure 3 Quiescent melanocyte stem cells are radio-sensitive.

3) Mechanisms for stem cell aging and quality control of stem cell pools.

Physiological hair graying is the most obvious outward sign of aging in mammals. We previously demonstrated that physiological hair graying is caused by incomplete self-renewal or maintenance of McSCs (Nishimura EK et al. 2005). However, it was still not known what causes the self-renewal of MsSCs to become defective during the course of aging. We have found that genotoxic stress abrogates renewal of melanocyte stem cells by triggering their differentiation but not stem cell apoptosis nor cellular senescence. Our findings indicated that a “stem cell renewal checkpoint” exists to maintain the quality of the melanocyte stem cell pool (Inomata K, Aoto T et al. Cell 2009) and similar checkpoint systems have been found in other stem cell systems more recently.

Highlight

Coupling of the radiosensitivity of melanocyte stem cells to their dormancy.

It is generally accepted that actively mitotic cells are the most sensitive to ionizing radiation, but it has not been clearly tested whether cycling stem cells are radiosensitive or not and whether the “stem cell checkpoint” system functions all the time or cyclically during a cell cycle or a hair cycle. We thus focused on hair graying to understand the stress-resistance of McSCs. We used Dct-H2B・GFP transgenic mice which enable the stable visualization of McSCs and an anti-Kit monoclonal antibody which selectively eradicate amplifying McSCs and found that quiescent McSCs are rather radiosensitive but the coexistence of quiescent and non-quiescent McSCs provide the stem cell pool with radioresistance (Figure 1, 3). Our data indicated that tissue radiosensitivity is largely dependent on the state of somatic stem cells under their local microenvironment. Furthermore, the coexistence of non-quiescent McSCs in the niche ensures the resistance of the McSC pool to different kinds of stresses to prevent hair graying (Ueno M et al. Pigment Cell Melanoma Res. 2014).

Publications

1. Okamoto N, Aoto T, Uhara H, Yamazaki S, Akutsu H, Umezawa A, Nakauchi H, Miyachi Y, Saida T, Nishimura EK.
A melanocyte-melanoma precursor niche in sweat glands of volar skin.
Pigment Cell & Melanoma Research. 27(6):1039-1050, 2014
2. Ueno M, Aoto T, Mohri Y, Yokozeki H, Nishimura EK.
Coupling of the radiosensitivity of melanocyte stem cells to their dormancy during a hair cycle.
Pigment Cell & Melanoma Research. 27(4):540-551, 2014

Invited lecture/presentation at international meetings

1. Emi K.Nishimura:Coupling of the stress sensitivity of melanocyte stem cells to their dormancy during a hair cycle:IPCC 2014: September 7, 2014, Singapore
2. Emi K.Nishimura:Hair follicle aging and stem cell regulation:8th World Congress for Hair Research: May 14-17, 2014, Jeju Island, Korea
3. Emi K.Nishimura:Stress sensitivity of melanocyte stem cells during a hair cycle:8th World Congress for Hair Research: May 14-17, 2014, Jeju Island, Korea

Oral Presentation at meetings

Hiroyuki Matsumura, Mohri Yasuaki, Nguyen Thanh Binh, Morinaga Hironobu, Emi K. Nishimua : Defective maintainance ob COL17A1 in hair follicle stem cells orchestrates hair follicle aging : The 12th Stem Cell Research Symposium : May 30th, 2014, Kyushu

Department of Immunology

Professor	Takeshi Tsubata, M.D., Ph.D.
Associate Professor	Takahiro Adachi, Ph.D.
Assistant Professor	Mitsuhiro Suzuki, Ph.D.
Assistant Professor	Naoko Matsubara, Ph.D.
	Chizuru Akatsu, Ph.D.
	Xu Miduo, Ph.D.
Researcher	Soha Gomaa Ramadan Abdel Salam, Ph.D.
	Liu Zhihong, Ph.D.
Lecturer	Ji-Yang WANG

Normal immune system removes pathogens and cancer cells but does not respond to non-microbial foreign substances or normal self-antigens. Immune responses to non-microbial foreign substances and self-antigens cause allergy and autoimmune diseases, respectively. Immune responses to non-protein antigens play crucial roles in host defense against pathogens such as tuberculosis bacilli and meningococci, and autoimmune diseases such as lupus and immuno-neurological disorders. The mechanisms for immune responses to non-protien antigens are distinct from those to protein antigens, but are largely unknown. Thus, immune responses to non-protein antigens constitute a remaining frontier in immunology research. Followings are our research subjects.

1) Elucidation of the mechanisms for humoral immune responses to glycans, glyco-lipids and nucleic acids-related antigens.
2) Elucidation of the role of glycan signals in the regulation of humoral immune responses, and development of modified glycan signals for therapy.
3) Elucidation of the mechanisms for autoantibody production in lupus and immuno-neurological disorders.
4) Role of cell stress such as reactive oxygen species (ROS) in B lymphocyte activation
5) Drug discovery

1. Elucidation of regulatory mechanisms for pathogenic autoantibody production in lupus.

Systemic lupus erythematosus is a prototype of systemic autoimmune diseases, and characterized by production of autoantibodies to various nuclear components such as DNA. Among these autoantibodies, anti-DNA antibodies and antibodies reactive to RNA-related antigens such as the Sm antigen play a pathogenic role. The previous studies on autoantibodies suggested that autoantibodies in SLE are generated from cross-reactive antibodies that react to the self-antigens weakly through somatic mutations of the immunoglobulin genes. We crossed the 56R mice transgenic for an immunoglobulin H chain of an anti-DNA antibody with CD40L-transgeni mice in which CD40L is overexpressed, and demonstrated in these mice that B cells producing such cross-reactive weakly self-reactive autoantibodies appear in the peripheral lymphoid organs and differentiate to plasma cells, resulting in autoantibody production. This result clearly demonstrates that weakly self-reactive crossreactive B cells escape from self-tolerance. CD40L-Tg 56R mice may be a useful model for the studies on the production of pathogenic autoantibodies.

2. Studies on the role of reactive oxygen species (ROS) in B lymphocyte activation

ROS play a various roles including in induction of cell activation and cell death. ROS is known to be produced in B cells upon ligation of the B cell antigen receptor (BCR). There are evidence suggesting that ROS play a role in BCR signaling, but also evidence suggesting that ROS are not involved in BCR signaling. Two major mechanisms for ROS production are NADPH oxidases and mitochondrial respiratory chain. We addressed mechanism for ROS production using inhibitors of NADPH oxidases and those of mitochondrial respiratory chain, and demonstrated that BCR ligation-induce ROS production involves NADPH oxidases but not mitochondria. Treatment with antioxidants markedly reduced B cell activation induced by BCR ligation, suggesting that ROS plays a crucial role in B cell activation. Among the 7 known NADPH oxidase isoforms, B cells predominantly express NOX2. In NOX2-deficient B cells, BCR ligation-induced ROS production was perturbed at the early phase but not late phase, and NOX2-deficient B cells were activated as efficiently as wild-type B cells. Thus, late phase ROS production plays a crucial role in B cell activation.

3. Development of sialic acid derivatives for immune regulation.

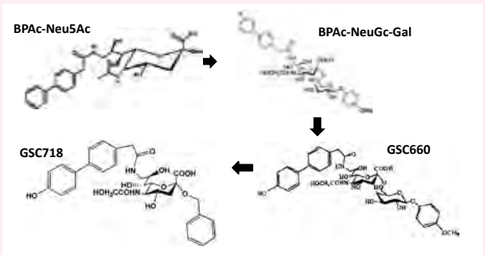
Although various immuno-modulating compounds have been developed, no such compound that targets B cells is available. We are developing the compounds that specifically regulate B cells by synthesizing sialic acid derivatives.

CD22,. Siglecs are expressed in various immune cells and recognize sialic acid as a ligand. As sialic acid is abundantly expressed in animal cells but rarely in microbes, Siglecs are thought to recognize sialic acid as a self motif and suppress autoimmune responses. Indeed, some Siglecs were shown to suppress immune response to sialic acid-containing antiges. In contrast, we demonstrated that CD22, a member of the Siglec family predominantly expressed in B cells, inhibits B cell response regardless whether antigens contain sialic acids or not. Thus, CD22 negatively regulates B cell responses to antigens in general. Moreover, we demonstrated that CD22-deficient B cells show markedly augmented antibody responses. These results strongly suggested that B cell responses can be augmented by reversing CD22-medicated suppression, and that CD22 is a suitable target molecule in developing a novel strategy to regulate immune responses. By collaborating with Profs. Kiso and Ishida at Gifu University, we developed a synthetic sialoside that binds to CD22 with high affinity and regulates B cell activation (see highlight). We are currently elucidating activity of this compound and also collaborating a pharma for drug development.

Highlight

Development of a synthetic silaoside that bind to CD22 with high affinity and regulates B cell activation.

CD22 is an inhibitory receptor predominantly expressed in B cells, and specifically recognizes α2,6 sialic acid as a ligand. Our results suggested that CD22 is a good target molecule to regulate immune responses. However, CD22 binds to α2,6 sialic acid only with low affinity (IC50~mM). Thus, we developed a synthetic sialoside that binds to CD22 with high affinity. Previously, Kelm et al. (2002) demonstrated that introduction of a biphenyl group at the C9 position of sialic acid increases the affinity to CD22 by around 100 fold. We then introduced galactose at the C2 position and further replace the galactose by a benzyl group, which increases the affinity to CD22 by 30 fold, resulting in a compound that binds to CD22 with IC50 ~100 nM. Moreover, this compound enhances proliferation of B cells in vitro, suggesting that this compound can regulate B cell activation.



(IC50 μM)	Me-Neu5Ac	BPAc-Neu5Ac	BPAc-NeuGcGal	GSC660	GSC718
hCD22	1400	35	3.7	0.90	0.128
mCD22	4689	123	6.2	1.70	0.101

(Kelm et al. 2002, Abdu-Allah et al. 2008)

Figure 1. Development of the synthetic sialoside GSC-718 that binds to CD22 with high affinity

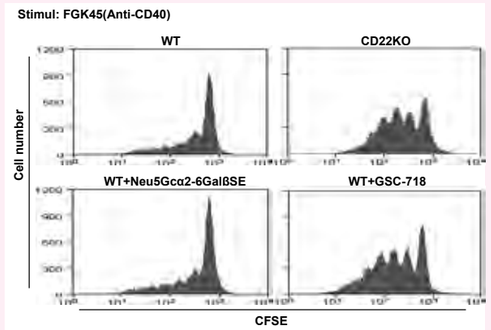


Figure 2. The synthetic sialoside GSC-718 augments B cell activation
B cell proliferation is analyzed by CFSE assay. Cell proliferation induces CFSE dilution resulting in reduction in CFSE fluorescence intensity.

Publications

[Original papers]

1. Aslam, M., Kishi, Y. and Tsubata, T. (2014): Excess CD40L does not rescue anti-DNA B cells from clonal anergy. *F1000Research* 2: 218.
2. Naito-Matsui, Y., Takada, S., Kano, Y., Iyoda, T., Sugai, M, Shimizu, A., Inaba, K., Nitschke, L., Tsubata, T., Oka, S., Kozutsumi, Y. and Takematsu, H. (2014): Functional evaluation of activation-dependent alterations in the sialogylcan composition of T cells. *J. Biol. Chem.* 289: 1564-1579.
3. Kawai, Y., Ouchida, R., Yamasaki, S., Dragone, L., Tsubata, T. and Wang, J.-Y. (2014): LAPTM5 promotes lysosomal degradation of intracellular but not the cell surface CD3ζ. *Immunol. Cell Biol.* 92:527-534.
4. Tsubata, T. (2014): Siglecs and B cell regulation. In: Taniguchi N., Endo T., Hart G., Seeberger P., Wong C. (Ed.) Glycoscience: Biology and Medicine: SpringerReference (www.springerreference.com). Springer-Verlag Berlin Heidelberg. 2014-04-01 06:48:34 UTC
5. Ouchida. R., Lu. Q., Liu. J., Li. Y., Chu. Y., Tsubata. T. and Wang, J.-Y.: FcμR interacts and cooperates with the B cell receptor to promote B cell survival. *J. Immunol.* 194:3096-101.
6. Muro. R., Nitta. T., Okada. T., Ideta. H., Tsubata. T. and Suzuki. H.: The Ras GTPase-activating protein Rasal3 supports survival of naive T cells. *PLoS ONE.* 10:e0119898.

Department of Molecular Pathogenesis

Professor **Akinori Kimura, M.D., Ph.D.**
Associate Professor **Takeharu Hayashi, M.D., Ph.D.**
Assistant Professor **Daisuke Sakurai, Ph.D.**
Research Associate **Taeko Naruse, Ph.D.**

Genetic factors, i.e. functional diversity of human genome mainly due to mutations or polymorphisms, are involved in the etiology and pathogenesis of human diseases. Contribution of gene mutations in the disease development is quite large in monogenic diseases showing simple Mendelian inheritance, while multiple gene mutations along with environmental factors are involved in the pathogenesis of multi-factorial diseases. Our goal is to decipher the disease-related genes for intractable or incurable diseases for which etiology and/or pathogenesis are not fully understood and, in addition, to develop novel therapeutic or preventive strategies for the intractable diseases based on the knowledge of disease-related genes.

1. Molecular mechanisms for hereditary cardiomyopathy

We have developed a screening system of mutations in the known 67 genes for hereditary cardiomyopathy. Among the sporadic children cases of hypertrophic cardiomyopathy, a considerable portion could be explained by *de novo* mutations in the sarcomere genes. In addition, by using iPS-derived cardiomyocytes it was revealed that hypertrophy and myofibrillar disarrays induced by ET-1 were more prominent in the cells from patients with hypertrophic cardiomyopathy irrespective of sarcomere gene mutations.

2. Molecular mechanisms for atherosclerosis

We generated a transgenic mouse line expressing coronary atherosclerosis-associated MKL1 under the CD68 promoter and found that the transgenic mice exhibited abnormality in function of macrophages. In addition, an international collaboration study revealed the impact of 9p21 locus on the longevity.

3. Molecular mechanisms for arrhythmia

We identified four *de novo CALM2* mutations in congenital long QT syndrome, which reduced the binding of calcium to calmodulin 2. We also identified an alpha-myosin heave chain gene (*MYH6*) mutation (delE933) in a case with sick sinus mutation. The *MYH6* mutation increased binding of alpha-myosin heavy chain and myosin binding protein C. Over-expression of the *MYH6* mutation impaired the sarcomere integrity in rat cardiomyocytes and decreased electrical velocity in HL-1 cells. In addition, involvement of *MYH6* mutation in bradycardia was proven in a zebrafish model.

4. Analysis of MHC and ULBP genes in human and old world monkeys

We have analyzed MHC class I diversities in macaque model for SIV vaccination in detail. In addition, divergence and diversity of *ULBP2* in primates, especially in the Old World monkey, both rhesus and cynomolgus macaques, were investigated. Phylogenic study suggested an evolutionary feature of *ULBP* gene families in the primates.

5. Genome diversity in association with HIV/AIDS

We have investigated natural selection on immune-related genes in the primate evolution. This year, we revealed that *APOBEC3H* polymorphisms were associated with the susceptibility to HIV-1 infection and progression to AIDS in Japanese.

Highlight

We have investigated diversity of ULBP2 genes in rhesus and cynomolgus macaques. It was revealed that there are two ULBP2 genes in the Old World monkeys, *ULBP2.1* and *ULBP2.1*. Both ULBP2 genes are highly polymorphic and the polymorphisms could be found at the contact sites of ULBP2 molecule and NKG2D receptor. A phylogenic analysis of primate *ULBP* gene family has revealed that the ULBP genes are evolved from an ancestor of *ULBP2* and the human-specific *ULBP6* has been diverged from *ULBP2* after the diversification of human and chimpanzee.

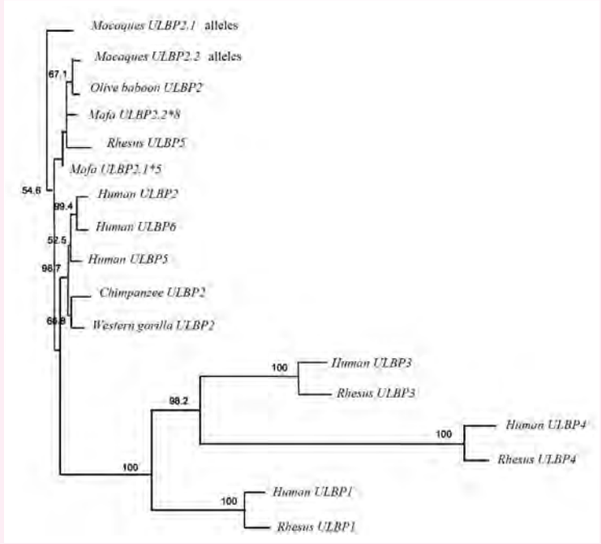


Figure Phylogenic tree of primate ULBP gene family
The phylogenic study suggested that ULBP2.2 gene was generated by duplication from ULBP2.1 along with ULBP5. On the other hand ULBP1, ULBP3, and ULBP4 were generated from an ancestral gene of ULBP2 and ULBP5. Of note was that ULBP6 was specifically present in humans, which was generated by duplication from ULBP2 after the diversification of human and other higher primates, chimpanzee and gorilla.

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3. Kimura A. Departure from the Hardy-Weinberg equilibrium. Gene 2014; 537(2): 357.
4. Katsuumi G, Shimizu W, Watanabe H, Noda T, Nogami A, Ohkubo K, Makiyama T, Takehara N, Kawamura Y, Hosaka Y, Sato M, Fukae S, Chinushi M, Oda H, Okabe M, Kimura A, Maemura K, Watanabe I, Kamakura S, Horie M, Aizawa Y, Makita N, Minamino T. Efficacy of bepridil to prevent ventricular fibrillation in severe form of early repolarization syndrome. Int J Cardiol. 2014; 172(2): 519-522.
5. Naruse TK, Akari H, Matano T, Kimura A. Divergence and diversity of ULBP2 genes in rhesus and cynomolgus macaques. Immunogenetics. 2014; 66(3):161-170.
6. Pinós T, Fuku N, Cámara Y, Arai Y, Abe Y, Rodríguez-Romo G, Garatachea N, Santos-Lozano A, Miro-Casas E, Ruiz-Meana M, Otaegui I, Murakami H, Miyachi M, Garcia-Dorado D, Hinohara K, Andreu AL, Kimura A, Hirose N, Lucia A. The rs1333049 polymorphism on locus 9p21.3 and extreme longevity in Spanish and Japanese cohorts. Age. 2014; 36(2): 933-943.
7. Okada S, Suzuki Y, Arimura T, Kimura A, Narumi H, Hasegawa S. A novel de novo mutation of beta-cardiac myosin heavy chain gene found in a 12-year-old boy with hypertrophic cardiomyopathy. J Genet. 2014; 93(2): 557-560.
8. Makita N, Yagihara N, Crotti L, Johnson CN, Beckmann BM, Roh MS, Shigemizu D, Lichtner P, Ishikawa T, Aiba T, Homfray T, Behr ER, Klug D, Denjoy I, Mastantuono E, Theisen D, Tsunoda T, Satake W, Toda T, Nakagawa H, Tsuji Y, Tsuchiya T, Yamamoto H, Miyamoto Y, Endo N, Kimura A, Ozaki K, Motomura H, Suda K, Tanaka T, Schwartz PJ, Meitinger T, Kääb S, Guichney P, Bhuiyan ZA, Shimizu W, Watanabe H, Chazin WJ, George AL. Novel calmodulin (CALM2) mutations associated with congenital arrhythmia susceptibility. Circ Cardiovasc Genet. 2014; 7(4): 466-474.
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10. Tanaka A, Yuasa S, Mearini G, Egashira T, Seki T, Kodaira M, Kusumoto D, Kuroda Y, Okata S, Suzuki T, Arimura T, Makino S, Kimura K, Kimura A, Furukawa T, Carrier L, Nobe K, Fukuda K. ET-1 induces myofibrillar disarray and contractile vector in hypertrophic cardiomyopathy-iPS cell-derived cardiomyocytes. J Am Heart Assoc. 2014; 3(6): e001263.
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Frontier Research Unit
Virus Research Unit

Associate Professor **Norio Shimizu, PhD**
Technical Assistant **Kiku Abe, Miki Igaue, Naomi Kojima, Hikari Shimada, Yuta Yunomae**

The goals of our research unit are: the elucidation of the development mechanism of Epstein-Barr virus (EBV) infection, the employment of immunodeficiency animals for the creation of virus research models and development of an exhaustive pathogenic microbial screening system.

1. Development of novel anti EBV drug

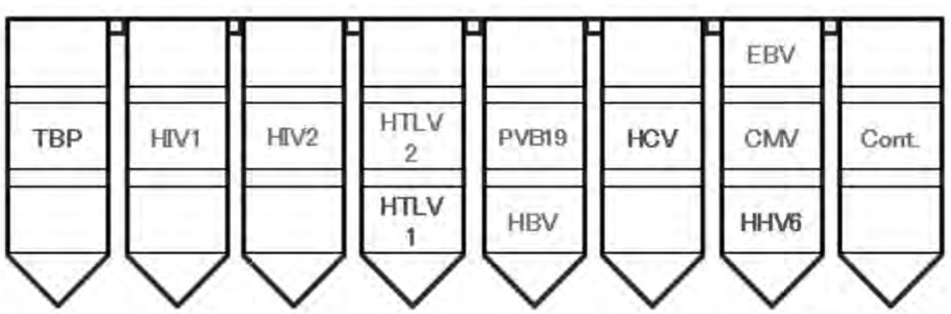
A novel nucleoside, 1-(2-deoxy-2-fluoro-4-thio-beta-D-arabino-pentofuranosyl) thymine (S-FMAU) was selectively cytotoxic to EBV-TK-transduced cells. S-FMAU blocked *ex vivo* EBV-induced transformation of B lymphocytes and reduced EBV copy numbers in the culture supernatant. EBV-infected T- and natural killer (NK) cells derived from Chronic active EBV infection (CAEBV) patients, which showed spontaneous EBV-TK expression, were much more susceptible to S-FMAU than to ganciclovir.

2. Development of an exhaustive pathogenic microbe screening system

We aim to establish an exhaustive pathogenic microbe screening system. We have modified our exhaustive virus screening system (capable of screening dozens of viruses simultaneously) which is currently under development so that in addition to viruses, it can also detect various other kinds of pathogens such as bacteria and protozoa. Other goals are to improve the sensitivity of the viral screening system and put it to practical use by conducting clinical microbiological investigations.

Highlight

Solid phase reagent for detection of 10 viruses



Publications

[Original papers]
1. Fujiwara S, Kimura H, Imadome K, Arai A, Kodama E, Morio T, Shimizu N, Wakiguchi H.: I Current research on chronic active Epstein-Barr virus infection in Japan. Pediatr Int. 56(2):159-166. (2014)
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3. Yoshimori M, Imadome K, Komatsu H, Wang L, Saitoh Y, Yamaoka S, Fukuda T, Kurata M, Koyama T, Shimizu N, Fujiwara S, Miura O, Arai A.: CD137 Expression Is Induced by Epstein-Barr Virus Infection through LMP1 in T or NK Cells and Mediates Survival Promoting Signals. PLoS One 9(11):e112564. (2014)
4. Ng SB, Ohshima K, Selvarajan V, Huang G, Choo SN, Miyoshi H, Shimizu N, Reghunathan R, Chua HC, Yeoh AE, Quah TC, Koh LP, Tan PL, Chng WJ.: EBV-associated T/NK-cell lymphoproliferative disorder in children and young adults has similar molecular signature to extranodal nasal NK/T-cell lymphoma but shows distinctive stem cell-like phenotype. Luek Lymphoma 10:1-27 [Epub ahead of print] (2014)

Division of Medical Genomics

Over the last decade, remarkable strides have been made in human genome science. The complete human genome sequences have been available through the public databases, and many high throughput technologies have been developed in the human genome project. In the post-sequence era we have excellent opportunities to identify genetic or epigenetic changes that are responsible for diseases. The missions of the Division of Medical Genomics are to understand genomic, epigenomic and proteomic changes underline the initiation and progression of human disease, and to elucidate the pathogenesis of intractable diseases. Our goal is to translate our research discoveries into the clinical setting.

【Molecular Cytogenetics】

The principal aim of our reserach is to understand the molecular basis underlying cancer and genetic diseases including chromosome aberration syndromes. We have contributed as follows;
1. Identification of novel genes including microRNAs responsible for cancer and unknown genetic diseases.
2. Understanding the pathogenesis of intractable cancers and genetic disorders based on the integrative omics approach including systems biology.
3. Establishment of practical tools for diagnosis in personalized medicine of cancer and genomic disorders in the clinical setting.

【Biochemical Genetics】

Our lab is focusing on basic transcriptional mechanism and its biological function and pathogenesis of human disease.
1. Role of stress response gene ATF3, a target of p53, in TRAIL-based pro-apoptotic cancer therapy. Further, the stress code of p53-ATF3 axis was investigated by genome-wide system biology.
2. Transcriptional properties of elongation factor Elongin A was elucidated in stress response and cranial nerve development.
3. Biochemical and biological role of FCP1, a causative gene for CCFDN, was studied and shown to be essential for transcription cycle.

【Molecular Genetics】

BRCAs, products of hereditary breast cancer genes, are associated with genome stability. We analyze functions of BRCAs and other related proteins to reveal the mechanism of breast carcinogenesis.
1. We aimed to establish novel synthetic lethal interactions between BRCA2 and chemical compounds provided by Chemical Biology Screening Center at TMDU.
2. We found that the enhancement of the ATPase activity of non-muscle myosin (NM)-IIC by BRCA2 was required for completion of the cytokinesis.
3. We analyzed the intramolecular BRCA2 region concerning the numerical integrity of centrosomes by an automated centrosome counting system.

【Molecular Epidemiology】

1. A risk calculation system for type2 Diabetes in Japanese has been constructed.
2. A single nucleotide polymorphism in the ryanodine receptor 3A (RYR3A) was found to associate with atherosclerosis.

【Genomic Pathology】

1. We are analyzing the global profiling of cancer-stromal interactions by massively-parallel sequencing of cancer xenograft transcriptome. We also started to examine patient-derived xenograft (PDX), where clinical cancer tissue is directly transplanted into immune-compromised mouse.
2. We are analyzing cancer immuno-genomics to discover biomarkers of cancer immunotherapy.
3. We performed genome-sequencing of diffuse-type (scirrhous-type) gastric cancer, and discovered recurrent RHOA driver mutation.

【Epigenetics】

1. We reported the existence of many LTR retrotranposon-derived genes in eutherian mammals, such as sushi-ichi-related retrotransposon homologue family of genes (*SIRH* family genes). Among them, we demonstrated that *Peg10*, *Peg11/Rtl1* and *Sirh7*, play essential eutherian-specific functions, namely, multiple aspects of placental function.
2. We have recently reported that distribution of *SIRH* genes and another LTR retrotranposon-derived genes, *PNMA* family genes, are much abundant in the eutherian mammals but only few in marsupial mammals, another group in mammals, suggesting that these LTR retrotransposon-derived genes deeply contributed to diversification and establishment of these two viviparous mammalian groups.
3. Assisted reproductive technologies, such as *in vitro* fertilization and intracytoplasmic sperm injection, become very popular, however, their effects on human development remain unclear. We have extensively examined pre-and postnatal epigenetic effects caused by such technologies.

【Bioinformatics】

1. We developed a new mathematical method to analyze topological and statistical properties of complex networks. By the method, we revealed that proteins with intermediate connectivities form a backbone of protein-protein interaction networks. Proteins in the backbone are tend to be drug targets, while almost no drug targets were found among hub proteins.
2. We conducted collaborative works with several research laboratories including following topics based on bioinformatics analysis: (1) identification of gene sets and their interaction networks associated with phenotypes and prognosis of hepatocellular carcinoma (HCC), (2) expression analysis of Aurora kinase B and alternative variant forms in HCC, (3) identification of IQGAP1 as a key regulator genes in naturally occurring hepatotumorigenesis induced by oxidative stress, and (5) identification of MUC12 as a prognosis marker in colorectal cancer.
3. We developed a new computational algorithm for inferring the dynamics of within-patient HIV evolution under anti-HIV therapy.
4. By conducting in silico and in vivo analyses, we revealed Hes1 is a master regulator to keep the stem cell undifferentiated state in the developmental process of taste receptor cells.

Department of Molecular Cytogenetics

Professor **Johji Inazawa M.D., Ph.D.**
Lecturer **Jun Inoue Ph.D.**
Assistant Professor **Tomoki Muramatsu Ph.D.**

The principal aim of Department of Molecular Cytogenetics is to understand the molecular mechanism underlying cancer and genetic diseases including multiple congenital anomalies and/or intellectual disability (MCA/ID). Our research interests are as follows; (1) Identification of genes responsible for cancer and unknown genetic diseases, (2) Development of innovative techniques for detection of genomic and epigenomic aberrations underlying the pathogenesis of cancer and genomic disorders, and (3) Establishment of practically useful modalities for diagnosis and therapy in personalized medicine in cancer and other intractable diseases. It is our goal to bridge the gap between basic and clinical research for the benefit of each of the patients.

1. Cancer omics research

For "tailor-made Medical treatment Program", we have explored tumor susceptible gene and cancer biomarkers of malignancy for esophageal squamous cell cancer (with Tokyo Med. Dent. Univ. and Aichi Cancer Center), breast cancer (with Cancer Institute Hospital and the Univ. of Tokushima), pulmonary cancer (Nagoya Univ. and Shiga Univ. of Medical Science), colorectal cancer (with CIH and Osaka Univ.), prostate cancer (with Kyoto Univ. and Iwate Medical Univ.) and gastric cancer (with National Cancer Center and UT) in order to establish a personalized cancer medicine. Furthermore, for "Project for development of innovative research on cancer therapeutics (P-DIRECT)", we have performed the integrative analysis of genomics, epigenomics, and gene expression in esophageal squamous cell cancers.

2. Function-based screening for identification of cancer-associated microRNAs having potential as therapeutic agents for cancer.

Recently, the Epithelial-Mesenchymal Transition (EMT) has been demonstrated to contribute to normal and disease processes including cancer progression. To explore EMT-suppressive microRNAs (miRNAs), we established a cell-based reporter system using a stable clone derived from a pancreatic cancer cell line, Panc1, transfected with a reporter construct containing a promoter sequence of CDH1/E-cadherin in the 5' upstream region of the ZsGreen1 reporter gene. Then, we performed function-based screening with 470 synthetic double-stranded RNAs (dsRNAs) mimicking human mature miRNAs using the system and identified miR-655 as a novel EMT-suppressive miRNA. Overexpression of miR-655 not only induced the upregulation of E-cadherin and downregulation of typical EMT-inducers but also suppressed migration and invasion of mesenchymal-like cancer cells accompanied by a morphological shift toward the epithelial phenotype. In addition, we found a significant correlation between miR-655 expression and a better prognosis in esophageal squamous cell carcinoma (ESCC). Moreover, ZEB1 and TGFBR2, which are essential components of the TGF-β signaling pathway, were identified as direct targets of miR-655, suggesting that the activation of the TGF-β-ZEB1-E-cadherin axis by aberrant downregulation of miR-655 may accelerate cancer progression.

3. Molecular basis for autophagy-based personalized cancer medicine.

It has been suggested that autophagy might contribute to tumor progression through cell survival of cancer cells in microenvironment and resistance to cancer therapy. However, we have found that autophagy was impaired by genetic or epigenetic aberrations in a part of cancer, suggesting that autophagy activity may be different in individual patient. Hence, it is important to develop molecular bisis for autophagy-based therapeutic concept. It has been considered that p62/SQSTM1, a substrate of autophagic degradation, may become to a molecular marker for the impaired autophagy. In 2014, we examined the expression status of this protein in 1258 cases from several tumor type and found that 358 of 1258 cases exhibit high expression of p62 protein, suggesting that autophagy activity may be low level in these cases. Furthermore, we found that high expression of p62 is associated with poor prognosis in ovarian cancers (Iwadate R et al. Acta Histochemica et Cytochemica. 2015 in press). Additionally, we are current going to identify small compounds or microRNAs, which are highly sensitive in autophagy-impaired cancer cells, for development of autophagy-based personalized cancer medicine.

4. Molecular cytogenetic investigation of congenital disorders

We have screened 646 patients with clinically uncharacterized multiple congenital anomalies and intellectual disability by several types of genomic microarray for three-stage screening. We have detected pathogenic copy number variants in 160 cases (24.8%). We also recruited patients with microcephaly with pontine and cerebellar hypoplasia (MICPCH) by a haploinsufficiency of the CASK gene, which have been established through the screening, and investigated their etiology. We detected various genetic aberrations suppressing the expression of CASK in 32 of 42 cases (76.2%) (Hayashi S et al. J Hum Genet. 2015 in press). For the remaining cases we applied target re-sequencing or whole exon sequencing by next generation sequencer to clarify comprehensively an etiology of MICPCH. We also constructed a the MCG CNV Database, which provides copy number variants (CNVs) and loss of heterozygosity (LOH) detected in 100 trios of healthy Japanese parents and one child by our in-house BAC arrays and SNP array (illumina), and released on the internet.

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Department of Molecular Genetics

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Our research is directed at understanding how cells recognize, assess and respond to lesions in DNA and the molecular mechanisms by which a damaged cell can distinguish between continued proliferation, permanent growth arrest and suicide. BRCAs, products of hereditary breast cancer genes, are associated with genome stability through DNA damage repair. We analyze functions of BRCAs and other related proteins to reveal the mechanism of breast carcinogenesis.

1. Loss of BRCA2 enhances PTX-induced microtubule stabilization

We aimed to identify potential synthetic lethality relationships between BRCA2 deficiency and chemical compounds provided by Chemical Biology Screening Center at TMDU in the hope that the use of chemotherapy in BRCA2-deficient breast cancer patients may be optimized on the basis of the tumor cell genotype. Synthetic lethality is a concept that is receiving increasing interest due to its potential exploitation in targeted cancer therapy. The chemical library (1,579 known compounds) was screened against capan-1 cells, which carries a naturally occurring 6174delT mutation in one BRCA2 allele accompanied by loss of the wild-type allele. Of the 53 leading hits in this screening, 18 compounds were confirmed as antineoplastic agents. In addition, antineoplastic agents, some of which inhibit cell proliferation by directly interfering with tubulin polymerization including paclitaxel (PTX).

Next, the effectiveness of the three-dimensional (3D) spheroid in BRCA2-siRNA knockdown cells as a model for synthetic lethality analysis was performed with well-known tubulin inhibitor, PTX. The monolayer culture exhibit an unnatural spread morphology, while cancer cells in 3D culture show a clustered, spheroid morphology that reflects in vivo tumors and provides better accuracy for anti-cancer drug testing. To verify a 3D spheroid assay, breast cancer cell line T-47D forming spheroids was evaluated. PTX significantly inhibited BRCA2-siRNA knockdown monolayer cell proliferation and 3D spheroid growth. To examine whether loss of BRCA2 affect the microtubule dynamics by the effect of PTX which induces microtubule stabilization, we performed a cell-based tubulin polymerization assay, in which T-47D cells were exposed to PTX following treatment of siRNA knockdown of BRCA2. The soluble tubulin dimers were separated from microtubule polymers by centrifugation. As shown in Figure 1, PTX treatment of BRCA2-siRNA knockdown cells resulted in a significant increase in microtubule polymer mass, from 34.5 ± 2.1% in untreated cells to 57.5 ± 1.4% in PTX-treated cells. In contrast, PTX treatment of control-siRNA cells resulted in an only modest increase in microtubule polymer mass, from 33.2 ± 1.2% in untreated cells to 41.9 ± 0.9% in PTX-treated cells (Fig.1). Our results indicate that loss of BRCA2 enhances microtubules more stabilization with PTX treatment suggesting that the PTX–tubulin interaction might be augmented under these conditions.

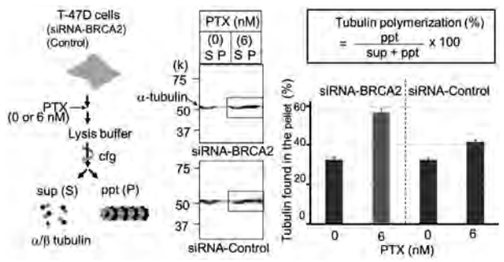

Figure 1. Tubulin polymerization assay in BRCA2-siRNA knockdown cells. Left panel: cells were treated with DMSO or 6 nM PTX for 24 h and subjected to tubulin polymerization assay. Following lysis and centrifugation the pellet fraction (ppt; P), containing polymerized microtubules, was separated from the supernatant fraction (sup; S), containing soluble tubulin dimers. Middle panel: The equal volumes of each were loaded on SDS–PAGE and immunoblotted using anti-α-tubulin antibody. Right panel: graphic display of percent tubulin in the pellet fraction, which was calculated as the densitometric ratio of the pellet fraction (P) divided by the total tubulin content (P+S) for each condition. Data show the average of three repeats.

2. Centrosomes at M phase act as a scaffold for the accumulation of intracellular ubiquitinated proteins

Centrosome size varies considerably during the cell cycle; it is greatest during metaphase, partly because of pericentriolar matrix recruitment and an increase in microtubule-organizing activity. However, the mechanism of centrosome maturation during M phase is poorly defined. In the present study, we identified and quantified centrosomal proteins during S and M phases using stable isotope labeling by amino acids in cell culture (SILAC) coupled with liquid chromatography–tandem mass spectrometry (LC–MS/MS). We identified 991 proteins, of which 310 and 325 proteins were upregulated during S and M phases, respectively. Ubiquitinated proteins containing K48- and K63-linked polyubiquitin chains accumulated in the centrosomes during M phase, although 26S proteasome activity in the centrosomes did not markedly differ between S and M phases. Conversely, cytoplasmic dynein, which transports ubiquitinated proteins to the centrosomes, increased 2-fold in the centrosomes during M phase relative to S phase. Furthermore, PYR-41, an ubiquitin E1 inhibitor, reduced centrosome size during metaphase, causing increased aneuploidy. RNA interference suppression of Ecm29, which inhibits proteasome activity, decreased the accumulation of ubiquitinated proteins in the centrosomes. These results show that accumulation of ubiquitinated proteins promotes centrosome maturation during M phase and further suggest a novel function of centrosomes as a scaffold temporarily gathering intracellular ubiquitinated proteins.

3. Periostin suppression induces decorin secretion leading to reduced breast cancer cell motility and invasion

The ability of cancer cells to metastasize is dependent on the interactions between their cell-surface molecules and the microenvironment. However, the tumor microenvironment, especially the cancer-associated stroma, is poorly understood. To identify proteins present in the stroma, we focused on phyllodes tumors, rare breast tumors that contain breast stromal cells. We compared the expression of proteins between phyllodes tumor and normal tissues using an iTRAQ-based quantitative proteomic approach. Decorin was expressed at reduced levels in phyllodes tumor tissues, whereas periostin was upregulated; this result was validated by immunohistochemical analysis of phyllodes tumors from 35 patients. Additionally, by immunoprecipitation and mass spectrometry, we confirmed that decorin forms a complex with periostin in both phyllodes tumors and BT-20 breast cancer cells. Following siRNA-mediated knockdown of periostin in T-47D cells, secreted decorin in the culture medium could be detected by multiple reaction monitoring (MRM). Furthermore, periostin knockdown in BT-20 cells and overexpression of decorin in MDA-MB-231 cells inhibited cell motility and invasion. Our results reveal the molecular details of the periostin–decorin complex in both phyllodes tumor tissues and breast cancer cells; this interaction may represent a novel target for anti-cancer therapy.

Our findings demonstrate that periostin is more abundant in phyllodes tumors than in normal tissues, and that it forms a complex with decorin. Previous work showed that decorin can delay tumor growth by blocking TGF-β, inhibiting inducers of angiogenesis such as VEGF, or interacting with E-cadherin. On the other hand, knockdown or neutralization of endogenous periostin results in inhibition of cell migration and invasion, although the mechanism remains unclear.

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Department of Molecular Epidemiology

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Many common chronic diseases are multifactorial in that they are caused by multiple genetic and environmental factors. By applying the technology and information of human genome to epidemiological studies, we aim to clarify the role of genetic polymorphisms, epigenetic changes, as well as their interaction with environmental factors, which may contribute to the development of these diseases.

1. Likelihood ratio-based integrated personal risk assessment of type 2 diabetes.

To facilitate personalized health care for multifactorial diseases, risks of genetic and clinical/environmental factors should be assessed together for each individual in an integrated fashion. This approach is possible with the likelihood ratio (LR)-based risk assessment system, as this system can incorporate manifold tests. We examined the usefulness of this system for assessing type 2 diabetes (T2D). Our system employed 29 genetic susceptibility variants, body mass index (BMI), and hypertension as risk factors whose LRs can be estimated from openly available T2D association data for the Japanese population. The pretest probability was set at a sex- and age-appropriate population average of diabetes prevalence. The classification performance of our LR-based risk assessment was compared to that of a non-invasive screening test for diabetes called TOPICS (with score based on age, sex, family history, smoking, BMI, and hypertension) using receiver operating characteristic analysis with a community cohort (n = 1263). The area under the receiver operating characteristic curve (AUC) for the LR-based assessment and TOPICS was 0.707 (95% CI 0.665-0.750) and 0.719 (0.675-0.762), respectively. These AUCs were much higher than that of a genetic risk score constructed using the same genetic susceptibility variants, 0.624 (0.574-0.674). The use of ethnically matched LRs is necessary for proper personal risk assessment. In conclusion, although LR-based integrated risk assessment for T2D still requires additional tests that evaluate other factors, such as risks involved in missing heritability, our results indicate the potential usability of LR-based assessment system and stress the importance of stratified epidemiological investigations in personalized medicine.

2. Association of the RYR3 gene polymorphisms with atherosclerosis in elderly Japanese population.

The Ryanodine receptor 3 gene (RYR3) encodes an intracellular calcium channel that mediates the efflux of Ca2+ from intracellular stores. Two single-nucleotide polymorphisms (SNPs) in the RYR3 gene have been shown to associate with stroke (rs877087) and carotid intima-media thickness (rs2229116) in two independent genome-wide association studies (GWAS) in Caucasian. We investigated the effect of these two SNPs as well as the 31.1 kilobases spanning region on atherosclerosis in Japanese population.

Atherosclerotic severity was assessed by carotid artery (n = 1374) and pathological atherosclerosis index (PAI) (n = 1262), which is a macroscopic examination of the luminal surfaces of 8 systemic arteries in consecutive autopsy samples. 4 tag SNPs in the 31.1 Kb region, rs877087, rs2132207, rs658750 and rs2229116, were genotyped and haplotypes were inferred to study the association with atherosclerotic indices.

rs877087 and rs2229116 were associated with PAI (OR = 2.07 [1.04-4.12] (95% CI), p = 0.038; and OR = 1.38 [1.02-1.86], p = 0.035, respectively). rs2229116 was also associated with common carotid atherosclerosis (OR = 1.45 [1.13-1.86], p = 0.003). The risk allele of rs2229116 was opposite from the original report. The haplotype block of this 31.1 Kb region was different between Caucasian and Japanese. Haplotype analysis revealed that only TAGG haplotype was associated with PAI (OR = 0.67 [0.48-0.94], p = 0.020) and atherosclerosis of common carotid artery (OR = 0.75 [0.58-0.98], p = 0.034). rs877087 and rs2229116 of RYR3 gene are associated with atherosclerosis severity in Japanese. The functional difference caused by rs2229116 needs to be investigated.

3. Association analysis of single nucleotide polymorphisms in miR-146a and miR-196a2 on the prevalence of cancer in elderly Japanese.

Single nucleotide polymorphisms (SNPs) affecting microRNA (miR) sequences may influence carcinogenesis. Our current study primarily aimed to confirm previously conducted association studies between rs2910164 found on miR-146a, and rs11614913 located on miR-196a2 polymorphisms and cancer phenotypes in the Japanese elderly population. rs2910164 (G/C) and rs11614913 (T/C) polymorphisms were determined by genotyping on the samples collected from 1,351 consecutive autopsy cases registered in the Japanese SNPs for geriatric research (JG-SNP) data base. Cancer samples were systematically reviewed, pathologically verified and assessed with respect to miR-146a and miR-196a2 genotypic variation. The current study covered 726 males and 625 females with a mean age of 80.3 ± 8.9 years. The study included 524 subjects without cancer and 827 subjects with at least one type of cancer, such as gastric (n=160), lung (n=148), colorectal (n=116) or others. Males with cancers (n=467) were more numerous than females (n=360). Both rs11614913 (CT: TT adjusted odds ratio (OR) 95% confidence interval (95%CI)=0.98 (0.75-1.28), p=0.873, CC: TT adjusted OR (95%CI)=1.06 (0.76-1.47), p=0.737, CT+CC: TT, adjusted OR (95%CI)=0.99 (0.77-1.29), p=0.990), and rs2910164 (CG: CC adjusted OR (95%CI)=1.12 (0.87-1.44), p=0.383, GG: CC adjusted OR (95%CI)=1.03 (0.71-1.48), p=0.887, CG+GG: CC adjusted OR (95%CI)=1.10 (0.87-1.39), p=0.446) polymorphisms did not show significant association with overall cancer in all subjects. However, "CC" genotype in rs11614913 polymorphism was significantly associated with increased gastric cancer (n=160) in all subjects (CC: CT+TT, adjusted OR (95%CI)=1.50 (1.02-2.22), p=0.040). We found that rs11614913 and rs2910164 do not pose general cancer risk, but rs11614913 may influence gastric cancer in Japanese elderly population. Confirmation of our study results requires further investigations with larger subject populations.

4. Epistasis effects of COMT and MTHFR on inter-individual differences in mental health: under the inverted U-shaped prefrontal dopamine model.

Higher cognitive performance, maintenance of mental health and psychological well-being require adequate prefrontal cortex (PFC) function. "Inverted U-shaped" dopamine model indicates optimal PFC dopamine level is important to attain its function while high or low levels have adverse effects. Catechol-O-methyltransferase (COMT) and methylenetetrahydrofolate reductase (MTHFR) may be involved in this complex non-linear PFC dopamine regulation. We addressed whether genetic variation reflecting COMT and MTHFR activities can explain the inter-individual mental health differences in healthy Japanese men (n=188). The mental health was measured by Mental Health Inventory (MHI)-5 score. The rs4633-rs4818-rs4680 haplotypes were used to represent the multilevel COMT activities, while for MTHFR, the functional single polymorphism, rs1801133 (C677T), was used. We examined the effectiveness of haplotype-based association analysis of COMT on mental health together with studying its interaction with MTHFR-C677T. As a result, the relation between activity-ranked COMT genotype and MHI-5 score showed a tendency to fit into an "inverted U-shaped" quadratic curve (P=0.054). This curvilinear correlation was significant in the subjects with MTHFR-CC (P<0.001), but not with MTHFR T-allele carriers (P=0.793). Our pilot study implies a potential influence of COMT and MTHFR genotypic combination on normal variation of mental health.

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Department of Biochemical Genetics

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Scope of research :

Transcription is one of the most important processes by which genome information is expressed from DNA to mRNA to protein. The faithful synthesis of mRNA is achieved by transcriptional machinery comprised of RNA polymerase II, basal factors and many co-factors, whose dysfunction is implicated in various human diseases. Our research interest is focused on the basic mechanism of transcription and its implication of immediate genetic events in determining cell fate in stress response and cancer.

Research 1 : Analysis of Pol II CTD phosphatase FCP1

The engine of the transcriptional machinery is Pol II. The largest Pol II subunit, Rpb1, has CTD, which consists of repetitive consensus heptapeptide YSPTSPS. Transcription is regulated by CTD phosphorylation/dephosphorylation and this is called "Transcription cycle". After transcription is terminated, Pol II falls off DNA template, but CTD should be dephosphorylated to be recycled for next transcription. FCP1 plays an important role in recycling of Pol II by dephosphorylating Ser2 preferentially in the CTD (Fig. 1). It has been reported that an autosomal recessive developmental disorder CCFDN is caused by a single-nucleotide substitution of *CTDP1*, encoding FCP1. Although CCFDN shows growth disorder and facial abnormality, its molecular mechanism remains to be poorly understood. We are focusing on the functions of FCP1 in order to elucidate the roles in transcription cycle and its implication in human disease.

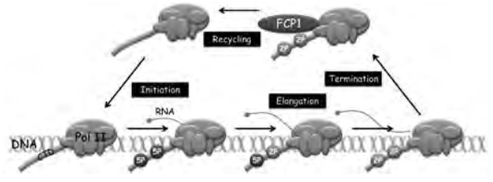

Figure 1. FCP1 contributes Pol II recycling in transcription cycle.

Research 2 : System biology approach to elucidate biological role of ATF3 in stress response

Activating transcription factor 3 (ATF3), an immediate early response gene, is known to play dichotomous role as oncogene or tumor suppressor in human cancers. We previously found that ATF3 is a target gene of p53 and inhibits p53 degradation to stabilize its expression level. Further, we generated genetically engineered mouse model of p53 and ATF3 gene double-knockout to unravel genetic codes of p53-ATF3 axis, and we performed genome-wide gene expression and ChIP-seq analysis. The genome-wide analysis of these mice is now revealing intriguing regulatory networks between these two transcription factors in cancer and stress response.

Research 3 : ASH1 and epigenetic mechanisms of facioscapulohumeral muscular dystrophy

We have shown that ASH1 specifically methylates histone H3 lysine 36 and plays a crucial role in activation of repeat elements in the chromosome 4 subtelomeric region of FSHD patients. Further we have successfully sequenced and assembled the highly GC rich repeats with PacBio RS paving a way to more convenient diagnosis. We have also shown that long non-coding RNA interacts with and recruites ASH1 to the repeat locus providing a novel therapeutic target for FSHD.

Highlight

1. FCP1 regulates cell cycle via p53 activation

We previous showed FCP1 knockdown induces cell growth arrest through p53 signaling pathway. Here, we revealed that FCP1 is involved G1 and M phase in cell cycle. First, FCP1 knockdown inhibits cell proliferation not only in p53 wild-type cells but also in p53 depletion cells, however p53 depletion cells grow faster and express lower level of p21 than p53 wild-type cells (Fig. 2). In cell cycle analysis by flow cytometry, FCP1 knockdown in p53 wild-type cells induces G1 arrest, but not in p53 depletion cells. Second, we unveiled FCP1 affects several genes via p53 activation implicated in G2/M transition and M phase of cell cycle by extensive gene expression profiling. Previous study demonstrated FCP1 plays a role in the mitosis exit by dephosphorylating crucial mitotic substrates, in addition we indicated FCP1 regulates G1 and M phase via p53 activation to regulate gene expression (Fig. 3).

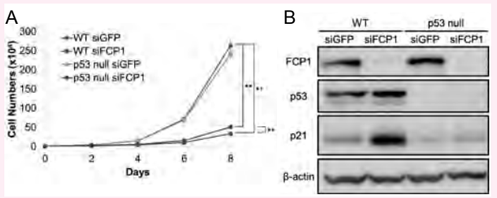

Figure 2. FCP1 knockdown inhibits cell proliferation via p53 activation.

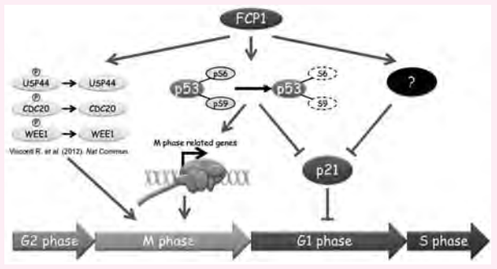

Figure 3. FCP1 regulates G1 & M phase in cell cycle.

2. ATF3 is involved in several stress response pathways including p53 pathway

We are trying to reveal the biological meaning of p53-ATF3 regulatory pathway. As a first step, we have performed microarray analysis using the doxorubicin treated embryonic fibroblasts from wild type, Atf3 KO, p53 KO and Atf3-p53 double KO mice. We illustrated heatmaps of stress response pathways included DNA damage response, unfolded protein response etc. (Fig. 4). The results show that most of these pathway genes were down-regulated by ATF3 KO, therefore implying that ATF3 generally up-regulates genes that are implicated in stress response.

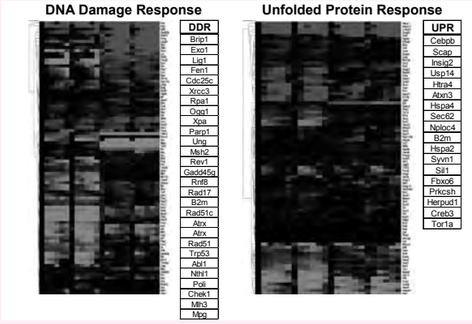

Figure 4. Heatmaps of stress response pathway

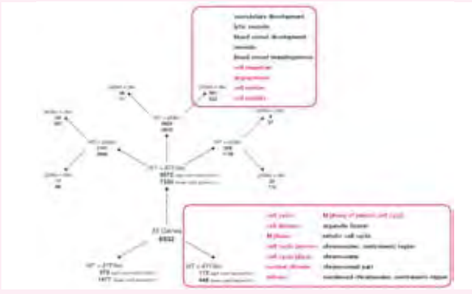

Figure 5. The role of ATF3 in the presence or absence of p53

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Research content

Tumor tissue is a complex system composed of tumor cells and multiple types of stromal cells. It is important for the understanding of developmental mechanisms of the disease to reveal the cell-cell interactions and interferences. Our purpose is to understand the dynamic multicellular interactions in such a complicated biological system by measuring a large amount of data at the genomic level, which leads to the identifications of therapeutic targets and biomarkers.

Furthermore, we also investigate the genomic approach for analyzing various intractable diseases. We are trying to reveal the molecular mechanism of such diseases by comprehensively genomic analysis of clinical samples.

Research introduction

1. Genomic approach for the cancer-stromal interaction

In the department of genomic pathology, we have developed a new method to analyze a wide range of cancer-stromal interactions in tumor tissues which are composed of various types of cells (tumor-stroma interactome, Fig 1). This kind of analysis has been technically difficult to be performed comprehensively and quantitatively. By obtaining the transcriptome data of tumor tissues from tumor bearing mouse, we create gene expression profiles of tumor cells (human cells) and stromal cells (mouse cells) by dividing the sequencing reads into human and mouse. Then, we reveal a global picture of the tumor-stroma interactions by incorporating the protein interaction database. We are going to identify more integrated interaction profiles using this method.

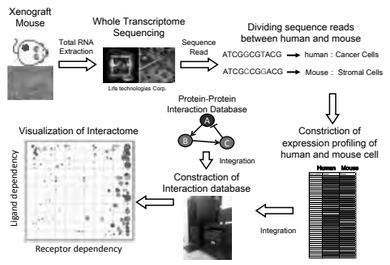

Fig 1. Genomic approach for cancer - stromal interaction

We are trying to reveal a global picture of interactions between cancer cells and stroma by this method and to identify inevitable signaling pathways on which the tumor microenvironments rely. We identified a number of important signals from stroma to cancer cells by using this method in pancreatic cancer xenograft mouse model. We identified WNT7B and WNT10A-FZD1 pathway from cancer to stroma by using interactome for Xenograft model of gastric and pancreatic cell lines (Fig. 2). To reveal the role for WNT signaling in stromal cells, we examined the effect of WNT signaling in the fibrobast activation and the inflammatory response of peritoneal macrophage. As a result, the activation of WNT signaling enhanced the TGF β-stimulated activation of fibroblast, and suppressed the inflammatory activation of peritoneal macrophage by LPS. These results indicate the possibility that WNT signaling is involved in the establishment of tumor stroma. We are examining the role for WNT signaling *in vivo*.

And we also confirmed the importance of these signals in clinical samples and in animal models by using chemical compounds. We are exploring new targets of drugs for cell-to-cell interactions by investigating a global view of the tumor-stroma interactome. Furthermore, by using a direct xenograft model (PDX: Patient Derived Xenograft, collaborated with Central Institute for Experimental Animals (CIEA), we are investigating the interactome analysis of multiple clinical tumors in order to make it possible to clarify the cell-cell interactome in primary human tumors.

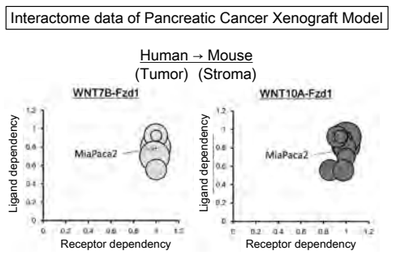

Fig 2. Identification of WNT signaling as Tumor-Stromal Interaction

2. Functional Genomics Screening

In the Department of Genomic Pathology, we are conducting various kinds of functional genomics screening by combining whole genomic shRNA libraries and next-generation sequencing technologies. Our goal is to identify novel therapeutic molecular targets against cancers, and to this end we are exploring possible candidate genes by developing a couple of different experimental settings. One example of our original screening strategy is a tumor implantation model in which shRNA-infected human fibroblasts and human cancer cells are co-inoculated into mice (Fig. 3). In this model, we can quantitatively characterize each of the shRNA-infected fibroblast clone in the *in vivo* tumor environments. We are now identifying genes which significantly activate and/or inactivate the growth rates of fibroblasts in specific environments of live tumor tissues in mice.

We performed numbers of functional genomics screening experiments targeting human gastric cancers, pancreatic cancers, colon cancers, liver cancers and so forth, having identified some candidate therapeutic target genes. We will continue these genomics screenings and will be trying to identify novel therapeutic molecular targets against otherwise devastating human cancers.

3. Genomics Analysis for Clinical Disease Tissues

In the department of genomic pathology, we have been investigating various clinical disease samples by genomics approaches. By utilizing massively-parallel sequencing, we are obtaining comprehensive data of transcriptome and whole exome sequencing of clinical tissue samples and trying to elucidate the pathogenic mechanism of the diseases defined by genomics aspects. In the diffuse gastric cancer (scirrhous carcinoma), by the deep exome sequencing, we identified the RHOA gene harboring somatic mutations with a high frequency of more than 20% of clinical samples. Verification experiments by using cancer cell lines suggested that this gene is an important driver gene for the diffuse gastric cancer (Highlight). In the department of genomic pathology, we will continue to investigate molecular mechanisms of *RHOA* gene identified in the diffuse gastric cancer. We are also going to further investigate various clinical disease samples by using genomics approach.

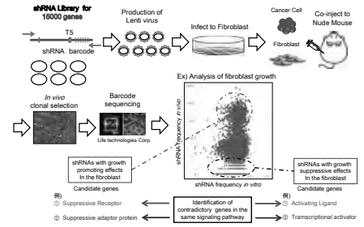

Fig 3. Functional Genomic Screening for elucidating the mechanism of Tumor stromal formation

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Department of Epigenetics

Professor	Fumitoshi ISHINO
Associate Professor	Takashi KOHDA
Assistant Professor	Ryuichi Ono
Tokunin Lecturer	Lee Jiyoung
Tokunin Assistant Professor	Mie Naruse, Masahito Irie
adjunct lecturer	Shin Kobayashi

Introduction of Department of Epigenetics

Epigenetics and Genetics are basics of biology that enables us to elucidate several 'genomic functions' in inheritance, development and evolution of the organisms including our human beings. Genomic imprinting is a mammalian-specific epigenetic mechanism that gives rise to functional differences between paternally- and maternally-derived genomes in development, behavior and growth. Somatic cloned animals give us unique chances to examine 'genetically identical but epigenetically diverged animals'. These studies show us how Epigenetics is important in mammalian biology. We focus on these mammalian-specific genomic functions to elucidate how these genomic functions work and have been evolved as new genomic functions during evolution. Our final goal is to contribute to the human biology as well as medicine in the 21st century by novel understanding of genomic functions.

Research subjects

1. Functional differences between paternally- and maternally-derived genomes in mammals (Genomic imprinting etc.)

Imprinted genes, such as paternally and maternally expressed genes (*PEG* and *MEG*) cause functional differences between parental genomes, thus, leading to several genomic imprinting diseases in humans. Much more DNA methylation differences than genomic imprints existed in sperms and oocytes may also play a role in mammalian early embryonic development. We focus on the biological functions of *PEG10* and *PEG11/RTL1* in mammalian development and evolution and on the role of differential gene expressions between paternal genomes in preimplantation development.

2. Roles of LTR-retrotransposon-derived genes in mammalian development and evolution

Two groups of genes, the *SIRH* (sushi-ichi retrotransposon homologues) and *PNMA* (paraneoplastic Ma antigen) family genes, exist in mammals. *PEG10* is a therian-specific genes, present in marsupials and eutherians but absent in monotremes while *PEG11/RTL1* and all the other genes are eutherian-specific. We are addressing their biological functions in current developmental system using KO mice as well as their roles in mammalian evolution as novel genes.

3. Biology of haploid ES cells in mammals

Mouse haploid cell lines provide us new tools for forward/reverse genetics as well as for addressing the relationship between ploidy and cell differentiation. We have already established several haploid ES cells from inbred strains, such as B6 and JF1.

4. New method of analyzing DNA methylation status in genomes

We have developed a new sequencing method, EnIGMA (Enzyme-assisted Identifidation of Genome Modification Assay), that distinguishes 5-methylcytosine (5mC) and 5-hydroxylmethylcytosines (5hmC) in single DNA fragments. As 5mC and 5hmC may play different roles in gene regulation, this method will provide us precise epigenetic information in the genome.

Highlight

Sirh7/Ldoc1 KO mice exhibit placental P4 overproduction and delayed parturition.

Sirh7/Ldoc1 (sushi-ichi retrotransposon homologue 7/Leucine zipper, down regulated in cancer 1) is one of the newly acquired genes from LTR retrotransposons in eutherian mammals. Interestingly, *Sirh7/Ldoc1* knockout (KO) mice exhibited abnormal placental cell differentiation/maturation, leading to an overproduction of placental progesterone (P4) and placental lactogen 1 (PL1) from trophoblast giant cells. Therefore, pregnant *Sirh7/Ldoc1* KO females also displayed delayed parturition associated with a low pup weaning rate (Figure 1). The placenta is an organ essential for mammalian viviparity and plays a major endocrinological role during pregnancy in addition to providing nutrients and oxygen to the fetus. P4 is an essential hormone in the preparation and maintenance of pregnancy and the determination of the timing of parturition in mammals, however, the biological significance of placental P4 in rodents have long been not properly recognized. Here we demonstrated that mouse placentas do produce P4 in mid-gestation coincident with a temporal reduction of the ovarian P4, suggesting that it plays a role in the protection of the conceptuses specifically in this period (Figure 2). All these results suggest that *Sirh7/Ldoc1* has undergone positive selection during eutherian evolution as a eutherian-specific acquired gene because it impacts reproductive fitness via the regulation of placental endocrine function.

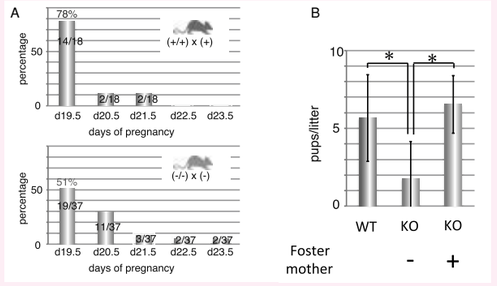

Figure 1. ***Sirh7/Ldoc1* KO mice exhibit delayed parturition and a low pup weaning rate.**
A Delayed parturition in *Sirh7/Ldoc1* null mice. Delivery days of (+/+) and (-/-) females mated with (+) and (-) males, respectively. B Decreased weaning rates. Average litter size (mean ± s. d.) for (+/+) females mated with (+) males and (-/-) females mated with (-) males, with or without foster mothers (postpartum females) (*: $p < 0.05$).

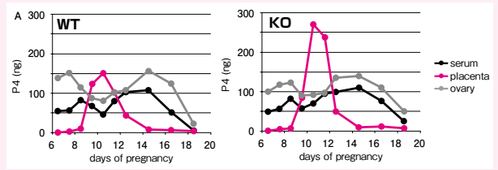

Figure 2. **Mouse placenta does produce progesterone during mid-pregnancy.**
The average amounts of P4 in the placenta (red) and ovary (gray) calculated as eight and two, respectively, and the serum P4 (black) calculated as 1.5 ml per individual of each pregnant (+/+) or (-/-) female on d6.5 to 18.5 are shown.

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Department of Bioinformatics

Professor **Hiroshi Tanaka**
Assistant Professor **Masaki Suimye Morioka**

Research Subjects

Our mission is "system-level understanding of biological systems" in molecular biology and evolution (systems evolution) and medicine (omics-based medicine, systems pathology). Recently, the whole genome sequences of diverse organisms have become available. Moreover, various "omics" information such as a proteome, transcriptome, and metabolome are currently accumulating. Our goal is to establish a grand-theory of biological sciences from the viewpoint of "evolving networks composed of biological molecules" by integrating omics information. Genomic and omics data are also utilized in the field of medicine. It has been revealed that most diseases are caused by the interaction among abnormalities of multiple genes, those at the tissue level, and environments. It is therefore possible to consider diseases as a system. From this standpoint, we try to establish the omics-based medicine and systems pathology.

Highlight

Network biology approach to epithelial-mesenchymal transition (EMT) in cancer metastasis: three stages of structural change of gene network in cancer EMT

The epithelial-mesenchymal transition (EMT) is a molecular program through which an epithelial cell loses its intercellular adhesion and acquires a migratory mesenchymal phenotype. Primary function of EMT is well-known as a necessary step in development. With accumulating evidences, EMT has now been widely acknowledged as a critical step in promoting metastasis in epithelium-derived carcinoma. In this study, we tried to clarify the essence of cancer EMT process by the network biology approach. We consider cancer EMT process is a biologically deep, overall structural change of the gene regulatory network (gene network or GRN, for abbreviation) in promoting cancer metastasis.

To conduct investigations on EMT from this point of view, we first defined 'state space of gene network' (GRN space), which is composed of all the possible activation patterns of gene network. Then, we introduced a quasi-potential distribution into this GRN space to indicate the relative stability of each state in GRN space. In doing so, we referred to the concept of Waddington's epigenetic landscape. Waddington proposed an epigenetic landscape as a metaphor for developmental process where a ball (cell) is rolling down a series of branching valleys separated by ridges on an inclined surface. In this study, we proposed a new method to create Waddington's landscape quantitatively, which is essentially empirical. We collected a number of samples of gene expression profiles, each of which shows a particular GRN activation pattern, from public databases of gene expression profiles. By using collected samples, we calculated an empirical frequency distribution (denoted by φ) of GRN states. The quasi-potential (denoted by ψ) is then obtained by applying a negative logarithm transformation to this empirical frequency distribution, if we assume the Boltzmann formula: $\psi = -k \log \varphi$ (k: const.) is applicable to GRN space. We thus consider that the obtained quasi-potential distribution on GRN space is the same as quantified Waddington's epigenetic landscape (qWEL). Cancer EMT process is supposed to draw a trajectory in this GRN space; starting from a basin of epithelial cell states, climbing over separating ridge or 'epigenetic barrier', then falling down to a basin of mesenchymal cell states. We depicted this EMT movement, by utilizing published experimental data (Takahashi et al., J Biol Chem. 285, 2010), where they used a cell line of retina pigment epithelial cell, inducing EMT in response to treatment of TGF α and TNF β. We traced the state movement of EMT in GRN space and found that cancer EMT process undergoes three subsequent stable stages, each of which forms the potential basin along the EMT trajectory.

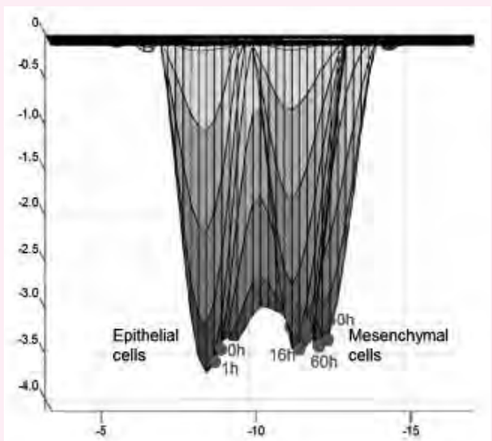

Figure 1. EMT trajectory on qWEL potential

To confirm three-staged transition of cancer EMT, we estimated structural changes of gene network during cancer EMT from the gene expression profiles data. We applied ARACNe algorithm for inference methods for gene network from the gene expression profile and applied the master regulator analysis to this gene network estimated by ARACNe, to identify the main regulations conducted by master regulators of the gene network. The main structure of estimated gene network during EMT was investigated by each group of major master regulators, which are alternatively active in subsequent time spans to cause the inlarge structural changes of GRN during cancer EMT.

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11. Nukaya S, Sugie M, Kurihara Y, Hiroyasu T, Watanabe K, Tanaka H : A noninvasive heartbeat, respiration, and body movement monitoring system for neonates. Artificial Life and Robotics, 19(4):414-419, 2014

[Conference Talks]

1. Hiroshi Tanaka: Attractor transition analysis of iPS cell and cancer metastasis in quantified Waddington epigenetic landscape (qWEL), AROB 19th, Oita, Japan, 2014.01.22
2. Takeshi Hase, Identification of drug-target modules in the human protein–protein interaction network, AROB 19th, Oita, Japan, 2014.01.22

Frontier Research Unit
Redox Response Cell Biology

Associate Professor Shun-Ichi Kurata

Since the living thing on the earth lives under oxygen existence, then they are put to a strong oxidative stress. The major cause of cellular oxidative stress is ROS (reactive oxygen species) produced by the mitochondrial electron-transfer system, and therefore, redox regulation and oxidative stress responses are essential for cell survival and homeostasis. Our research deals with molecular mechanisms of redox responses, focusing on mitochondrial biochemical reactions directly linked to 1)cellular signaling pathways to transcriptional control and 2) apoptosis induction. In addition, we also investigate p63, a member of the tumor suppressor p53 family, for stress-response ability and pathophysiological significance of its high-level expression in squamous cell carcinomas.

1. Positive and negative regulation of Wnt target genes by p63 (TP63)

p63 (TP63, p51) is expressed in the TA and ΔN isoforms and plays transcription regulatory functions in various mechanisms. p63 RNA silencing in squamous cell carcinoma cell lines suggested that some of the Wnt target genes including MMP7 are suppressed, but other genes including SNAI2 (SLUG) are activated by p63. Luc reporter gene expression analyses with pGL3-OT and pGL3-OF indicated that only ΔNp63α, among different p63 isoforms, when transfected with TCF-4 and b-catenin, strongly activate the promoter dependent on the Wnt response element (WRE). Furthermore, ΔNp63α was co-immunoprecipitated with flag-TCF-4, but not with flag-β-catenin, from DNA-free soluble nuclear extracts. To analyze mutual interaction between p63 and TCF/β-catenin on the DNA, chromatin immunoprecipitation (ChIP) with anti-p63, anti-TCF-4 and anti-b-catenin antibodies were carried out in p63-knockdown and the control cells. Although we failed to detect association of p63 with WREs of MMP7, β-catenin-WRE binding was enhanced by p63-silencing. This implies that transcription of MMP7 was repressed by the ΔNp63α-TCF/LEF interaction. In contrast, multiple p53/p63 consensus sequences exist close to WREs of SNAI2, to which p63-binding was detected. In this context, β-catenin-WRE binding was not enhanced by p63-silencing. These results indicate that p63 can positively and negatively control WRE target genes depending on the cis arrangement of p53/p63-elements and WREs in the enhancer region.

2. A Detection of the import of procaspase-9 into the mitochondrial intermembrane space

The intermembrane space (IMS) of mitochondria (MT) was found to contain plenty of cytosolic proteins by recent proteomic studies. We previously detected procaspase-9 (procasp-9), an initiator of apoptosis, in IMS to suggest an apoptosis-inducing mechanism involving caspase-9 activation in IMS. The protein importing mechanism across the mitochondrial outer membrane remains obscure in mammals, while disulfide (S-S)-mediated IMS import mechanism with Tom40 and Mia40 has recently been proposed for MT of Saccharomyces cerevisiae. In this study, we *in vitro* assessed IMS import of procasp-9 using MT obtained from HeLa cells. Either bacterially expressed or *in vitro*-synthesized Procasp-9-Flag protein was incubated with purified MT. After sedimentation, proteins outside the MT were digested with proteinase K. Import of Procasp-9-Flag was evident in normal MT, but not in CCCP-treated (Δψm-disrupted) MT. The protein import was significantly facilitated by glutathione. Furthermore, the Procasp-9-Flag import was blocked by RNA interference of *CHCHD4* encoding Mia40. Interestingly, the Procasp-9 was found to have a twin CX_8C motif corresponding to the mitochondria IMS-sorting signal. These results imply that procaspase-9 is imported by the mechanism with Mia40, the central component of the protein import and assembly machinery of mitochondrial IMS.

Publications

1. Katoh I and Kurata S. Association of endogenous retroviruses and long terminal repeats with human disorders. Frontiers in oncology 11(3) 234-237 2015

Frontier Research Unit
Laboratory of Gene Expression

Associate Professor Hidehito KUROYANAGI
Project Assistant Professor Yumiko YAMASAKI-KATO (June, 2014-)

Based on recent transcriptome analysis, >90% of human multi-exon genes produce multiple mRNA isoforms. Regulation of the splice site choice through so called "splicing codes" provide a versatile mechanism for controlling gene expression and for generation of the proteome diversity. We are trying to decipher the splicing codes in living organisms.

1. A Fluorescence Reporter System Offers a Path to Alternative Splicing Codes *in Vivo*.

We have recently developed a transgenic fluorescence alternative splicing reporter system that enables visualization of alternative pre-mRNA processing patterns *in vivo* (Nat Meth, 2006; Nat Protoc, 2010). With this reporter system, we have visualized spatiotemporal profiles of tissue-specific and/or developmentally regulated alternative splicing events in living nematode worms C. *elegans*. By isolating and analyzing mutant worms defective in the color profiles, we have identified *trans*-acting factors and *cis*-elements involved in the splicing regulation (Mol Cell Biol, 2007; Genes Dev, 2008; PLoS Genet, 2012; PLoS Genet, 2013). We have solved solution structure of two RNA-binding proteins cooperatively recognizing their target RNA stretch by sandwiching a hydrophobic guanine base (Figure) (Nat Struct Mol Biol, 2014). Through these studies, we now realize that molecular mechanisms of the alternative splicing regulation are conserved throughout metazoan evolution.

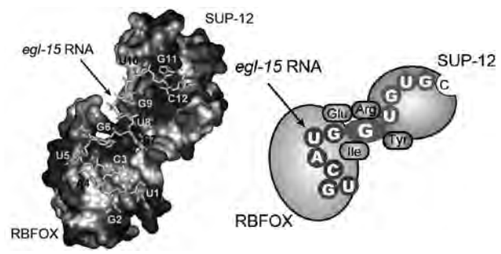


Figure Solution structure of ASD-1 and SUP-12 RRM domains cooperatively recognizing the *egl-15* pre-mRNA.

2. Regulation Mechanisms of Cardiac Muscle-Specific Alternative Splicing of the *TTN* Gene in Vertebrates.

Dilated cardiomyopathy (DCM) is caused by mutations in sarcomere protein genes including *TTN*. Titin, encoded by the *TTN* gene, is a huge protein; passive tension of myofibers is mainly attributed to the titin protein. The *TTN* gene consists of 363 exons and its pre-mRNA splicing patterns and apparent molecular weight of the titin proteins are developmentally regulated and vary between cardiac muscles and skeletal muscles. In DCM models, the ratio of the titin protein isoforms are affected, suggesting correlation between the titin isoform change and DCM pathology. We constructed a fluorescence reporter minigene to successfully visualize the heart-specific splicing regulation of the *TTN* gene. By utilizing this reporter, we found that some point mutations in an RNA-binding protein RBM20 disrupt its function as a splicing factor for the heart-type *TTN* pre-mRNA splicing.

Publications

1. Kuwasako K, Takahashi M, Unzai S, Tsuda K, Yoshikawa S, He F, Kobayashi N, Güntert P, Shirouzu M, Ito T, Tanaka A, Yokoyama S, Hagiwara M, Kuroyanagi H, Muto Y. RBFOX and SUP-12 sandwich a G base to cooperatively regulate tissue-specific splicing. **Nature Structural & Molecular Biology**. 21, 778–786, 2014.
2. Kuroyanagi H, Takei S, Suzuki Y. Comprehensive analysis of mutually exclusive alternative splicing in *C. elegans*. **Worm**. 3: e28459, 2014.

Symposium Presentations

1. Hidehito Kuroyanagi. Cooperative regulation of tissue-specific alternative splicing by multiple splicing factors determines ligand-binding specificity of FGF receptors. The 9th International Symposium of the Institute Network, Osaka University, Suita, Osaka, June, 2014.
2. Hidehito Kuroyanagi. RBFOX and SUP-12 cooperatively regulate muscle-specific alternative splicing to determine ligand-binding specificity of FGF receptors in *C. elegans*. RIKEN Symposium "Noncoding RNA regulation", RIKEN, Wako, Saitama, October, 2014.

Project Research Unit
Affiliated Institutes

Project Research Unit

Pathophysiology

Associate Professor　Tokio Yamaguchi

Summary : Bilirubin, an efficient antioxidant, is shown to scavenge reactive oxygen species (ROS) produced by oxidative stress in vivo. We ndicated that psychological stress contributed to the oxidative conditions, and the oxidative conditions, and the subsequent increase of the urinary concentration of biopyrrins provoked by the reaction of bilirubin with ROS, and that biopyrrins could be useful marker of psychologicalstress.

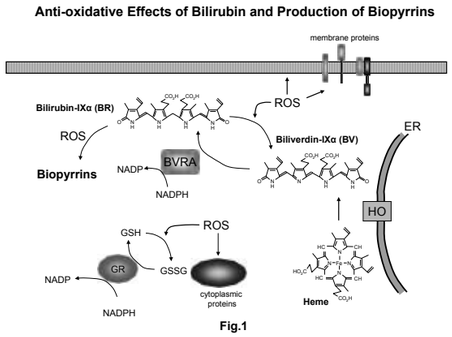


Fig.1

Research projects

1. Induction of heme metabolic enzyme-systems and production of reactive oxygen species provoked by oxidative stress (pathophysiological significance of bilirubin as an antioxidant)
2. Development of the stress-checker using biopyrrins (oxidative metabolites of bilirubin) as a stress marker by the immuno-chromato assay.

Publications

1. Determination of the epitope of anti-bilirubin monoclonal antibody 24G7 by kinetic analysis. Takuya Iwabuchi, Makoto Suematsu, Akiko Sugimoto, Tokio Yamaguchi. In submission (**Biochem Biophys Res Commun**)
2. Effects of lifestyle factors on urinary oxidative stress and serum antioxidant markers in pregnant Japanese women : A cohort study. Matsuzaki M., Haruna M., Ota E., Murayama R., Yamaguchi T., Shioji I., Sasaki S., Yamaguchi T., Murashima S. **BioScience Trends.** 2014. 8(3), 176-184.
3. Complex of branched cyclodextrin and lidocaine prolonged the duration of peripheral nerve block. **Journal of Anesthesia** 2009. 23, 295-297. Suzuki R., Arai YCP., Hamayasu K., Fujita K., Hara K., Yamaguchi T., Sasaguri S.
4. Monitoring of urinary biopyrrins after rat cardiac transplantation. **Journal of Surgical Research** 2009. 151(2), 266. Maeda H., Yamamoto M., Radhakrishnan G., Nakagawa A., Yamamoto M., Okada H., Yamaguchi T., Sasaguri S.
5. Biphasic elevation of bilirubin oxidation during myocardinal ischemia reperfusion. **Circulation Journal** 2008. 72(9), 1520-1527. Yamamoto. M., Maeda H., Hirose N., Yamamoto M., Nakagawa A., Radhakrishnan G., Nakagawa A., Yamamoto M., Okada H., Yamaguchi T., Sasaguri S.

Medical Genomics

Associate Professor　Michinori Kubota

Neural activities to frequency-modulated (FM) sounds with different FM sweep rates were investigated in the primary auditory cortex using optical imaging with a voltage-sensitive dye (RH795). FM sounds were applied at different sweep rates (0.5-16.5 kHz in 16-400 ms duration). Peak amplitudes to a 0.5 kHz tone in the 0.5 kHz frequency band (FB) did not change much with respect to the duration. Those in the other FBs also showed the similar function of duration with smaller amplitudes. Peak amplitudes to the FM sounds in the 8 and 16 kHz bands showed a function of duration with the maximal amplitude at 16 ms duration and decreased beyond 64 ms one. This relation of peak amplitudes to duration tended to be salient in the dorsal regions of FBs but not in the ventral ones, nor in the lower FBs.

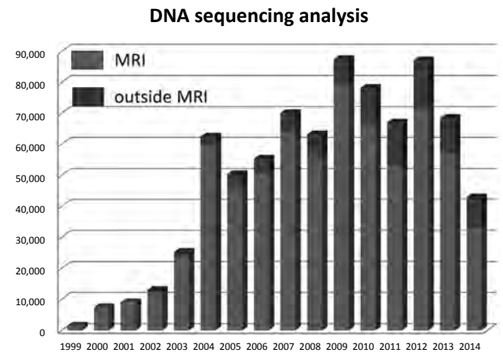
Publications

Hosokawa Y, Kubota M, Sugimoto S, Horikawa J. Neural activities to frequency-modulated sounds in the frequency bands of the primary auditory cortex of guinea-pigs observed by optical recording. J Physiol Sci, Vol. 64, Suppl. 1, S250 (2014).

Advanced Technology Laboratories

Genome Laboratory

This laboratory is established to support the education and research in the Medical Research Institute and Graduate School of Biomedical Science. Sequencing analyses requested by researchers belonging to the Medical Research Institute and those to the other Departments of Tokyo Medical and Dental University are done in this laboratory. In addition, several equipment including Flow Cytometer and Plate Reader are under management of the laboratory. Followings are the achievements in 2014.



1. Sequencing analyses

A total of 42,909 samples from 2,985 researchers were sequenced in the year of 2014. Among them 10,689 (24.5%) samples were requested by researchers outside the medical Research Institute (see below). Analysis by using next generation sequencing equipment (Ion Torrent PGM) has been started in 2013 and 35 runs were done.

2. Equipment under the management of the Genome Laboratory.

DNA sequencer (ABI3130xl) × 2, Next generation sequencer (Ion Torrent PGM), PCR machine (ABI7900) × 5, Flow Cytometer, Luminescent Plate Reader, Ice Machine, Centrifuge, Ion One Touch system, DNA/RNA shearing system, and others.

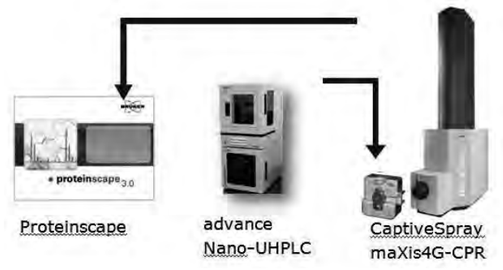
3. Introductory seminars

Introductory seminars were done for use of instruments (3 times).

Laboratory of Cytometry and Proteome Research

This laboratory will help researchers to separate specific cells and analyze the proteome expressed in the cells. we set up equipments written below.

For proteome analysis, we have a 2D-electrophoretic system, LC-MSMS system and Biacore system in this laboratory. We can accept the consignment analysis of proteins with the two dimension electrophoresis and the mass spectrometry by request of researchers of this university. In addition, we can give technical advices on cytometry and proteome researches to young scientists who wish to start own research.



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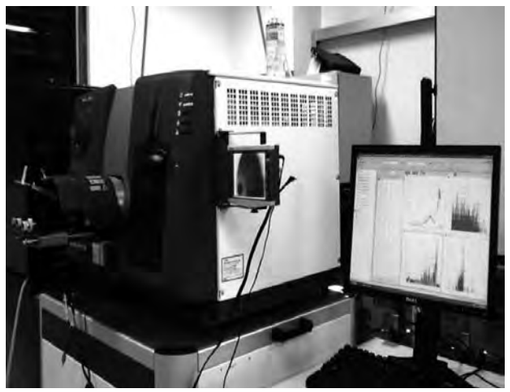
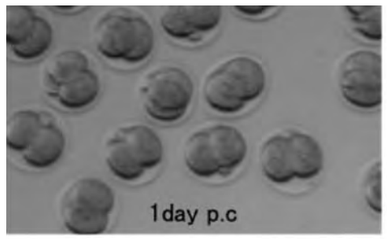


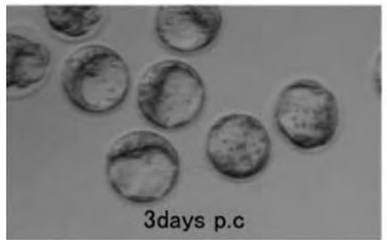
Qtrap5500 ABSCIEX

Laboratory of Recombinant Animals

Recombinant animals including transgenic, knock-out and knock-in mice are now essential for the medical and biological studies and education in graduate schools. Those animals are also essential for the research on the pathogenesis and pathophysiology of intractable diseases, and development of new treatments for these diseases. In the Recombinant Animal Laboratory, excellent technical staffs generate, clean up, maintain and store recombinant mice to facilitate the biomedical research in Medical Research Institute.

Medical Research Institute runs this laboratory as a part of the core facility. The committee of this laboratory composed of faculty members of Medical Research Institute regulates the laboratory by educating new users, and controling generation and introduction of new recombinant mice.





Laboratory of Anatomy and Cell Function

Laboratory of anatomy and cell function (LACF) has a support system for histological technologies at Medical Research Institute (MRI). We equip basic technical instruments and materials for analyzing research of cells and tissue available for common use. LACF provides technological assist for all research laboratories at MRI as shown in the following list and facilities for study.

<Common equipment>

- Confocal laser microscope
- Fluorescence microscope
- Cryostat
- Rotary microtome
- Spin-tissue-processor
- Tissue-embedding-station
- Real-time PCR
- Laser microdisection
- X-ray System

Bioresource Laboratory

Bioresource Lab. of Medical Research Institute was established in 2004, to support researchers and help postgraduates in cell culture. The center safely supply domestically or internationally well-recognized cultured cell lines as research materials. All of the cell lines handled are collected after exchanging MTA with original developers. EB-virus transformed cell lines are also established with B-lymphocytes from patients with intractable diseases including genomic disorders after written informed consent from each of the patients or their parents and also with approval of the Internal Review Board on ethical issues.

Laboratory of Structural Biology

The Laboratory of Structural Biology is a small member of the Facilities and equipped with a high-brilliancy X-ray generator and an image plate X-ray detector. The Laboratory is also equipped with a dynamic light scattering (DLS) instrument, enabling the measurements of the particle size (thus oligomeric state and aggregation) of protein molecules. An introductory practical course was held for students of Graduate School. The laboratory joined the Joint Usage/Research Program of the Institute this year and is now open for users from the outside of the university.

Stem Cell Laboratory

In order to assist researchers working on stem cell-related subjects across Medical Research Institute, Stem Cell Laboratory was established as of December, 2009. Now the Laboratory is equipped with basic and state-of-the-art research facilities. For instance, we have high-speed cell sorters (MoFlo Legacy and MoFlo XDP), time-lapse confocal laser scanning microscope, sonicator, and hybridization oven.

This Laboratory is managed by the Operating Committee composed of five Professors and three Associate Professors in the Institute, and the services are provided by one Technical Staff and one Technical Assistant.

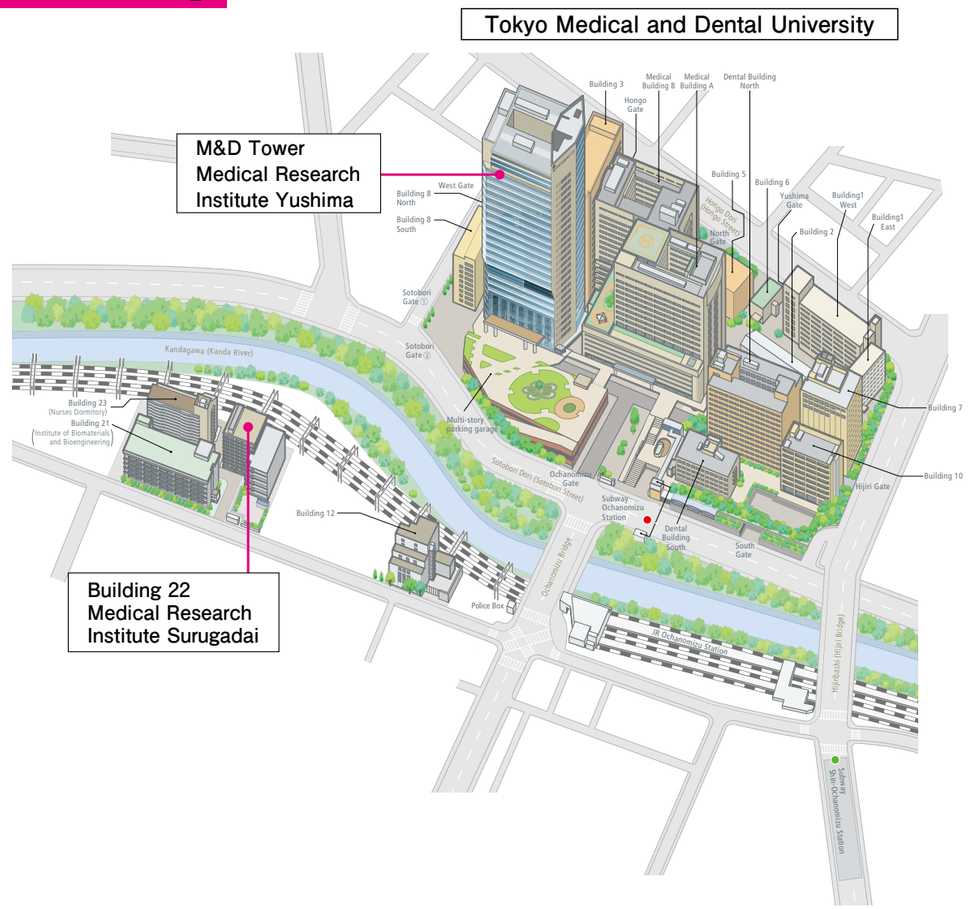
From August 1, 2013, the use of the equipment and services is opened newly to researchers in other departments within the University and those outside. Moreover, sorting service has started. The number of users is increasing gradually.

Throughout 2014, the number of overall use cases was 516, which is 188% of that in 2013. We held 6 short courses for beginners to help them use the equipment.

Advisory Committee Members

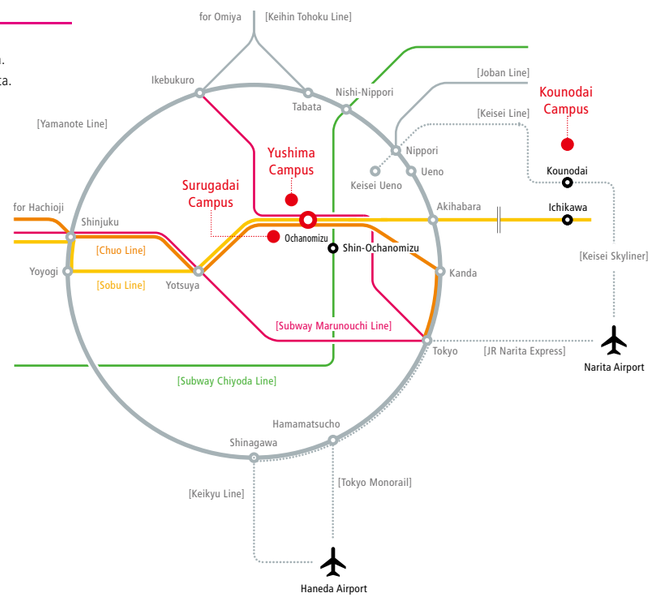
GO Mitiko	External Executive Director Research Organization of Information and Systems
SASAZUKI Takehiko	University Professor Kyushu University
TANAKA Takaharu	President Hoshi University
TANIGUCHI Masaru	Director RIKEN Center for Integrative Medical Sciences
NAGAI Ryozo	President Jichi Medical University
NAKAGAMA Hitoshi	Director National Cancer Center Research Institute
NAGANO Tetsuo	Director Pharmaceuticals and Medical Devices Agency
NISHIKAWA Shin-ichi	Advisor JT Biohistory Research Hall

Access Map



Nearest Stations

- JR Line Ochanomizu Sta.
- Subway Marunouchi Line Ochanomizu Sta.
- Subway Chiyoda Line Shin-Ochanomizu Sta.



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