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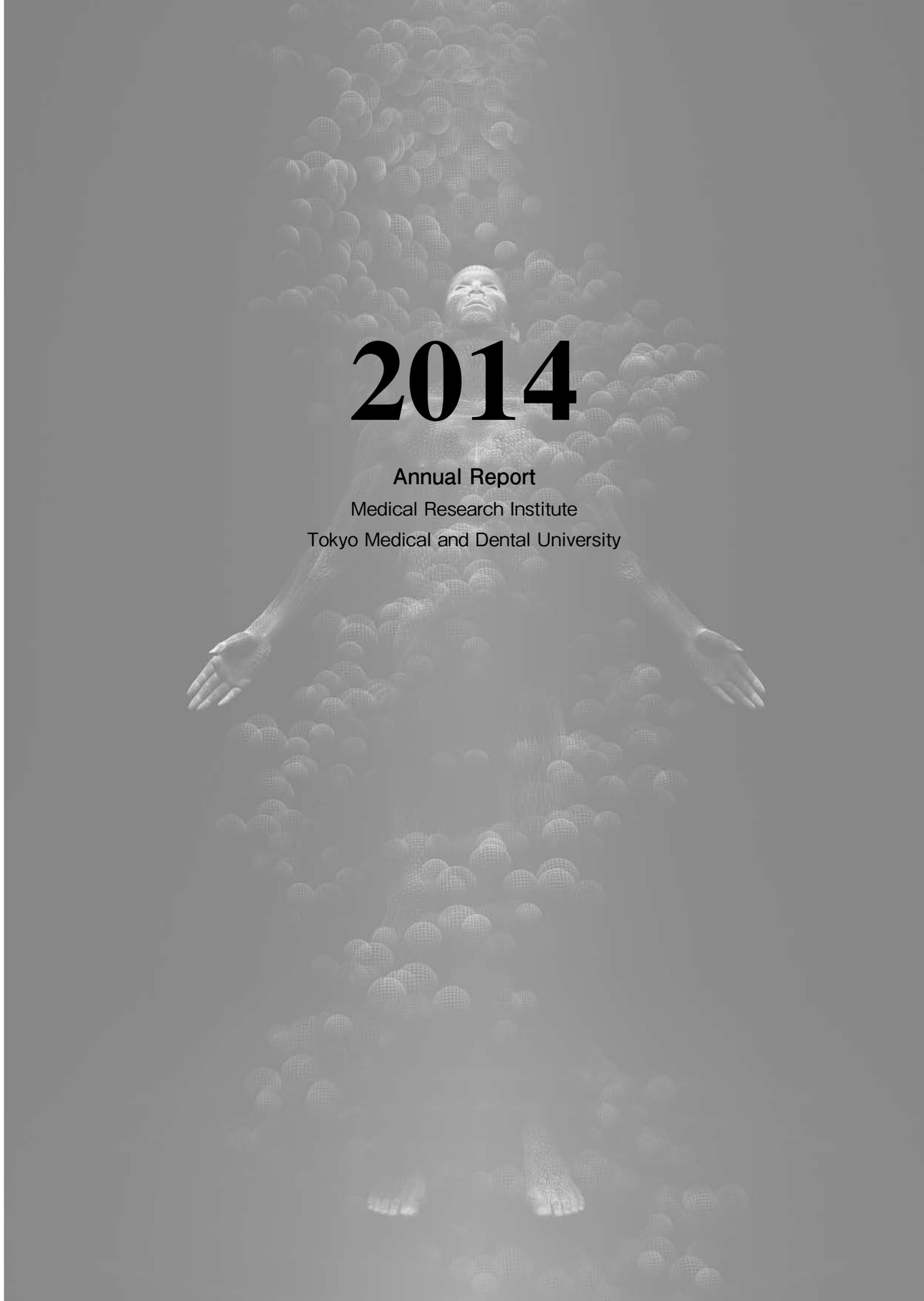
ANNUAL REPORT 2014

Tokyo Medical and Dental University

Medical Research Institute / Tokyo Medical and Dental University

Annual Report 2014



2014

Annual Report

Medical Research Institute

Tokyo Medical and Dental University

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Yushima Area

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

Medical Research Institute
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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
	Department of Molecular Neuroscience	Professor	Kohichi Tanaka
	Department of Biodefense Research	Professor	Toshiaki Ohteki
	Department of Bio-informational Pharmacology	Professor	Tetsushi Furukawa
	Department of Stem Cell Regulation	Professor	Tetsuya Taga
	Department of Structual Biology	Professor	Nobutoshi Ito
	Frontier Research Unit Laboratory of Oxygen Biology		
	Tenure Track Research Unit Department of Cellular and Molecular Medicine		
Division of Pathophysiology	Department of Neuropathology	Professor	Hitoshi Okazawa
	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
	Frontier Research Unit Virus Research Unit		
	Project Research Unit		
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	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	Professor	Hiroshi Tanaka
	Frontier Research Unit Redox Response Cell Biology		
	Frontier Research Unit Laboratory of Gene Expression		
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Laboratory for Integrated Research Projects on Intractable Diseases			
Division of Integrative Research	System Biology for Intractable Diseases	Visiting Professor	Satoru Miyano
Advanced Technology Laboratory	Genome Laboratory		
	Laboratory of Cytometry and Proteome Research		
	Laboratory of Recombinant Animals		
	Laboratory of Anatomy and Cell Function		
	Bioresource Laboratory		
	Laboratory for Structure Analysis		
	Information Support Office		
	Stem Cell Laboratory		
Administrative Office	General Affairs Section		

Highlight

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

– A new avenue for vaccine development and autoimmune disease treatment –

Onai, N. et al., A clonogenic progenitor with prominent plasmacytoid dendritic cell developmental potential. ***Immunity*** 38, 943-57 (2013).

Our research group, led by Prof. Toshiaki Ohteki at the Department of Biodefense Research, Medical Research Institute, has discovered a novel source of dendritic cells (DCs), considered the "control tower" of the immune system. DCs consist of conventional DCs (cDCs) and plasmacytoid DCs (pDCs), both of which play critical regulatory roles in the immune system. In particular, pDCs are characterized by their capacity to produce large amounts of type I interferons (IFNs). This function could be important in medical applications for treating viral infections and autoimmune diseases. In 2007, in collaboration with a research group in Switzerland, we identified progenitor cells committed to the DC lineage for the first time. However, these progenitors gave rise to many more cDCs than pDCs, implying that there must be another unidentified type of DC progenitor that serves as a major source of pDCs.

We focused on finding progenitor cells that serve as a major source of pDCs and that were closely related to the previously identified ones. After a long search, we identified a new type of DC progenitors with prominent pDC developmental potential. The number of pDCs generated from each of the new DC progenitor cells is several times higher than that from the previously reported DC progenitor. Importantly, the new DC progenitor highly expresses E2-2, an essential transcription factor for pDC development, whereas its Id2 expression, critical for cDC development, is relatively low. In addition, this progenitor never gives rise to cells outside the DC lineage. Since both the previous and newly identified DC progenitors strictly give rise to DCs, we designate them together by the term, common DC progenitors (CDPs) (Fig. 1). DCs have recently received much attention as a potential target for vaccine development against infectious diseases and cancer. The identification of DC progenitors that produce 500-1,000 DCs and no other hematopoietic cells may be valuable in the development of therapeutic applications for infectious diseases, cancers, and autoimmune diseases.

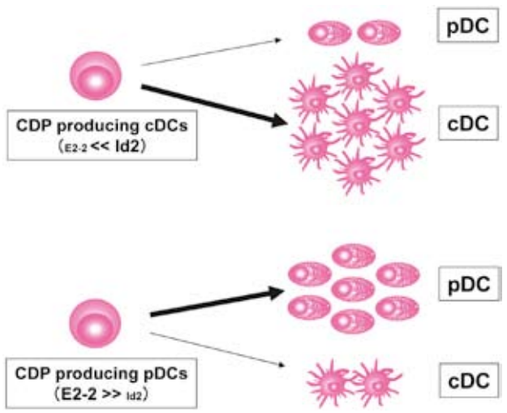


Fig. 1 CDPs are divided into two subpopulations.

Discovery of a novel function of dendritic cells to fine-tune excessive immune responses

– A new avenue of treatment for infectious and autoimmune disease –

Ohyagi, H., Onai, N. et al., Monocyte-derived dendritic cells perform hemophagocytosis to fine-tune excessive immune responses. ***Immunity*** 39, 584-98 (2013).

Our research group, led by Prof. Toshiaki Ohteki at the Department of Biodefense Research, Medical Research Institute, in collaboration with Prof. Kenichi Sawada of Akita University Graduate School of Medicine, has discovered a novel function of dendritic cells (DCs) for fine-tuning excessive immune responses *in vivo*. In addition to conventional DCs (cDCs) and plasmacytoid DCs (pDCs), DCs may also be derived from inflammatory monocytes (monocyte-derived DCs, Mo-DCs), especially under inflammatory conditions. The present study demonstrated that, during severe viral infections, Mo-DCs engulfed apoptotic erythroid cells in a process called hemophagocytosis, which is a characteristic of hemophagocytic syndrome (HPS). Importantly, hemophagocytosis was required for Mo-DCs to produce interleukin-10 (IL-10), an important immunoregulatory cytokine, thereby fine-tuning the immune responses to limit self-damage and ensure the host's survival (Fig. 2).

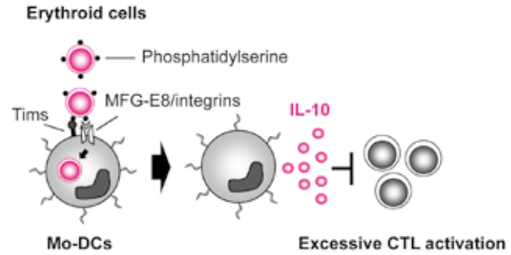


Fig. 2 Hemophagocytosis and its immunological relevance.

Hemophagocytic syndrome (HPS), which is characterized by fever, hepatosplenomegaly, cytopenia, hypertriglyceridemia and/or hypofibrinogenemia, and hemophagocytosis, is a severe, often fatal inflammatory disease. HPS is generally divided into primary and acquired forms. Our research group initially found that high doses of Toll-like receptor (TLR) ligands, such as CpG and poly I:C, when injected into wild-type (WT) mice, produced symptoms of HPS and induced hemophagocytosis, which is typically defined as the engulfment of erythroid cells by Mo-DCs in the blood, spleen, and bone marrow (Fig. 3, left panel). Most viruses that induce HPS in humans establish a chronic infection. Thus, to examine the cellular and molecular events underlying hemophagocytosis, we used the lymphocytic choriomeningitis virus (LCMV) variant clone 13 (C13), which also elicits a chronic infection in mice. As expected, LCMV C13 infection effectively induced hemophagocytosis in WT mice (Fig. 3, right panel). In this context, LCMV infection-induced type I interferons (IFNs) were necessary for both the erythroid cell expression of the apoptosis indicator phosphatidylserine (PS), and the Mo-DC expression of the PS receptors. Importantly, injecting antibodies (Abs) against the Mo-DC PS receptor, which blocks hemophagocytosis, effectively reduced production of the regulatory cytokines, IL-10 and TGF-β1 in LCMV C13-infected WT mice. This finding suggested that the Mo-DC production of IL-10 in response to C13 infection is hemophagocytosis-dependent. To examine the physiological relevance of the hemophagocyte-derived IL-10 in viral infection, *Cd11c-cre Il10*$^{fl/fl}$ (CKO) mice, in which the hemophagocytes cannot produce IL-10, were infected with LCMV C13. Importantly, the CKO mice showed excessive cytotoxic T lymphocyte (CTL) activity, tissue damage, and mortality. Taken together, these results point to hemophagocytosis as a mechanism that ensures the host's survival by preventing excessive immune response-mediated damage (Fig. 2), instead allowing the virus to persist in the host under conditions of severe viral infection.

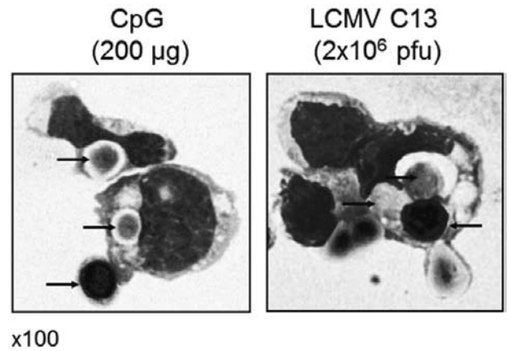


Fig. 3 Hemophagocytosis by Mo-DCs.

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】

- Elucidation of novel role of CNOT3 in regulation of osteoporotic bone mass via mRNA stability.
- Identification of essential role of TRPV4 in mechanical stress-induced intracellular calcium oscillation.
- We found that Nck1 deficiency accelerates unloading-induced bone loss.

【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】

- Both GluRN2B and GluRN2D are involved in the degeneration of retinal ganglion cells induced by excitotoxicity.
- Dock3 prevents glaucomatous retinal degeneration in GLAST KO mice by suppressing the surface expression of both GluRN2B and GluRN2D.
- The lateral habenula lesion shortens REM sleep duration.
- Highly efficient *in vivo* genome editing in mice.

【Biodefense Research】

- Discovery of a novel source of dendritic cells, a control tower of immune system.
  – A new point of view on vaccine development and treatment of autoimmune disease –
- Discovery of a novel function of dendritic cells to fine-tune excessive immune responses.
  – A new avenue of treatment for infectious and autoimmune disease –

【Bio-informational Pharmacology】

- Genome-wide association study (GWAS) identified 8 atrial fibrillation-associated SNP. Algorithm based on these 8 SNP yielded atrial fibrillation-prediction model with 55% sensitivity and 72% specificity.
- Gene mutations and variants confer familiar and common cardiac arrhythmias.
- Human iPS cell-derived cardiomyocytes (hiPSC-CM)-based drug screening system and diseased hiPSC-CM models were established.

【Stem Cell Regulation】

- Sox17 contributes to the maintenance of hematopoietic cell clusters containing HSCs in the midgestation AGM region.
- We proposed a new model for the molecular regulation of neural stem cell self-renewal, in which the stem cell growth promoting signals, like FGF2 and Wnts, interfered with the neuronal and glial differentiation.
- We identified some proteins as the candidates of cancer stem cell niche factors by utilizing a synthetic polymer Pol10.

【Structural Biology】

- The complex structure of a signaling protein with a phosphopeptide was determined.
- Interactions between the Alzheimer's disease-related tau protein and PPIase were investigated.
- Crystal structures of vitamin D receptor in complex with various ligands were also determined.

Department of Molecular Pharmacology

Professor	Masaki Noda, M.D., Ph.D.
Associate Professor	Yoichi Ezura, M.D., Ph.D.
Assistant Professor	Tadayoshi Hayata, Ph.D.
Research Instructor	Takuya Notomi, 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 conducted 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 mechanisms of regulation of the development, differentiation, and function of each group of these cells.

Research Projects

1. Nck1 deficiency accelerates unloading-induced bone loss (Aryal AC, Hayata T, Ezura Y, Noda M).

Mechanical stress is an important signal to determine the levels of bone mass. Unloading-induced osteoporosis is a critical issue in bed-ridden patients and astronauts. Many molecules have been suggested to be involved in sensing mechanical stress in bone, though the mechanisms involved in this phenomenon are not fully understood. Nck1 is an adaptor protein known to mediate signaling from plasma membrane-activated receptors to cytosolic effectors regulating actin cytoskeleton remodeling. Nck1 has also been implicated in cellular responses to endoplasmic reticulum stress. In vitro, in case of cell stress the actin cytoskeleton is disrupted and in such cases Nck1 has been reported to enter the nucleus of the cells to mediate the nuclear actin polymerization. However, the role of Nck1 in vivo during the bone response to mechanical stimuli is unknown. The purpose of this study is to examine the role of Nck1 in unloading-induced bone loss in vivo. Sciatic and femoral nerve resection was conducted. Neurectomy-based unloading enhanced Nck1 gene expression in bone about twofold. Using the Nck1 deficient mice and control Nck1+/+, effects of neurectomy-based unloading on bone structure were examined. Unloading reduced bone volume in wild type mice by 30% whereas the levels in bone loss were exacerbated to 50% in Nck1 deficient mice due to neurectomy after 4 weeks. These data demonstrate that Nck1 gene deficiency accelerates the mechanical unloading-induced bone loss suggesting Nck1 to be a crucial molecule in mechanical stress mediated regulation in bone metabolism (J Cell Physiol, 2013).

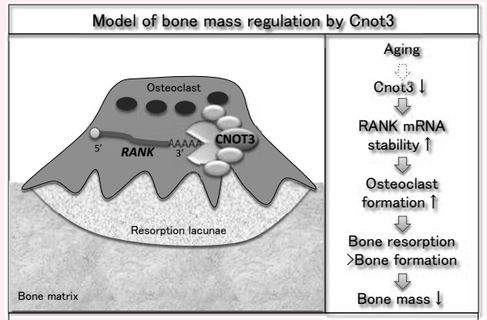
2. Osteoblastic differentiation enhances expression of TRPV4 that is required for calcium oscillation induced by mechanical force (Suzuki T, Hayata T, Ezura Y, Noda M).

Mechanical stress is known to alter bone mass and the loss of force stimuli leads to reduction of bone mass. However, molecules involved in this phenomenon are incompletely understood. As mechanical force would affect signaling events in cells, we focused on a calcium channel, TRPV4 regarding its role in the effects of force stimuli on calcium in osteoblasts. TRPV4 expression levels were enhanced upon differentiation of osteoblasts in culture. We found that BMP-2 treatment enhanced TRPV4 gene expression in a dose dependent manner. BMP-2 effects on TRPV4 expression were suppressed by inhibitors for transcription and new protein synthesis. In these osteoblasts, a TRPV4-selective agonist, 4α-PDD, enhanced calcium signaling and the effects of 4α-PDD were enhanced in differentiated osteoblasts compared to the control cells. Fluid flow, as a mechanical stimulation, induced intracellular calcium oscillation in wild type osteoblasts. In contrast, TRPV4 deficiency suppressed calcium oscillation significantly even when the cells were subjected to fluid flow. These data suggest that TRPV4 is involved in the flow-induced calcium signaling in osteoblasts (Bone, 2013).

Highlight

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

Osteoclastogenesis is under the control of posttranscriptional and transcriptional events. However, posttranscriptional regulation of osteoclastogenesis is incompletely understood. CNOT3 is a component of the CCR4 family that regulates mRNA stability, but its function in bone is not known. Here, we show that Cnot3 deficiency by deletion of a single allele induces osteoporosis. Cnot3 deficiency causes an enhancement in bone resorption in association with an elevation in bone formation, resulting in high-turnover type bone loss. At the cellular level, Cnot3 deficiency enhances receptor activator of NF-κB ligand (RANKL) effects on osteoclastogenesis in a cell-autonomous manner. Conversely, Cnot3 deficiency does not affect osteoblasts directly. Cnot3 deficiency does not alter RANKL expression but enhances receptor activator of NF-κB (RANK) mRNA expression in bone in vivo. Cnot3 deficiency promotes RANK mRNA stability about twofold in bone marrow cells of mice. Cnot3 knockdown also increases RANK mRNA expression in the precursor cell line for osteoclasts. Anti-CNOT3 antibody immunoprecipitates RANK mRNA. Cnot3 deficiency stabilizes luciferase reporter expression linked to the 3'-UTR fragment of RANK mRNA. In contrast, Cnot3 overexpression destabilizes the luciferase reporter linked to RANK 3'-UTR. In aged mice that exhibit severe osteoporosis, Cnot3 expression levels in bone are reduced about threefold in vivo. Surprisingly, Cnot3 deficiency in these aged mice further exacerbates osteoporosis, which also occurs via enhancement of osteoclastic activity. Our results reveal that CNOT3 is a critical regulator of bone mass acting on bone resorption through posttranscriptional down-regulation of RANK mRNA stability, at least in part, even in aging-induced osteoporosis (Proc Natl Acad Sci USA, 2014).



Aging reduces gene expression of Cnot3 in bone. In Cnot3 hetero knockout mice, RANK mRNA stability is increased, osteoclastogenesis is enhanced and bone resorption exceeds bone formation, which results in bone loss.
Our study revealed that Cnot3 is a regulator of aging-induced osteoporosis. We also demonstrated that the presence of Cnot3 is effective on maintenance of bone mass. Hereafter, our results are expected to contribute to development of the novel diagnosis and therapy against osteoporosis.

Significance

Osteoporosis is a highly prevalent disease affecting nearly 20 million people in the United States and is life-threatening in elderly patients. However, underlying pathophysiology regarding the posttranscriptional control of bone resorption is incompletely understood. 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.

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 bone mass via CNOT3. ***Proc Natl Acad Sci U S A*** 111:2692-7, 2014.
2. Komatsu K, Shimada A, Shibata T, Wada S, Ideno H, Nakashima K, Amizuka N, Noda M, Nifuji A. Alendronate promotes bone formation by inhibiting protein prenylation in osteoblasts in rat tooth replantation model. ***J Endocrinol*** 219:145-58, 2013.
3. Suzuki T, Notomi T, Miyajima D, Mizoguchi F, Hayata T, Nakamoto T, Hanyu R, Kamolratanakul P, Mizuno A, Suzuki M, Ezura Y, Izumi Y, Noda M. Osteoblastic differentiation enhances expression of TRPV4 that is required for calcium oscillation induced by mechanical force. ***Bone*** 54:172-8, 2013.
4. Aryal AC, Miyai K, Hayata T, Notomi T, Nakamoto T, Pawson T, Ezura Y, Noda M. Nck1 deficiency accelerates unloading-induced bone loss. ***J Cell Physiol*** 228:1397-403, 2013.

Department of Molecular Cell Biology

Professor Hiroshi Shibuya
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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.

Roles of IQGAP1 on the canonical Wnt signaling.

Wnt signaling plays important roles in multiple developmental events during embryogenesis. Canonical Wnt signaling is initiated by binding of the Wnt ligand to the cell-surface Frizzled and transmembrane LRP complex. This leads to the membrane recruitment and activation of Dishevelled (DVL), which inactivates the APC/Axin/GSK-3 complex in the cytoplasm, responsible for the degradation of β-catenin. As a result, β-catenin accumulates in the cytoplasm, translocates to the nucleus and associates with Tcf transcription factors, which activate the Wnt target genes. DVL plays an additional role in the Wnt signaling pathway, by localizing to the nucleus and binding a complex containing β-catenin and Tcf, which in turn activates Wnt target genes in the nucleus. The subcellular localization of DVL, either on the cell membrane or in the nucleus, is important for understanding its function in Wnt signaling.

To identify novel proteins that may bind to DVL, we performed a high-throughput analysis of proteins that co-immunoprecipitated with human DVL1 in HEK 293 cells using direct nanoflow liquid chromatography-coupled tandem MS (LC-MS/MS). We identified several known DVL-binding proteins, such as CK1, CK2, Strabismus, Par1, Axin and PP2C. In addition, we identified IQGAP1 as a candidate protein that may physically interact with DVL1. IQGAP1 contains multiple protein-interacting domains: the CH (calponin homology) domain binds to F-actin, the WW domain binds to ERK2, the IQ repeat motifs bind to calmodulin and myosin light chain, and the Ras GAP-like domain binds to Cdc42 and Rac1. IQGAP1 is also known to bind to E-cadherin and β-catenin, and is involved in cytoskeltal reorganization and cell adhesion. On the other hand, IQGAP1 stimulates β-catenin-mediated transcriptional activation.

We investigated roles of IQGAP and DVL in the canonical Wnt signaling pathway, and we have already obtained the following results: [1] xIQGAP1, xDVL2 and β-catenin can form a complex, and each protein contributes to the nuclear localization of each other under the Wnt stimulation. [2] Depletion of xIQGAP1 by antisense morpholino oligonucleotides (*xIQGAP1*-MO) reduced expression of Wnt target genes induced by *Xwnt-8*. [3] Importin-β5 and Ran, which directly bind to IQGAP1, contribute to canonical Wnt signaling pathway, playing a role in nuclear localization of DVL and β-catenin.

We performed more analyses to further elucidate the mechanism of nuclear localization of Wnt components with IQGAP1, and obtained the following new results.

1. The expression of xIQGAP1 in HEK 293T cells increased GTP-bound active form of xRan1 in the same way as the effect of xRanGEF.
2. xIQGAP1 bound preferably to GDP-bound inactive form of xRan1 rather than GTP-bound active form.
3. The hydrolysis of the xRAN1 by xRanGAP was reduced by xIQGAP1.
4. The expression of xIQGAP1 inhibited the interaction between active form of xRAN1 and xRanGAP.
5. The GTP-bound form of xRan1 was promoted by xRanGEF, but not by xIQGAP1 *in vitro*.
6. The expression of xIQGAP1 did not inhibit the interaction between inactive form of xRAN1 and xRanGEF.

These results suggest that the direct interaction between xIQGAP1 and xRan1 inhibits xRanGAP function, and is required for nuclear import of DVL, IQGAP1 and β-catenin in Wnt signaling pathway.

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

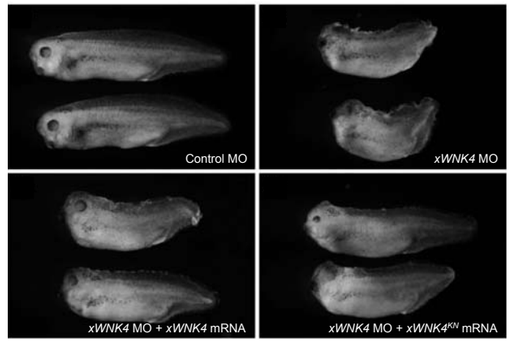
WNK (with no lysine (K)) kinase family that has been recently identified serine/threonin protein kinase family conserved among many species. There are four mammalian WNK family members, and mutations in two of them, WNK1 and WNK4 have been linked to a hereditary form of human hypertension known as Pseudohypoaldosteronism 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.



WNK4 plays an inportant role in anterior formation in *Xenopus*.

Publications

1. Sato, A. and Shibuya, H. (2013). WNK Signaling Is Involved in Neural Development via Lhx8/Awh Expression. **PLoS One** 8, e55301.

2. Shimizu, M., Goto, T., Sato, A. and Shibuya, H. (2013). WNK4 is an essential effector of anterior formation in FGF signaling. **Genes Cells** 8, 442-449.

3. Goto T., Michiue T., Ito Y., Asashima M. (2013). Characterization of CXC-type chemokine molecules in early Xenopus laevis development. **Int. J. Dev. Biol.** 57, 41-47.

4. Goto, T., Sato, A., Shimizu, M., Adachi, S., Satoh, K., Iemura, S, Natsume, T and Shibuya, H. (2013). IQGAP1 Functions as a Modulator of Dishevelled Nuclear Localization in Wnt Signaling. **PLoS One**, 8, e60865.

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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	Ning Bai
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 previously demonstrated that glial glutamate transporter GLAST knockout mice, a model of normal tension glaucoma (NTG), showed retina ganglion cell (RGC) degeneration due to glutamate neurotoxicity. Glaucomatous retinal degeneration in GLAST KO mice was significantly improved by deletion of GluRN2B or GluRN2D. Furthermore, we demonstrate that Dock3, a guanine nucleotide exchange factor, binds to both GluRN2B and GluRN2D and reduce their surface expression, thereby protecting RGCs from excitotoxicity. These findings raise the possibility that GluRN2B, GluRN2D and Dock3 might be potential therapeutic targets for treating neurodegenerative diseases such as NTG and Alzheimer's disease.

2. A novel role of the habenula in regulation of sleep

After we begin to sleep, non-rapid eye movement (REM) sleep dominates first and then, REM sleep follows. Alternation between non-REM and REM sleep lasts several times until we wake up in the morning. Previous studies showed that sleep pattern in the patients with depression is peculiar in that REM sleep appears earlier after the sleep onset with showing more frequent rapid eye movement during REM sleep. It remains, however, unclear how such a characteristic changes in sleep pattern occurs in the patients with depression.

Since REM sleep occurs more frequently in the animals with lesion of the serotonergic neurons, we hypothesize that the lateral habenula, which regulates the serotonergic activity in the brain, may modulate the REM sleep, whose pattern alters in the patients with depression. Thus, present study tries to address whether the lateral habenula is essential in REM sleep and how the lateral habenula acts during sleep.

We focused on the activity called theta rhythm which appears in the hippocampus during REM sleep. Sleep analysis showed that the rats with habenular lesion showed reduced theta rhythm in the hippocampus and shortened REM sleep duration. This inhibitory effect of the lateral habenular lesion on the REM sleep disappeared when the serotonergic neurons in the midbrain were lesioned. Thus, results indicated that the lateral habenula is essential for maintenance of the theta rhythms in the hippocampus associated with REM sleep.

These results indicated that the lateral habenula regulates the maintenance of REM sleep in rat via serotonergic modulation. Current study reveals a novel role of the lateral habenula linking the serotonin, altered metabolism of which is reported in depression, with REM sleep, suggesting that the hyperactivated habenula in the patients with depression may cause altered REM sleep. This possibility will be addressed more directly by future study which examines whether the animals with hyperactivation of the lateral habenula shows depressive behaviors and sleep disturbance with up-regulation of REM sleep.

3. Highly efficient and ultra-rapid in vivo genome editing in mice

The knockin mouse models carrying precisely modified human single nucleotide variants (SNVs) provide a unique and direct opportunity to investigate those functional consequences *in vivo*. The recent success of direct gene targeting in mouse zygotes by genome editing technologies enabled rapid and convenient knockin mouse production without embryonic stem cells or targeting vectors. However, the knockin efficiency is still quite low. We show a nearly 25-fold improvement in the *in vivo* genome editing efficiency in mice with highly active Platinum TALENs and oligo nucleotide donor. Single microinjection is sufficient to produce several germline-competent knockin founders carrying a precisely modified human SNV without off-target modification. Taken together, our Platinum TALENs technology provides a fast and efficient approach to the production of genetic mouse models that reproduce the disease SNVs of complex diseases and brings about drastic developments in the field of genome editing, leading to a boost in functional genomic research.

Publications

[Original papers]

1. Schreiner, AE., Durry, S., Aida, T., Stock, MC., Ruther, U., Tanaka, K., Rose, CR., Kafitz, KW. Laminar and subcellular heterogeneity of GLAST and GLT-1 immunoreactivity in the developing postnatal mouse hippocampus. *J Comp Neurol* 522. 204-224, 2014.
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6. Aizawa H, Yanagihara S, Kobayashi M, Niisato K, Takekawa T, Harukuni R, McHugh TJ, Fukai T, Isomura Y, Okamoto H. The synchronous activity of lateral habenular neurons is essential for regulating hippocampal theta oscillation. *J Neurosci*. 33. 8909-21, 2013.
7. Isomura Y, Takekawa T, Harukuni R, Handa T, Aizawa H, Takada M, Fukai T. Reward-modulated motor information in identified striatum neurons. *J Neurosci*. 33. 10209-20, 2013.
8. Aoki T, Kinoshita M, Aoki R, Agetsuma M, Aizawa H, Yamazaki M, Takahoko M, Amo R, Arata A, Higashijima S-I, Tsuboi T, Okamoto H. Imaging of neural ensemble for the retrieval of a learned behavioral program. *Neuron* 78. 881-894, 2013.

[Reviews]

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2. Aizawa, H., Cui, W., Tanaka, K., Okamoto, H. Hyperactivation of the habenula as a link between depression and sleep disturbance. *Front Hum Neurosci* 7. 826, 2013.
3. Aizawa H. Habenula and the asymmetric development of the vertebrate brain. *Anat Sci Int*. 88. 1-9, 2013.
4. Okamoto H and Aizawa H. Fear and anxiety regulation by conserved affective circuits. *Neuron* 78. 411-413, 2013.

Department of Biodefense Research

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Our research projects focus on understanding the dynamic maintenance and transfiguation 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, 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. Differentiation and function of dendritic cells

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. We have discovered a novel source of dendritic cells (DCs), considered the "control tower" of the immune system (***Immunity*** 38, 943-57 (2013)). DCs consist of conventional DCs (cDCs) and plasmacytoid DCs (pDCs), both of which play critical regulatory roles in the immune system. In particular, pDCs are characterized by their capacity to produce large amounts of type I interferons (IFNs). This function could be important in medical applications for treating viral infections and autoimmune diseases. In 2007, in collaboration with a research group in Switzerland, we identified progenitor cells committed to the DC lineage for the first time. However, these progenitors gave rise to many more cDCs than pDCs, implying that there must be another unidentified type of DC progenitor that serves as a major source of pDCs. This time, we successfully identified a DC progenitor with prominent pDC differentiation potential. The number of pDCs generated from the new DC progenitor cells is several times higher than that from the previously reported DC progenitor. Importantly, the new DC progenitor highly expresses E2-2, an essential transcription factor for pDC development. In addition, this progenitor never gives rise to cells outside the DC lineage. Based on our findings, we designate them together by the term, common DC progenitors (CDPs) (Fig. 1). The identification of DC progenitors that produce 500-1,000 DCs and no other hematopoietic cells may be valuable in the development of therapeutic applications for infectious diseases, cancers, and autoimmune diseases.

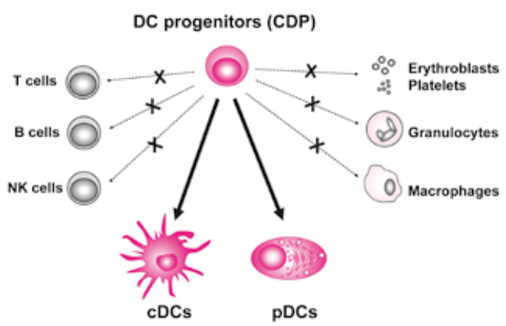

Fig.1 CDP give rise strictly to cDCs and pDCs.

2) Discovery of a novel function of dendritic cells to fine-tune excessive immune responses

An immune response is a double-edged sword that simultaneously defends and injures the host; the more severe the infection, the greater the regulatory control must be. We have discovered a novel function of dendritic cells (DCs) for fine-tuning excessive immune responses *in vivo*. In addition to cDCs and pDCs, DCs may be derived from inflammatory monocytes (monocyte-derived DCs, Mo-DCs), especially under inflammatory conditions. The present study demonstrated that, during severe viral infections, Mo-DCs engulfed apoptotic erythroid cells in a process called hemophagocytosis, which is a characteristic of hemophagocytic syndrome (HPS). Importantly, hemophagocytosis was required for Mo-DCs to produce interleukin-10 (IL-10), an important immunoregulatory cytokine, thereby fine-tuning the immune responses to limit self-damage and ensure the host's survival (***Immunity*** 39, 584-98 (2013)). These results point to hemophagocytosis as a mechamism that ensures the host's survival by preventing excessive immune response-mediated damage, instead allowing the virus to persist in the host under conditions of severe viral infection. In summary, our findings indicate that hemophagocytosis is induced by TLR ligands or viruses in sequential steps (Fig. 2) to suppress potentially damaging immune responses.

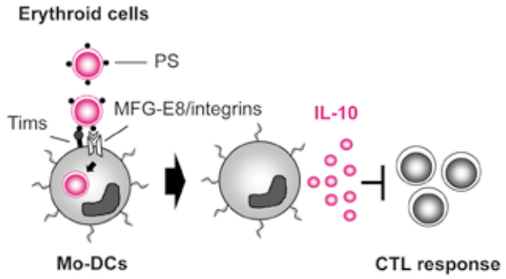

Fig.2 Hemophagocytosis and its immunological relevance.

2. 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*** 15, 696-700 (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*** 121, 3263-73 (2013)).

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2. Ohyagi H, Onai N, Sato T, Yotsumoto S, Liu J, Akiba H, Yagita H, Atarashi K, Honda K, Roers A, Muller W, Kurabayashi K, Hosoi-Amaike M, Takahashi N, Hirokawa M, Matsushima K, Sawada K, and Ohteki T. Monocyte-derived dendritic cells perform hemophagocytosis to fine-tune excessive immune responses. ***Immunity*** 39, 584-98, 2013.
3. Sato T, Kitawaki T, Fujita H, Iwata M, Iyoda T, Inaba K, Ohteki T, Hasegawa S, Kawada K, Sakai Y, Ikeuchi H, Nakase H, Niwa A, Takaori-Kondo A, Kadowaki N. Human CD1c^{+} myeloid dendritic cells acquire a high level of retinoic acid-producing capacity in response to vitamin D3. ***J Immunol*** 191, 3152-60, 2013.
4. Hayashi A, Sato T, Kamada N, Mikami Y, Matsuoka K, Hisamatsu T, Hibi T, Roers S, Yagita H, Ohteki T, Oshimura A, Kanai T. A Single strain of *Clostridium butyricum* induces intestinal IL-10-producing macrophages that suppress acute colitis. ***Cell Host Microbe*** 13, 711-22, 2013.
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7. Ichikawa A, Kuba K, Morita M, Chiba S, Tezuka H, Hara H, Sasaki T, Ohteki T, Ranieri V.M , dos Santos C C, Kawaoka Y, Akira S, Luster A D, Lu B, Penninger J M, Uhlig S , Slutsky A S, and Imai Y. CXCL10-CXCR3 enhances the development of neutrophil-mediated fulminant lung injury of viral and non-viral origin. ***Am J Respir Crit Care Med*** 187, 65-77. 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. Gender-specific medicine (GSM) for cardiovascular diseases

In the past few years, we have shown that non-genomic regulation of cardiac ion channels by sex hormones underlies, at least in part, gender difference in cardiac electrophysiology, and thus susceptibility to arrhythmias. This year, we used FRET imaging and LC/MS technology to show that non-genomic regulation of cardiac ion channels by sex hormones cross-talks with β-adrenergic receptor signaling specifically in the raft micro-domain.

2. 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. CHARGE study found 10 SNPs associated with AF: among them, 6 SNPs were associated with both European/American and Japanese, and 4 with European/American but not with Japanese.

(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, and found a novel pathway generating abnormal automaticity in the pulmonary vein myocardium, which is the main triggering factor of atrial fibrillation.

(3) Risk stratification

Another sales-point of GWAS is the risk stratification of the diseases and its use for future personalized medicine. Based on GWAS data, we calculated AF risk score and classified them into 4 quartile groups. The highest risk group has 5.5 higher risk of AF development relative to the lowest risk group (left panel in Figure 1). The risk stratification yielded around 60% sensitivity and specificity (right panel in Figure 1), which are not enough for personalized medicine, and further studies to increase odds ratio are needed.

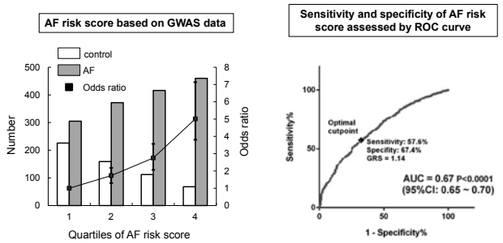

Fig.1 Left panel:AF risk score and odds ration calculated from GWAS data. Right panel:ROC curve yielding sensitivity and specificity of the risk stratification estimated from GWAS data.

3. Pathogenesis of ventricular fibrillation (VF) and sudden cardiac death

Despite extensive effort by many researchers for years, VF remains the main cause of sudden death, and the biggest challenge in arrhythmia research. Last year, we showed that genetic deletion of the His-Purkinje system-specific transcription factor in mice exhibited exercise-related ventricular tachyarrhythmias. This year, we searched for genetic disturbance of this transcription factor in patients with idiopathic VF, and found that the mutations of this factor are responsible for idiopathic VF, and a common variant is a modifier of causative gene mutations for idiopathic VF.

4. Use of iPS cells for arrhythmia research

In the past few years, we have aimed to use human iPS-derived cardiomyocytes (hiPS-CMs) for drug screening. hiPS-CMs include various types of cardiomyocytes, such as atrial, ventricular, and nodal types of cardiomyocytes, and exhibit relatively immature electrophysiological properties of cardiac cells, hindering high-quality drug screening. In order to generate mature ventricular-like hiPS-CMs, we over-expressed a gene into hiPS-CMs. The genetically-altered hiPS-CMs exhibited mature forms of action potentials and drug sensitivity. Our novel technique would be useful for evaluation of drug-induced alternation of repolarization processes in the human cardiomyocytes.

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

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 drugs. This year, we applied to examine cardiac toxicity of anti-cancer drugs.

Publications

[original articles]

1. Asayama M, Kurokawa J, Shirakawa K, Okuyama H, Kagawa T, Okada J, Sugiura S, Hisada T, Furukawa T. Effects of an hERG activator, ICA-105574, on electrophysiological properties of canine hearts. *J. Pharmacol. Sci.* 2013;121:1-8.
2. Kurokawa J, Furukawa T. Non-genomic action of sex steroid hormones and cardiac repolarization. *Biol. Pharm. Bull.* 2013:36:8-12.
3. Furukawa T, Ebana Y. Current overview of genetic background of atrial fibrillation: possible genetically therapeutic targets for the treatment of atrial fibrillation. *J. Arrhyhm.* (in press)
4. Okata S, Yuasa S, Yamane T, Furukawa T, Fukuda K. The generation of induced pluripotent stem cells from a patient with *KCNH2* G603D, without LQT2 disease associated symptom. *J. Med. Dent. Sci.* 2013;60:17-22.
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[Book]

1. Tetsushi Furukawa. Ion Channel Expression and Function of iPSC-derived Cardiomyocytes. In:Cardiac Regeneration using Stem Cells. (eds.) Keiichi Fukuda, Shinsuke Yuasa. CRC Press, 2013.

[Review Articles]

1. Kurokawa J, Furukawa T. Non-genomic action of sex steroid hormones and cardiac repolarization. ***Biol. Pharmacol. Bull***, 2013;36:8-12.

Department of Stem Cell Regulation

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Research Student	Kazuo TERASHIMA

Research Outline

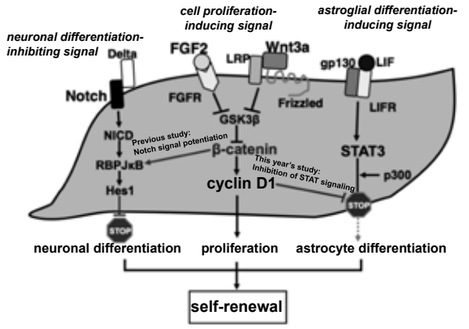
Our research has been conducted to elucidate the mechanisms by which stem cells are regulated. The major focus has been on neural stem cells, hematopoietic stem cells, and cancer stem cells. The study is aimed to understand development, maintenance, and regeneration of the central nervous system and 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. Studies on molecular mechanisms that regulate self-renewal and differentiation of neural stem cells

Neural stem cells (NSCs) are self-renewing multipotent cells that generate neurons, astrocytes and oligodendrocytes. Their fate decisions deeply involve cell-external signals, such as growth/differentiation factor and extracellular matrices, and cell-intrinsic programs based on the activities of transcription factors and epigenetic modifications. Self-renewal of NSCs is an essential event to expand the NSC pool, and should be built on the tight linkage between the promotion of cell proliferation and the inhibition of neuronal and glial differentiations. However, its molecular basis has remained unclear. We have previously demonstrated that fibroblast growth factor 2 (FGF2) and Wnt signals cooperate to promote NSC self-renewal through nuclear accumulation of β-catenin that promotes proliferation by LEF/TCF-mediated cyclin D1 expression (central part in Figure) on one hand, and on the other, inhibits neuronal differentiation by potentiation of Notch signal activity (left part in Figure). In this year, we have newly reported that the cylcin D1 protein whose expression is up-regulated by FGF2 and Wnt inhibits the interaction between p300 and STAT3 and reduces the promoter activity of the astroglial specific gene *GFAP* (right part in Figure). Thus, we have newly proposed a new model that explains how the growth promoting signals, like FGF2 and Wnts, inhibit the neuronal and glial differentiation to help NSCs self-renewal.

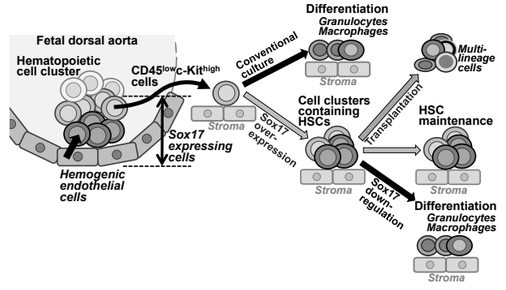
Signal Interaction Supporting Neural Stem Cell Self-Renewal



Self-renewal of NSCs *in vivo* involves the signals from their microenvironment (niche). However, the overall picture of the niche has not yet been uncovered. To elucidate the molecular basis of the neural stem cell niche, we have been conducting a new approach with synthetic polymer arrays. In collaboration with the University of Edinburgh, we have tested 400 different synthetic polymers for their ability to maintain NSC properties, and successfully identified hit polymers. We have further analyzed the mechanisms of action of these polymers, and detected several candidate proteins that bind specifically to the polymers.

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

In the aorta-gonad-mesonephros (AGM) region of midgestation mouse embryo, hematopoietic cell clusters containing hematopoietic stem/progenitor cells appear inside the dorsal aorta. Sry-related high mobility group box (Sox) 17 is a transcriptional factor known as a marker of endodermal cells. It was recently reported that Sox17 conditional knockout mice displayed a decrease in the number of hematopoietic stem cells in the fetal and neonatal, but not adult mice. In the present study, we examined the function of Sox17 in the hematopoiesis of the AGM region. Sox17 was expressed in endothelial cells and the hematopoietic cell clusters in the dorsal aorta. Overexpression of the Sox17 protein in CD45^{low}c-Kit^{high} AGM cells, which are a component of hematopoietic cell clusters, followed by the coculture with stromal cells, led to the formation and long-term passages of cell clusters with the hematopoietic activity in vitro (Figure). We transplanted these Sox17-overexpressing cells to irradiated mice. After 4 months of transplantation, myeloid cells in peripheral blood (PB), bone marrow (BM) and spleen (SP), erythroid cells in PB, BM and SP, and T-lymphocytes in thymus were repopulated, but repopulation of B-lymphocytes in PB, BM and SP was very faint. Based on these results, we conclude that the Sox17-overexpressing cells have the long-term repopulating ability in vivo, with less differentiation preference into B-lymphocytes. In such Sox17-overexpressing cell-transplanted mice, common myeloid progenitors (CMPs) were abundantly observed in BM. The data raises a possibility of oncogenic function of Sox17 when its expression is sustained.

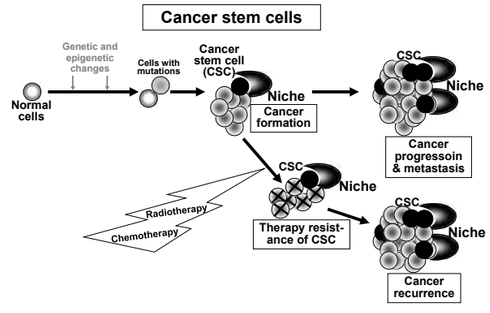


3. Characterization of cancer stem cells and their niche

"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 preferentially supports the proliferation of SP over MP cells, suggesting Pol10 mimics the native niche. SP cells incubated on Pol10 showed dramatically higher tumorigenic activity 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 several candidates of niche factors for CSCs.

This 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. Kusunoki S, Kato K, Tabu K, Inagaki T, Okabe H, Kaneda H, Suga S, Terao Y, Taga T and Takeda S: The inhibitory effect of salinomycin on the proliferation, migration and invasion of human endometrial cancer stem-like cells. Gynecol. Oncol., 129: 598-605, 2013.
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[Review and Book]

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Department of Structural Biology

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Assistant Professor	**Nobutaka Numoto**
Technical Assistant	**Michiko Hattori, Marina Ohno**
Postgraduate Student	**Kenrou Shinagawa, Michika Miyashita**

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. It has been reported that CD100, which belongs to the semaphorin family, interacts with CD72, whereas some carbohydrate molecules are also 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 *E. coli* and but severe aggregation of CD72-CTLD prevented us from further purification. After much effort, including testing a number of constructs and point mutants to overcome the aggregation, we have succeeded in purification of soluble CD72-CTLD. We also tried to overexpress fused CD72-CTLD with a highly soluble tag-protein. The fusion CD72-CTLD was successfully harvested in the soluble fraction and then tag-protein was cleaved by the protease. Both the refolding and tag-cleaved CD72-CTLD was suitable for crystallization experiments. We have obtained some crystals of CD72-CTLD (Fig. 1) after crystallization trials under hundreds conditions. X-ray diffraction experiments were performed at synchrotron radiation facility and the crystal structure analysis is in progress.

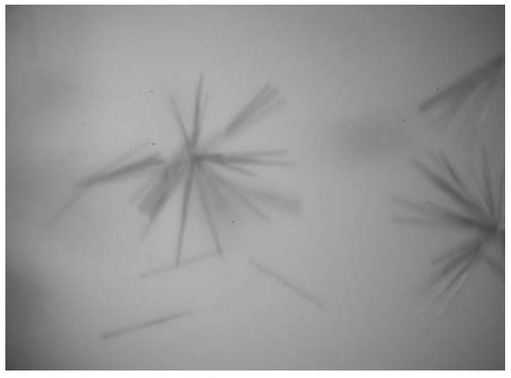


Fig.1 Crystals of CD72-CTLD

2. Crystal structure analyses of oxygen-binding intermediates of the giant hemoglobin

Since there are no well-accepted blood substitutes, many approaches have been experimented to develop an oxygen-carrying blood substitute, including cross-linking, polymerization, and encapsulation of hemoglobin (Hb). On the other hand, short storage lifetime of the blood for transfusion due to autoxidation of Hb is also remaining problem needed to be overcome.

Extracellular giant Hbs occur in some annelids (invertebrate). One of those giant Hbs is composed of 24 subunits, which is 6 times more than that of human Hb, and the molecular mass is about 400 kDa (Fig. 2). This 24meric giant Hb shows strong resistance to autoxidation. Therefore, molecular architecture, detailed mechanisms of oxygen-transporting, and resistance to autoxidation of the giant Hbs can provide useful information for developing blood substitutes.

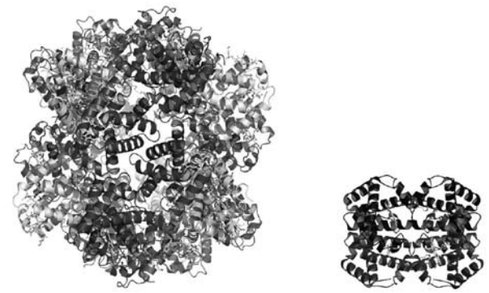


Fig.2 Structures of the giant (left) and human (right) hemoglobins

We have determined the crystal structures of the giant Hb in both oxygenated (oxy) and deoxygenated (deoxy) forms (Fig. 3). In this process, we found that the crystals of the oxy form could be transformed to the deoxy form without disrupting the crystals and are now trying to obtain crystals in which Hb forms various intermediate states between fully oxygenated and deoxygenated states. No intermediate structures have ever been reported for any vertebrate and invertebrate Hbs to date. Thus these intermediate structures would provide much for understanding fundamental processes of the cooperative oxygen-binding of all Hbs. The diffraction data of several intermediate states have already been obtained, and the preliminary structural analyses indicate some particular regions are prone to structural changes between the oxy and deoxy states.

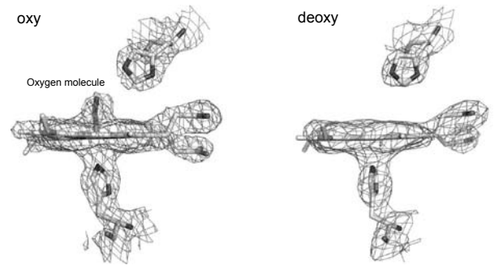


Fig.3 Models and electron densities around the oxygen binding pockets of the giant hemoglobin

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, contributes to the continuous effort by wwPDB to improve PDB.

Publications

[Research Papers]

1. Masuno† H, Ikura† T, Morizono D, Orita I, Yamada S, Shimizu M, Ito N: Crystal structures of complexes of vitamin D receptor ligand-binding domain with lithocholic acid derivatives. J Lipid Res 54: 2206-2213, 2013. (†These authors contributed equally to this work.)

2. Ikura T, Ito N: The peptidyl-prolyl isomerase activity of FK506 binding protein 12 prevents tau peptide from aggregating. Protein Eng Des Sel, 26: 539-546, 2013.

3. Nakabayashi M, Tsukahara Y, Iwasaki-Miyamoto Y, Mihori-Shimazaki M, Yamada S, Inaba S, Oda M, Shimizu M, Makishima M, Tokiwa H, Ikura T, Ito N: Crystal structures of hereditary vitamin D-resistant rickets-associated vitamin D receptor mutants R270L and W282R bound to 1,25-dihydroxyvitamin D3 and synthetic ligands. J Med Chem, 56: 6745-6760, 2013.

4. Higo K, Ikura T, Oda M, Morii H, Takahashi J, Abe R, Ito N: High resolution crystal structure of the Grb2 SH2 domain with a phosphopeptide derived from CD28. Plos One, 8, e74482: 1-6, 2013.

5. Numoto N, Shimizu K, Matsumoto K, Miki K, Kita A: Observation of the orientation of membrane protein crystals grown in high magnetic force fields. J Cryst Growth, 367: 53-56, 2013.

6. Nagamatsu Y, Takeda K, Kuranaga T, Numoto N, Miki K: Origin of Asymmetry at the Intersubunit Interfaces of V_1-ATPase from *Thermus thermophilus*. J Mol Biol, 425: 2699-2708, 2013.

Frontier Research Unit
Laboratory of Oxygen Biology

Associate Professor　Koh Nakayama, 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, 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 altering multiple cellular functions including metabolism, respiration, and cell growth. HIF-α is actively degraded during normoxic condition, whereas it is stabilized and activated under hypoxic conditions. PHD is a HIF-prolyl hydroxylase which hydroxylates and regulates the expression of HIF-α. There are 3 PHDs indentified, 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 sensor molecule(s) and 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 possibly as an oxygen sensor. Ultimately, we challenge to invent a tool which would suppress the progression of hypoxic tumor by modifying the oxygen sensor.

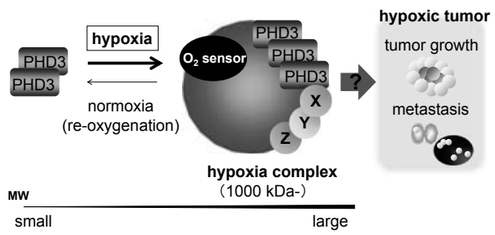


Figure Characterization of Hypoxia Complex

Publications

1. Nakayama K.* CREB and NF-κB are activated during prolonged hypoxia and cooperatively regulate the induction of matrix metalloproteinase MMP1. ***J. Biol. Chem***. 288, 22584-22595, (2013).

2. Arima N., Uchida Y., Yu R., Nakayama K., Nishina H. Acetylcholine receptors regulate gene expression that is essential for primitive streak formation in murine embryoid bodies. ***Biochem. Biophys. Res. Commun***. 435, 447-453, (2013).

3. Muramatsu S., Tanaka S., Mogushi K., Adikrisna R., Aihara A., Ban D., Ochiai T., Irie T., Kudo A., Nakamura N., Nakayama K., Tanaka H., Yamaoka S., Arii S. Visualization of stem cell features in human hepatocellular carcinoma enlightened in vivo significance of tumor-host interaction and clinical implication. ***Hepatology*** 58, 218-228, (2013).

4. Nakayama K., Nangaku M. Hypoxia-inducible factor and signal transducer and activators of transcription 3: two central regulators meet to regulate kidney pathophysiology. ***Clin. Exp. Pharmacol. Physiol.*** 40, 251-252, (2013).

Tenure Track Research Unit
Department of Cellular and Molecular Medicine

Associate professor　Yumiko Tanaka MD, PhD
Assistant professor　Daiki Taneichi PhD
Research Technician　Sayuri Shikata

Research outline

Cardiovascular disease, as a consequent of the obesity related metabolic syndrome, remains a significant cause of morbidity and mortality in industrialized societies. 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 resolution, 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 poly 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 NFκB kicks off both pro-inflammatory and anti-inflammatory signaling pathway. TLR4 activation rapidly, and transiently inhibits Liver X receptor (LXR) signaling through NFκB, 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 plays an important role in the regulation of the fatty acid synthesis to regulate homeostasis. By elucidating the crosstalk 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.

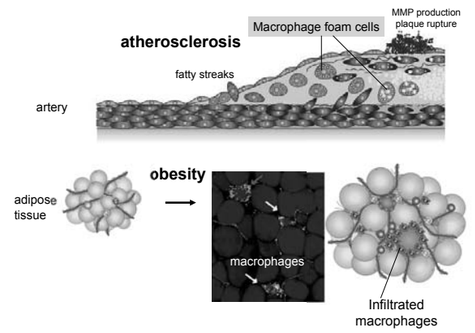


Fig.1 Macrophage is important for a chronic inflammation.

Publications

1. Shen H, Eguchi K, Kono N, Fujiu K, Shibata M, **Oishi-Tanaka Y**, Komuro I, Arai H, Nagai R, and Manabe I. The saturated fatty acid palmitate aggravates neointima formation by promoting smooth muscle phenotypic modulation. ***Arterioscler Thromb Vasc Biol*** 33, 2596-2607, 2013.

2. Lam M, Cho H, Lesch H, Heinz S, **Oishi-Tanaka Y**, Benner C, Kaikkonen M, Salim A, Rosenfeld M, Ecans R, and Glass CK. Rev-Erbs negatively regulate macrophage gene expression by repressing enhancer-directed transcription. ***Nature*** 498, 511-515, 2013.

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】

- Elucidation of pathological phosphorylation signaling in dementias.
- Discovery of common pathology mediator genes in polyglutamine diseases.

【Pathological Cell Biology】

- Identification of Atg5-independent macroautophagy in yeast.
- Identification of autophagic cell death *in vivo*.

【Developmental and Regenerative Biology】

- Summary of studies on non-alcoholic acid liver disease using fish models.
- Discovery of a novel role of acetylcholine receptors in mouse ES cell differentiation.

【Stem Cell Biology】

- Identification of melanocyte stem cells in sweat glands of volar skin.
- Mechanisms of aging-associated hair loss.

【Immunology】

- Elucidation of the distinct tolerance mechanisms for SLE-related anti-Sm and anti-DNA autoantibody producing B lymphocytes.
- Development of high affinity CD22-binding compounds for regulation of antibody production.

【Molecular Pathogenesis】

- HLA-linked NFKBIL1 regulate immunity and infection through modulation of alternative splicing of human and viral genes.
- Deciphering molecular basis of gender difference in LMNA-linked dilated cardiomyopathy: Activation of androgen receptor-FHL2 axis in activation of SRF.

【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
Associate Professor	Kazuhiko Tagaawa
Adjunct Lecturer	Nobuyuki Nukina, Masaki Sone, Toshiki Uchihara
Assistant Professor	Takuya Tamura
Project Assistant Professor	Toshikazu Sasabe, Chisato Yoshida, Kyota Fujita, Xigui Chen, Hidenori Honma, Junko Taniguchi
Technicians	Tayoko Tajima, Chiharu Mizoi, Yuko Uyama, Kimiko Ibagawa
Secretary	Asami Ohashi
Graduate Students	Ying Mao, Shigenori Uchida, Kanou Kondo
Research Trainees	Juliana Bosso Taniguchi

Research contents

Our research aim are: 1) to elucidate molecular mechanisms underlying neurodegenerative diseases and to develop effective therapeutic approach based on the information obtained; 2) to uncover the mechanisms of mental retardation (MR) influenced by a key regulator of neurodegenerative diseases, PQBP1; 3) to study mechanisms of stem cell differentiation through characterization of a transcription factor, Oct-3/4. Progress along 1) in this year will be described in the following.

1. VCP, a causative gene of FTLD controls the common pathology of polyglutamine diseases

More than 10 years ago, we originally demonstrated that VCP binds to a polyglutamine-tract by a screening with yeast two-hybrid method (Imafuku et al., BBRC 1998). Thereafter, involvement of VCP in the pathologies and the binding between VCP and a polyglutamine disease protein (Ataxin-3) were reported by another group (Hirabayashi et al., Cell Death and Differ. 2001). These results suggested that VCP might be a molecule involved in multiple polyglutamine diseases. However, this question had not been directly addressed and the molecular mechanisms mediated by VCP in the pathogenesis had remained unclear. Beyond polyglutamine diseases, VCP, a multi-functional protein belonging to AAA ATPase family and carrying functions in membrane trafficking, ER protein degradation and DNA repair etc, is known to be a causative gene for a form of frontotemporal lobar degeneration (FTLD), IBMPD.

We hypothesized that there would be a common pathology among polyglutamine diseases mediated by VCP. In order to clarify impaired function of VCP in the common pathogenesis, we first tested the bindings between VCP and polyglutamine disease proteins (Ataxin1, Ataxin7 Hunthintin and AR). We found that VCP binds to all 4 types of polyglutamine disease proteins, and surprisingly both normal and mutatant forms interact with VCP almost equally. The binding was dependent on polyglutamine tract sequence because the mutant forms of polyQ proteins lacking polyglutamine tract sequence did not bind to VCP. We also found the co-localizations of VCP and polyglutamine disease proteins in nuclear aggregates by immunostaining. Moreover, we found VCP was localized in the nucleus of neurons in contrast to cytoplasmic localization in the other types of cells. These findings suggested that VCP dysfunction in the nucleus of neurons might contribute to the common pathology. We reported that DNA double strand break (DSB) is involved in polyglutamine disease pathogenesis (Enokido et al., JCB 2010). Therefore, we tested the effect of polyQ disease proteins via VCP on DNA damage repair.

In drosophila and mouse models of HD and SCA1, signals of gamma H2AX or H2Av, DNA-DSB markers, were increased in neurons. Furthermore, VCP overexpression in transgenic models recovered the lifespan shortening and reduced gamma H2AX and H2Av signals. Micro irradiation method demonstrated mutatant Ataxin1 and Huntingtin perturbed intracellular dynamics of VCP, and inhibited VCP accumulation to the DNA damage foci.

Collectively, our results suggested that any mutatant polyQ disease protein can bind to VCP and will modify its dynamics. Consequently, DNA repair function is impaired and DNA-DSB is increased. (Fuiita et al., Nature Commun 2013)

2. RPA1, a DNA repair gene controls SCA1 pathogenesis

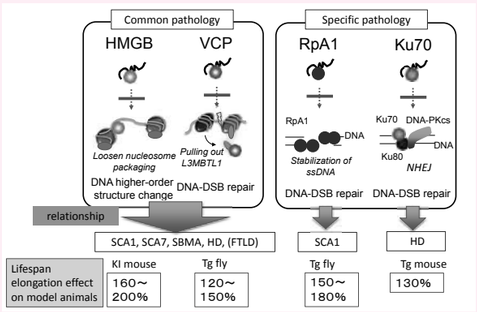
We have made a novel SCA1 model drosophila which has shortened lifespan. We performed *in vivo* screening of the DNA repair genes by testing their effects on the lifespan shortened by expression of mutant ataxin-1 protein in motor neurons. We found 8 genes recovered lifespan and 12 genes that worsened lifespan. To elucidate the molecular networks, we further analyzed functional connectivity among the recovering worsening genes with IPA software. The result suggested that RPA1 and Chk1 are the core gene in each network. We confirmed the roles of each gene in SCA1 pathogenesis by using compound eye degeneration model fly. As expected, RPA1 overexpression recovered and suppression worsened the eye phenotype. Again, as expected, Chk1 overexpression worsened and suppression recovered the phenotype. Overexpression of RPA1 recovered not only the lifespan shortening but also the increased DNA-DSB. We demonstrated the binding between Ataxin1 and RPA1. Mutant Ataxin1 had stronger interaction with RPA1. Micro irradiation method demonstrated mutated Ataxin1 modified the intracellular dynamics of RPA1, and prevent RPA1 accumulation to the local damaged area.

Taken together, our results suggest that mutant Ataxin1 binds to RPA1 and impairs its dynamics. Consequently, DNA repair function is impaired and DNA-DSB is increased. (Barclay et al., Hum Mol Genet 2014)

Highlight

"DNA repair molecules in polyglutamine diseases"

Multiple unbiased approaches elucidated the importance of DNA repair in polyglutamine diseases. VCP may be related common pathogenesis and RPA may be related SCA1 specific pathogenesis. Combinational therapeutics of the gens is hopeful.



Publications

1. Fujita, K., Nakamura, Y., Oka, T., Ito, H., Tamura, T., Tagawa, K., Sasabe, T., Katsuta, A., Motoki, K., Shiwaku, H., Sone, M., Yoshida, C., Katsuno, M., Eishi, Y., Murata, M., Taylor, JP., Wanker, EE., Kono, K., Tashiro, S., Sobue, G., La, Spada, AR., and Okazawa, H. (2013) A functional deficiency of TERA/VCP/p97 contributes to impaired DNA damage repair in multiple polyglutamine diseases. ***Nature Commun.*** *4*:1816. doi: 10.1038/ncomms2828
2. Li, C., Ito, H., Fujita, K., Shiwaku, H., Yunlong Qi, Y., Tagawa, K., Tamura, T., Okazawa, H. (2013) Sox2 transcriptionally regulates Pqbp1, an Intellectual Disability-Microcephaly causative gene, in neural stem progenitor cells. ***PLOS ONE*** *8*, .e68627. doi: 10.1371/journal.pone.0068627
3. Shiwaku, H., Yagishita S., Eishi Y., Okazawa, H. (2013) Bergmann glia are reduced in spinocerebellar atasia type 1. ***Neuroreport.*** *24*, 620-625. doi: 10.1097/WNR.0b013e32836347b7.
4. Ikeuchi, Y., de la Torre, L., Matsuda, T., Steen, H., Okazawa, H., Bonni, A. (2013) The XLID protein PQBP1 and the GTPase dynamin 2 define a signaling link that orchestrates ciliary morphogenesis in postmitotic neurons. ***Cell Reports*** *4*, 1–11. doi:10.1016/j.celrep.2013.07.042
5. Barclay, S.S., Tamura, T., Ito H., Fujita, K., Tagawa, K., Shimamura, T., Katsuta, A., Shiwaku, H., Sone, M., Imoto, S., Miyano, S. and Okazawa, H. Systems biology analysis of *Drosophila in vivo* screen data elucidates core networks for DNA damage repair in SCA1.
Hum Mol Genet 2014 Mar 1;23(5):1345-64. doi: 10.1093/hmg/ddt524

Department of Pathological Cell Biology

Professor	Shigeomi SHIMIZU
Junior Associate Professor	Tatsushi YOSHIDA
Tokunin Junior Associate Professor	Masatsune TSUJIOKA
Assistant professor	Satoko ARAKAWA
Tokunin Assistant Professor	Michiko MUROHASHI, Shinya HONDA, Hirofumi YAMAGUCHI, Tsutomu HASHIDUME, Min Kyoung SHIN

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 that the budding yeast *Saccharomyces cerevisiae*, which lacks Atg5, can form macroautophagic structures (autophagosomes and autophagic bodies) and undergoes autophagy-mediated protein degradation. This finding indicated that alternative macroautophagy machinery is phylogenetically conserved from yeasts to mammals.

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.

The Bcl-2 family of proteins are well-characterized regulators of apoptosis, among which Bax and Bak act as a mitochondrial gateway. Although embryonic fibroblasts from Bax/Bak double-knockout (DKO) mice are resistant to apoptosis, we have previously shown that these cells still die with autophagic structures in response to various types of cellular stress stimuli. In this year, we generated Atg5/Bax/Bak triple-knockout (TKO) mice to elucidate the physiological role of autophagic cell death. Embryonic fibroblasts and thymocytes from TKO mice showed far less autophagy and better viability than DKO cells. The formation of interdigital web of DKO embryos occurred at embryonic day 15.5 (one day after that of wild type mice) with enhanced autophagy. In contrast, that of TKO embryos occurred at later day (day 16.0) without any autophagic manifestations. These data indicate that autophagic cell death occurs for compensation of apoptosis in mouse embryo.

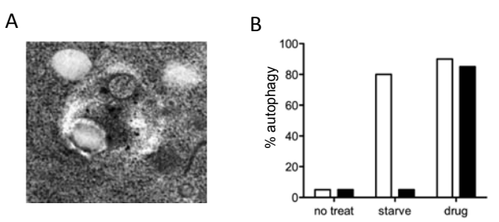


Fig.1 Induction of macroautophagy in *atg5Δ* yeast cells
(A) Induction of macroautophagic structures in *atg5Δpep4Δ* cells. Representative image of substituted thin section is shown. An autophagosome containing a ribosome and Golgi granule is present in the cytosol. (B) Accumulation of autophagic bodies by drug in *atg5Δ* cells. Wild-type (open columns) and *atg5Δ* cells (closed columns) were starved for 3 h or treated with drug. The number of cells containing autophagic bodies was counted.

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.

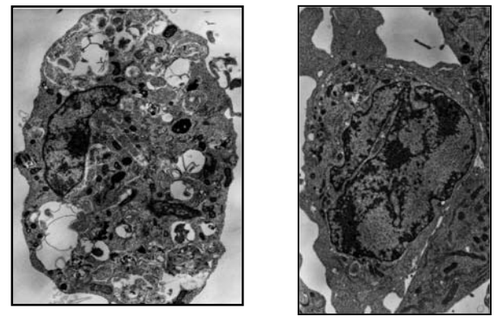


Fig.2 Electron micrographs of Bax/Bak DKO MEFs and Atg5/Bax/Bak TKO MEFs treated with etoposide.
Representative features of etoposide-treated MEFs are shown. A large number of autolysosomes were observed in DKO MEFs, but not in TKO MEFs.

List of Publications

[Original paper]

1. Mizushima T, Arakawa S, Sanada Y, Yoshino I, Miyazaki D, Urushima H, Tsujimoto Y, Ito T, Shimizu S.: Inhibition of epithelial cell death by Bcl-2 improved chronic colitis in IL10 KO mice. ***Am J Pathol.*** 183, 1936-44 (2013)
2. Shimizu S, Yoshida T, Tsujioka M, Arakawa S.: Autophagic Cell Death and Cancer. ***Int. J. Mol. Sci.*** *in press*
3. Shimizu S, Honda S, Arakawa S, Yamaguchi H.: Alternative Macroautophagy and Mitophagy. ***Int. J. Biochem. Cell Biol.*** *In press*

[Review paper]

1. Shimizu S, Arakawa S, Nishida Y, Yamaguchi H, Yoshida T.: Mammalian autophagy can occur through an Atg5/Atg7-independent pathway. ***AUTOPHAGY:*** *Cancer, Other Pathologies, Inflammation, Immunity, and Infection.*Vol. 2 (Edit MA Hayat) Academic Press, 49-59 (2013)

Department of Developmental and Regenerative Biology

Professor Hiroshi Nishina, Ph.D.
Associate Professor Jun Hirayama, Ph. D.
Assistant Professor Yoichi Asaoka, Ph.D.
JSPS Research Fellow Norio Miyamura, 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 NH_2-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

Non-alcoholic fatty liver disease (NAFLD) is a condition in which excessive fat accumulates in the liver of a patient who has not consumed excessive alcohol (Fig. 1). Non-alcoholic steatohepatitis (NASH), a severe form of NAFLD, can progress to hepatic cirrhosis and/or hepatocellular carcinoma (HCC). NAFLD/NASH is considered to be a hepatic manifestation of metabolic syndrome, and its incidence has risen worldwide in lockstep with the increased global prevalence of obesity. Over the last decade, studies in rodents have yielded an impressive list of molecules associated with NAFLD/NASH pathogenesis. However, the identification of novel metabolic factors using mammalian model organisms is inefficient and expensive compared with studies using other vertebrate models such as zebrafish (*Danio rerio*) and medaka (*Oryzias latipes*). Significant advances in unraveling the molecular pathogenesis of NAFLD/NASH have recently been achieved through unbiased forward genetic screens employing small fish models. Furthermore, these easily manipulated organisms have been used to great advantage to evaluate the therapeutic effectiveness of various chemical compounds for NAFLD/NASH treatment. In this review, we summarize the aspects of NAFLD/NASH pathogenesis previously revealed by rodent models, and discuss how small fish are increasingly being used to uncover new factors that contribute to normal hepatic lipid metabolism. We describe the various types of mutation, transgenic, and dietary fish models in use for this purpose, and contrast them with rodent models. The utility of small fish in identifying novel potential therapeutic agents for the treatment of NAFLD/NASH is also addressed (Fig. 2).

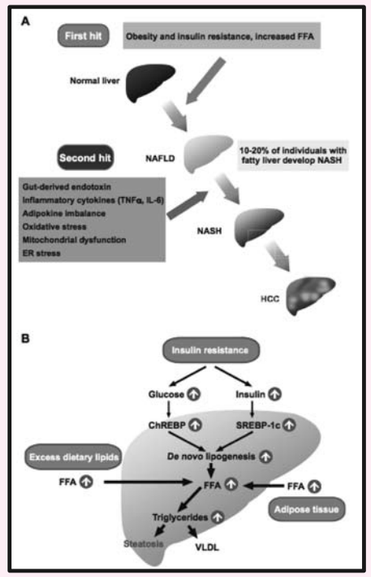


Fig.1. Mechanisms of NASH: The Two-hit Theory.

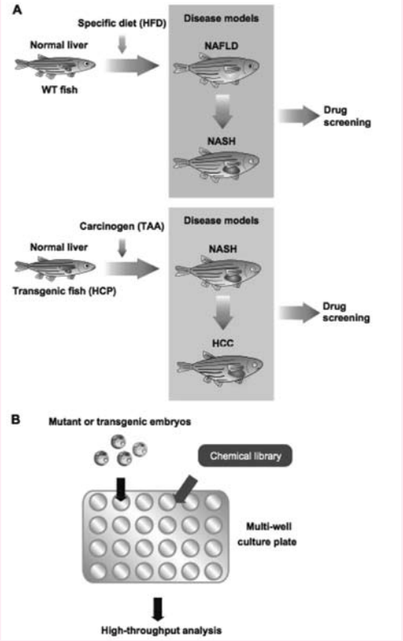


Fig.2. Drug Screening Strategy using Fish Disease Models

Publications

1. Norie Arima[1], Yoshimi Uchida[1], Ruoxing Yu, Koh Nakayama and Hiroshi Nishina (2013) Acetylcholine Receptors Regulate Gene Expression that Is Essential for Primitive Streak Formation in Murine Embryoid Bodies. ***Biochem. Biophys. Res. Commun.*** 435, 447-453 ([1] Contributed equally).

2. Menno J. Oudhoff, Spencer A. Freeman, Amber L. Couzens, Frann Antignano, Ekaterina Kuznetsova, Paul H. Min, Jeffrey P. Northrop, Bernhard Lehnertz, Dalia Barsyte-Lovejoy, Masoud Vedadi, Cheryl H. Arrowsmith, Hiroshi Nishina, Michael R. Gold, Fabio M.V. Rossi, Anne-Claude Gingras, and Colby Zaph (2013) Control of the Hippo pathway by Set7-dependent methylation of Yap. ***Dev. Cell*** 26, 188-194.

3. Yoichi Asaoka, Shuji Terai, Isao Sakaida and Hiroshi Nishina (2013) [review] The expanding role of fish models in understanding non-alcoholic fatty liver disease (NAFLD). ***Disease Models & Mechanisms*** 6, 905-914.

Department of Stem Cell Biology

Professor	Emi K. Nishimura, M.D., Ph. D.
Assistant Professor	Hiroyuki Matsumura, Ph. D.
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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 ageing. We further aim to apply this knowledge to regenerative medicine using somatic stem cells and the treatment of cancer as well as other age-associated diseases.

1) Identification of stem cells in the skin: follicular melanocyte stem cells vs. volar melanocyte stem cells.

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 cell population every hair cycle. We previously identified the source of those melanocytes, "melanocyte stem cells" (McSC), which are located in the hair follicle bulge and supply mature melanocytes required for hair pigmentation (Nishimura EK et al. Nature 2002) (Figure 1). We are currently trying to identify melanocyte stem cells in hairless areas of skin by generating Dct-H2B・GFP transgenic mice in which melanocyte stem cells can be stably visualized. We have discovered an unprecedented melanocytic population in the mouse footpad skin using the transgenic mice. We are currently trying to identify and characterize the population and test whether those cells satisfy the criteria for somatic stem cells and whether the population can be an origin of melanoma in the acral volar skin which contain abundant eccrine sweat glands instead of hair follicles.

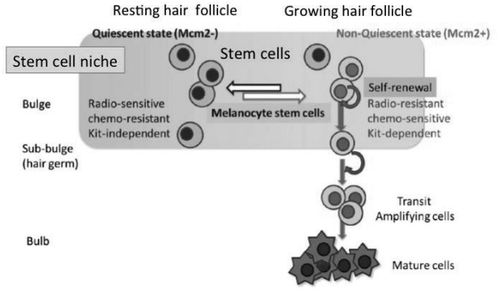

Figure 1 Schematic of different McSC states and their stress-resistance for self-renewal.
Aactivated McSCs are chemosensitive and radioresistant, while quiescent McSCs are chemoresistent and rariosensitive .The coexistence of quiescent and activated stem cells in anagen hair follicles provides strong stress-resistance for maintenance of the stem cell pool to prevent their depletion and resultant hair graying.

2) Mechanisms of stem cell maintenance

We have demonstrated that the progressive hair graying phenotype is caused by incomplete maintenance of McSCs. The phenotype is characteristically seen in some coat color mutants such as *Bcl2* deficient mice and *Mitf-vit* mutant mice. *Mitf* encodes a transcription factor of the bHLH Zip type and is known as a master regulator of melanocyte development. *Bcl2* is one of the target genes of MITF. *Mitf* and *Bcl2* turned out to be essential intrinsic genes involved in McSC maintenance to prevent hair graying (Nishimura EK et al. Science 2005). While we previously found that the niche microenvironment plays a dominant role in MSC fate determination (Nishimura EK et al. 2002), the identity of the niche cells and the underlying molecular mechanisms have been largely unknown. Melanocyte stem cells (McSC) and hair follicle stem cells (HFSC), which are originally derived from a completely different developmental origin, are located in the bulge area of mammalian hair follicles. Our study revealed that HFSCs provide a functional niche for MCSCs through transforming growth factor β (TGF-β) signaling to prevent premature hair graying (Tanimura S et al. Cell Stem Cell 2011).

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

Physiological hair graying is the most obvious outward sign of aging even in normal mammals. We previously demonstrated that physiological hair graying is caused by incomplete self-renewal/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. Stem cell differentiation but not stem cell apoptosis nor senescence turned out to be the major fate of McSCs under irreparable/excessive genotoxic stress or with aging. 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).

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 radiosenitive or not and whether the stem cell checkpoint functions all the time in stem cells 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. The irradiated quiescent McSCs prematurely differentiate in the niche upon their activation without sufficiently renewing themselves nor providing mature melanocytes to the hair bulb for hair pigmentation. These data indicate 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. in press).

Publications

1. Ueno M, Aoto T, Mohri Y, Yokozaki H and Nishimura EK. Coupling of the radiosensitivity of melanocyte stem cells to their dormancy during the hair cycle. Pigment Cell Melanoma Res. in press
2. Morinaga H, Takenaka T, Hashiya F, Kizaki S, Hashiya K, Toshikazu B and Sugiyama H. Sequence-specific electron injection into DNA from an intermolecular electron donor. Nucleic Acids Res. 41(8):4724-4728. 2013

Invited lecture/presentation at international meetings

1. Emi K. Nishimura : Hair Follicle aging and stem cell regulation : The 23rd Hot Spring Harbor Internatinal Symposium jointly with The 3rd "Grants for Excellent Graduate Schools" International Symposium: November 5, 2013, Kyushu University
2. Emi K. Nishimura : DNA damage and melanocyte stem cells: Montagna Symposium on the Biology of Skin : October 10, 2013, Washington, USA
3. Emi K. Nishimura: Melanocyte Stem Cells Maintenance, Survival and Differntiation: International Pigment Cell Development Workshop: May 7th, 2013, Edinburgh, UK
4. Emi K. Nishimura: Mechanisms of Hair Follicle Aging and Stem Cell Regulation: 7th World Congress for Hair Research: May 5th, 2013, Edinburgh, UK

Oral Presentation at meetings

1. Yasuaki Mohri, Nguyen Thanh Binh, Hiroyuki Matsumura, Yuko Tadokoro, Mayumi Ito, Jan Hoeijmakers and Emi K. Nishimura : The fate switch of hair follicle stem cells to the epidermis underlies baldness due to hair follicle aging : The 11th Stem Cell Research Symposium : May 5th, 2013, Tokyo

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.
	Soha Gomaa Ramadan Abdel Salam, Ph.D.
Lecturer	Ji-Yang WANG

The nature of immune responses depends on whether they respond to protein or non-protein antigens because T lymphocytes recognize only protein antigens. Normal immune system removes pathogens and cancer cells but does not respond to non-microbial foreign substances or self-antigens. Immune responses to non-microbial foreign substances and self-antigens cause allergy and autoimmune diseases, respectively. How immune system distinguishes pathogens from non-microbial antigens and self-antigens is already clarified for protein antigens. However, little is know about such distinction for non-protein antigens. 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. 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) 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. Among these autoantibodies, anti-DNA antibodies and antibodies reactive to RNA-related antigens such as the Sm antigen play a pathogenic role. Self-reactive B lymphocytes (B cells) that produce autoantibodies are regulated by various self-tolerance mechanisms including deletion and inactivation so that autoantibodies are not produced in healthy individuals. Anti-DNA B cells are tolerized in the bone marrow where B cells are generated the precursors at the immature B cell stage. Some of the anti-DNA B cells that are functionally inactivated (anergized) migrate to the T cell zone and red pulp in peripheral lymphoid tissues and undergo rapid apoptosis. In collaboration with Professor Weigert at Chicago University, we found that anti-Sm B cells migrate to the marginal zone in peripheral lymphoid tissues and regulated by apoptosis in marginal zone (Kishi et al. PNAS, 2012). We further demonstrate that tolerance of anti-Sm but not anti-DNA B cells is reversed by overexpression of CD40L (Kishi et al. 2012, Aslam et al. 2013), a member of TNF family involved in survival and activation of CD40-expressing B cells and dendritic cells. This suggests that marginal zone deletion is a distinct tolerance mechanism, and that tolerance of anti-Sm B cells may be broken in SLE as patients with SLE show excess CD40L.

2. Genetic factors that regulate autoantibody production in SLE and immuno-neurological disorders.

Guillain-Barre syndrom (GBS), an immuno-neurological disease, is often triggered by H. influenzae and Campylobactor infection, but may involve genetic factors. Patients with GBS often produce autoantibodies to gangliosides, sialic acid-containing glycolipids. We are analyzing the Siglec family genes encoding negative regulators of cell activation expressed in various immune cells in patients with GBS in collaboration with Professor Kusunoki at Kinki University.

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, a member of the Siglec family that predominantly express in B cells, negatively regulates signaling through B cell antigen receptor. Although CD22 specifically binds to a2,6 sialic acid, CD22 inhibits B cell response to antigens regardless of whether the antigen contains sialic acids. In collaboration with Professors Kiso and Ishida at Gifu University, we developed a sialic acid derivate GSC-718 that binds to CD22 with affinity 10000 fold higher than the natural ligand (Fig. 1). We are currently analyzing its biological activities for drug discovery in collaboration with a pharma.

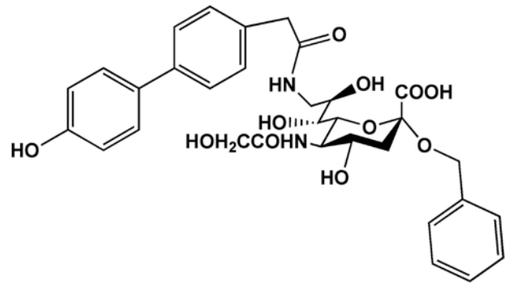

Figure 1. Structure of GSC-718

Highlight

Distinct tolerance mechanisms for lupus-related self-reactive B lymphocytes depending on antigen-specificity.

Systemic lupus erythematosus (SLE) is characterized by production of autoantibodies to various nuclear components including DNA and protein-RNA complex such as the Sm antigen. Autoantibody-transgenic mice in which all the B cells express the transgene-encoded autoantibody is a useful tool to study tolerance of self-reactive B cells. We crossed anti-DNA H chain transgenic mice 3H9 and 56R established by Dr. Weigert in Chicago University with our CD40L-transgenic mice in which CD40L is overexpressed as is the case for patients with SLE and its animal models. Excess CD40L has been suggested to be involved in development of lupus by inducing survival and activation of CD40-expressing B lymphocytes and dendritic cells.

The immunoglobulin composed of the 3H9 H chain and l light chain reacts to DNA, whereas that composed of the 56R H chain and Vk38C reacts to the Sm antigen. Both 3H9/l and 56R/Vk38C-expressing B cells appear in the peripheral lymphoid organs but rapidly undergo apoptosis, although these B cells are located at different regions in the peripheral lymphoid organs: 3H9/l-expressing B cells reside in T cell zones and red pulp whereas 56R/Vk38C-expressing B cells are located in the marginal zone. To further demonstrate that these B cells with different antigen specificity are tolerized by distinct mechanisms, we addressed whether excess CD40L breaks tolerance of these B cells. Excess CD40L perturbed tolerance of 56R/Vk38C-expressing B cells, leading to survival of these B cells and production of autoantibody, whereas excess CD40l did not perturb tolerance of 3H9/l-expressing B cells. This finding supports the notion that distinct tolerance mechanism regulate anti-DNA and anti-Sm B cells.

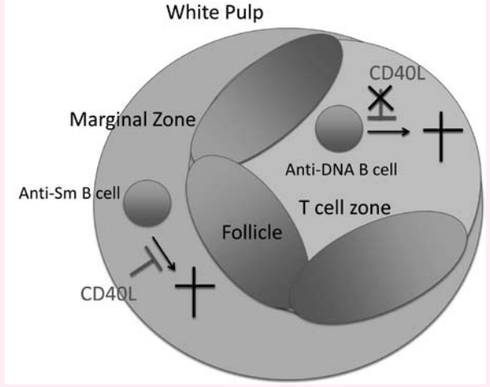

Figure 2. Tolerance of self-reactive B cells and its break down
Although both anti-Sm B cells and a part of anti-DNA B cells undergo apoptosis in peripheral lymphoid tissues, anti-Sm B cells are located in marginal zone and their tolerance is perturbed by excess CD40L. In contrast, tolerance of anti-DNA B cells that are located in T cell zone and red pulp is not perturbed by excess CD40L.

Publications

[Original papers]

1. Shimoda, M., Bolduc, A., Takezaki, M., Amtani, Y., Huang, L., Nutt S. L., Kamanaka, M., Flavell, R. A., Mellor A. L, Tsubata, T. andKoni, P. (2013): Constitutively CD40-activated B cells regulate CD8 T cell inflammatory response by IL-10 induction. *J. Immunol.* 190: 3189-3196.
2. Xu, M., Hou, R., Sato-Hayashizaki, A., Man, R., Zhu, C., Wakabayashi, C, Hirose, S., Adachi, T. and Tsubata, T. (2013): *CD72*^{c} is a modifier gene that regulates *Fas*^{lpr}-induced autoimmune disease. *J. Immunol.* 190: 5436-5445.
3. Aslam, M., Kishi, Y. and Tsubata, T. (2013): Excess CD40L does not rescue anti-DNA B cells from clonal anergy. *F1000 Research* 2:218.
4. 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 sialoglycan composition of T cells. *J. Biol. Chem.* 289: 1564-1579.
5. Kawai, Y., Ouchida, R., Yamasaki, S., Dragone, L., Tsubata, T. and J.-Y. Wang (2014): LAPTM5 promotes lysosomal degradation of intracellular but not the cell surface CD3ζ. Immunol. Cell Biol. (in press).

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 less 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 searched for mutations of FHOD3 in patients with dilated cardiomyopathy (DCM), who have no mutations in the known disease genes. A disease-associated mutation, Y1249N was identified in a patient. Functional analysis revealed that the mutation decreased actin dynamics-dependent activation of serum response factor in dominant-negative manner. In addition, we revealed molecular mechanisms for gender-difference in heart failure found in LMNA-mutation derived DCM in both human and mouse model: nuclear accumulation of androgen receptor and FHL2-SRF complex. Furthermore, hypertrophic cardiomyopathy-associated ANKRD1 mutations were found to differentially affect the cardiac muscle contractility. On the other hand, Apelin was revealed to act as a regulator of ACE2 in cardiac failure.

2. Molecular mechanisms for atherosclerosis

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

3. Molecular mechanisms for arrhythmia

We identified a mutation of SCN3B, V110I, in 3 out of 178 Japanese patients with Brugada syndrome, who had no mutations in the known disease genes. Functional study showed that the SCN3B mutation impaired intracellular trafficking of Nav1.5 channel and decreased inward currents.

4. Analysis of MHC in human and old world monkeys

We revealed that an HLA-linked autoimmune disease-associated NFKBIL1 gene regulated alternative splicing of human and viral genes, via interaction with Clk1 and ASF/SF2 in the presence of hnRNP (see Highlight). On the other hand, 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 Old World monkey, was investigated.

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 TIM1 and TRIM5a polymorphisms were associated with HIV/AIDS in humans.

Highlight

We have previously reported that promoter polymorphisms in NFKBIL1 encoding IkBL were associated with the susceptibility/resistance to autoimmune diseases and chronic inflammatory diseases. This year we revealed that IkBL bound Clk1. Because Clk1 was known to regulate alternative splicing, we investigated splicing events of mini-genes for CD45, CTLA4 and CD72. It was found that IkBL inhibit the alternative splicings of these immune-related genes, compete with Clk1 for splicing and binding to RRM domain of ASF/SF2. In addition, we found that the alternative spicing of these genes were independent from kinase domain of Clk1. Moreover, IkBL inhibit Influenza viral M gene. These findings provide us with novel insights into the functional impact of NFKBIL1 in regulation of both immunity and infection.

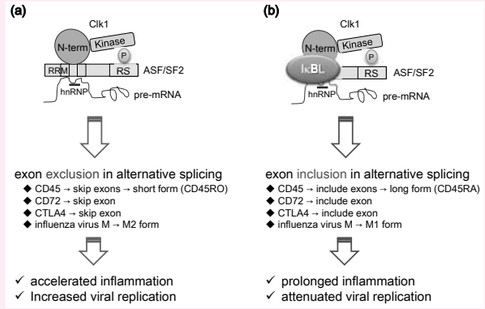

Figure. Involvement of IkBL in the CLK1-mediated alternative splicing
CLK1-mediated alternative splicing process is schematically represented. (a) In the absence of IkBL, pre-mRNA binds RRMs of ASF/SF2 and undergoes alternative splicing induced by hnRNP. CLK1 usually affects the alternative splicing process by phosphorylating RS domain of ASF/SF2. The process results in skipping exons of human immune-related genes and influenza virus M gene. (b) In the presence of IkBL, CLK1-mediated alternative splicing is attenuated. IkBL binds both RRMs of ASF/SF2 and N-terminal domain of CLK1. CLK1-mediated phosphorylation of RS domain of ASF/SF2 is not inhibited by IkBL. The attenuated splicing process may result in the inclusion of exons, leading to prolonged inflammation and attenuated viral replication.

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Frontier Research Unit
Virus Research Unit

Associate Professor　Norio Shimizu, PhD

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

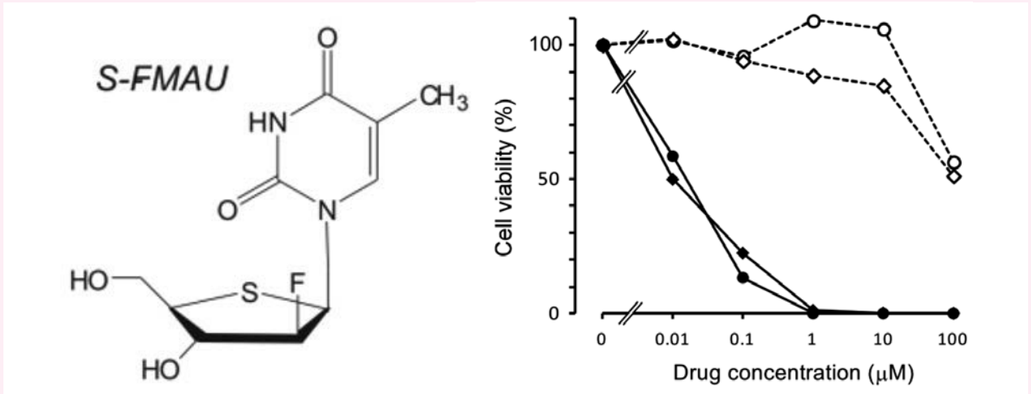
A novel nucleoside, 1-(2-deoxy-2-fluoro-4-thio-beta-D-arabino-pentofuranosyl) thymine (S-FMAU) was selectively cytotoxic to EBV-TK-transduced cells. S-FMAU blocked ex vivo EBV-induced transformation of B lymphocytes and reduced EBV copy numbers in the culture supernatant. EBV-infected T- and natural 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

A Nucleoside Analog S-FMAU Selectively Eliminates Epstein Barr Virus-Infected Cells



Left panel: Structures of the S-FMAU, Right panel: Inhibitory effects of S-FMAU on EBV infected cells.

Publications

[Original papers]

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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 systems 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. We identified a polymorphism in the ATP10D gene associates with serum HDL level atherosclerosis of the coronary and brain arteries.
2. We identified that CDKN2A/B, ADTRP, and PDGFD polymorphisms associate with coronary atherosclerosis and there may be an additive effect among these risk alleles.

【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 frequent gain-of-function 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 are 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 gene in naturally occurring hepatotumorigenesis induced by oxidative stress, and (4) 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 that Hes1 was 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.
Tokunin Lecturer Shin Hayashi M.D., 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 mental retardation (MCA/MR). Our research interests are as follows; (1) Identification of genes responsible for cancer and unknown genetic disorders, (2) Development of innovative techniques for detection of genomic and epigenomic aberrations underlying the pathogenesis of cancer and genetic 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. Identification of genes responsible for cancer by integrative genomics and epigenomics

For the last decade we performed Comparative genomic hybridization (CGH) analysis in over 2000 cases of various types of cancer and cell lines, and we constructed CGH database that is available through the internet (http://www.cghtmd.jp/cghdatabase/index.html). Through those CGH analyses we detected a number of novel and nonrandom amplifications in various tumors and identified target genes within the amplicons, such as *GASC1* (Gene Amplified in Squamous Cell Carcinoma 1) and *cIAP1* in esophageal squamous cell carcinomas (ESCCs), respectively. The former is a demethylase for tri- or dimetylated lysine 9 on histone H3. Some of them are being now focused as the target for the molecular target therapy.

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

Recently, the epithelial-to-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. Identifying SIX1 target genes based on Next Generation Sequencing technology.

In recent years, SIX1 has been implicated in tumor initiation and tumor progression in a variety of cancers. We performed ChIP-seq analysis to identify SIX1 binding sites genome-widely and obtained several SIX1 candidate target genes. Now we are validating these candidates using experimental and computational approaches.

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

By measuring the autophagy activity in several human cancer cell lines, we found the autophagy-impaired cancer cell lines accompanied by gene aberration of autophagy-related genes. Importantly, while it has been known that NRF2, a transcipriona factor, is constitutively activated in autophagy-impaired cancer cells, we identified 4 miRNAs *(miR-507, -634, -450a, -129-5p)* that can negatively regulate transcriptional activity by directly targeting NRF2. Furthermore, we demonstrated tumor growth inhibition by administration of miRNA *in vivo*. These findings provide important information for development of autophagy-based personalized cancer medicine.

5. Molecular cytogenetic investigation of MCA/MR

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 147 cases (22.6%). 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 30 of 40 cases (75.0%). 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

Professor	Yoshio Miki, MD. Ph.D.
Associate Professor	Akira Nakanishi, Ph.D.
Tokunin Lecturer	Koichi Nagasaki, Ph.D.
Assistant professor	Katsuya Takenaka, Ph.D.
Assistant professor	Ken Miyaguchi, Ph.D.

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. Synthetic lethality effect for chemotherapy using BRCA2-deficient breast cancers

Tumor suppressor genes BRCA1 and BRCA2 function in a complex gene network that regulates homologous recombination and DNA double-strand break repair. Inhibitors of poly (ADP-ribose) polymerase (PARP)-mediated DNA repair have shown promise in early clinical studies in the treatment of breast cancer. Synthetic lethality is a concept that is receiving increasing interest due to its potential exploitation in targeted cancer therapy. Current breast cancer treatment is defined by clinical and pathological characteristics, and recurrence and resistance remain a problem. Disruption of the BRCA-network through gene mutation, deletion, or RNAi-mediated silencing can sensitize cells to small molecule inhibitors of PARP.

In this study, we aimed to establish novel synthetic lethal interactions between BRCA2 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. In order to identify potential synthetic lethality relationships between chemical compounds and BRCA2, 4-[3-(4-iodophenyl)2-(4-nitrophenyl)2H-5-tetrazolio]1,3-benzene disulfonate-1 (WST-1)-based cytotoxicity assays were performed on capan-1 cells, which carries a naturally occurring 6174delT mutation in one BRCA2 allele accompanied by loss of the wild-type allele, and chemical compounds. WST-1 produces a formazan salt by a reducing reaction that is dependent upon mitochondrial dehydrogenase enzymes. As such mitochondrial activity is present only in living cells, formazan production acts as a directly proportionate measure of cell viability. Two concentrations of chemical compounds were simultaneously screened for on 96-well plates containing capan-1cells. The chemical library (1,230 known compounds) was screened against capan-1 cells. Of the 40 leading hits in this screening, 28 compounds were confirmed as antibacterial and antineoplastic agents.

2. Enhancement of the ATPase activity of non-muscle myosin (NM)-IIC by BRCA2

BRCA2 is localized to the midbody during cytokinesis and interacts with the human nonmuscle myosin heavy chain (NMHC) IIC. The biochemical activity of NMHC IIC originates from its actin-activated MgATPase activity. However, the specific function of BRCA2 in regulating the biochemical activity of NMHC IIC in the midbody is unclear.

Treatment of A549 cells with blebbistatin, an inhibitor of myosin II ATPase activity, caused decay of the IIC-ring surrounding a-tubulin and imperfections in ring formation. MgcRacGAP, which is a marker of the midbody, was observed in the decay of the IIC-ring. To explore the function of BRCA2 in IIC-ring formation, we analyzed the effect of BRCA2 on the actin-dependent ATPase activity of NM-IIC. The actin-dependent ATPase activity of NM-IIC was measured following incubation of the immunoprecipitated NMHC-IIC-HA in the presence or absence of BRCA2-FLAG. We confirmed that introduction of the plasmid encoding NMHC-IIC into cells leads to binding of the exogenous NMHC-IIC to the 12A and 12B isoforms of endogenous light chain. The ATPase was activated when both proteins (BRCA2-FLAG and NMHC-IIC-HA) were present. ATPase activation following incubation of NMHC-IIC and BRCA2 occurred in a dose-dependent manner. The light chain was phosphorylated following the addition of BRCA2-FLAG to the immunoprecipitated NMHC-IIC. Furthermore, in the presence of IIC (A1)-HA, which inhibits the binding of BRCA2 to NM-IIC, ATPase activation was inhibited. The absence of F-actin from the reaction mixture reduced ATPase activity significantly. The localization of F-actin at the Flemming body was observed by immunofluorescence microscopy. A mutant NMHC-IIC (AA)-HA in which Lys204 and Thr205 in the ATP-binding site (GESGAGKT; 198–205) were substituted with alanine did not exhibit ATPase activity in response to incubation with BRCA2. These results suggested that the ATPase activity is increased by NM-IIC–BRCA2 association.

In this study, the interaction of BRCA2 with NM-IIC was shown to be required for the activation of NM-IIC ATPase activity. We speculate that a kinase present in the BRCA2 immunoprecipitate might phosphorylate the MLC (myosin light chain), resulting in the activation of ATPase activity. ROCK (Rho-associated coiled-coil-forming kinase), which phosphorylates Ser19 of smooth muscle MLC2, was detected in the anti-BRCA2 immunoprecipitates by mass spectrometry. This result raises the possibility that ROCK1 may phosphorylate and activate myosin IIC. This issue should be addressed in future studies. We identified the subcellular localization of each isoform during cytokinesis and found that only the NM-IIC isoform co-localizes and interacts with BRCA2 at the Flemming body. The interaction of NM-IIC with BRCA2 at the Flemming body allows the activation of NM-IIC ATPase activity followed by IIC-ring formation (Fig. 1).

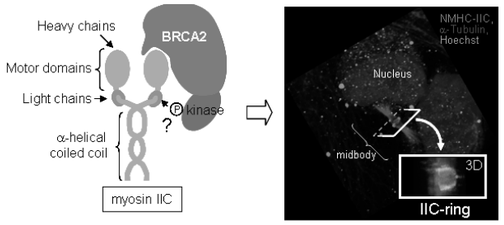

Fig.1. Enhancement mechanism of NM-IIC ATPase activity by BRCA2.

3. Analysis of intramolecular BRCA2 region concerning the numerical integrity of centrosomes by an automated centrosome counting system

Besides a role in DNA damage repair, BRCA2 also maintains the numerical integrity of centrosomes. For further analysis of intramolecular region of BRCA2 responsible for the centrosome regulation, we divided BRCA2 into small pieces and tried to see which region would function in the regulation. To date, centrosome numbers were widely counted by human eyes looking at random microscopic images. However, this method would not be applicable for BRCA2 regions since thousands of centrosomes could not be counted avoiding human biases. We are developing a computerized automated centrosome counting system, which will soon be applicable for accurate counting of thousands of centrosomes without human biases. Intramolecular region of BRCA2 and other molecules responsible for the centrosome integrity would be determined near future.

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Department of Molecular Epidemiology

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Assistant Professor **Shinobu Ikeda, DMD. Ph.D.**

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. Associations between the CDKN2A/B, ADTRP and PDGFD Polymorphisms and the Development of Coronary Atherosclerosis in Japanese Patients

Genome-wide association studies have identified a series of susceptibility loci for coronary artery disease (CAD). The present study attempted to replicate the results for eight of these loci, CDKN2A/B (rs1333049), ADTRP (rs6903956), PDGFD (rs974819), TCF21 (rs12190287), COL4A1-A2 (rs4773144), HHIPL1 (rs2895811), ADAMTS7 (rs4380028) and UBE2Z (rs46522), in patients with pathologically defined atherosclerosis of the coronary arteries. Autopsy cases of elderly Japanese subjects were enrolled in the JG-SNP study (n=1,536). Polymorphisms were genotyped, and their associations with the coronary stenosis index (CSI) and incidence of pathological myocardial infraction (MI) were investigated. The potential combinatorial effects of the susceptibility loci were also assessed.
Results: Among the eight loci tested, three exhibited signs of positive associations. CDKN2A/B showed the most robust associations with CSI and MI (p=0.007 and OR=1.843, 95% CI 1.293- 2.629, p=0.001, for CC+CG vs. GG). In addition, ADTRP demonstrated associations with CSI and MI, although the risk allele was opposite from that observed in the original report (p=0.008 and OR=1.652, 95% CI 1.027-2.656, p=0.038 for GG vs. AA+AG). Meanwhile, PDGFD displayed a suggestive association with CSI in women, but not men (p=0.023). CDKN2A/B and ADTRP were also found to be significantly associated with the severity of the CSI in a case-control setting. The cumulative risk allele counting of CDKN2A/B, ADTRP and PDGFD indicated an increased number of risk alleles to be associated with a higher CSI (p=4.61E-05).
The present study confirmed the association between CDKN2A/B and CAD and identified a different associated risk allele of ADTRP. PDGFD was found to exhibit a gender-specific association with CAD. The combination of multiple risk alleles may be associated with a higher risk of CAD.

2. A gene variant in the ATP10D gene associates with atherosclerotic indices in Japanese elderly population.

ATP10D belongs to a subfamily of P-type ATPases implicated in phospholipids translocation from the exoplasmic to the cytoplasmic leaflet of cellular biological membrane. Previous genome-wide association study (GWAS) identified that a variant in ATP10D gene (rs2351791) associates with serum lipid profile and myocardial infarction. The objective of this study is to assess the effect of this variant on atherosclerosis in Japanese elderly population.
Consecutive autopsy cases registered in JG-SNP study were recruited (n = 1536). The samples were pathologically assessed for atherosclerosis using macroscopic examination of the formalin-fixed arteries, and coronary stenotic index (CSI), intracranial atherosclerotic index (ICAI) and pathological atherosclerotic index (PAI), which represent systemic arteries were calculated. The variant rs2351791 (G/T) in Atp10d gene was genotyped by Taqman genotyping assay and association determined.
Both CSI and ICAI were significantly higher in GG genotype than GT genotype and TT genotype (p = 0.003 and p = 0.001, respectively). Both associations remained significant in minor allele dominant model after adjusting for age, hypertension, diabetes, HDL, smoking and drinking (p = 0.001 and p = 0.001, respectively). PAI was not associated with this variant. Consistent with the previous report, plasma HDL cholesterol level was lower in GG genotype compared to GT + TT genotypes (p = 0.001).
The rs2351791 SNP in the Atp10d gene affects the susceptibility for cardiac and intracranial vascular stenosis in the elderly Japanese population.

3. Association of the G-protein β3 subunit gene polymorphism with the incidence of cardiovascular disease independent of hypertension

Association of the C825T G-protein β3 subunit (GNB3) gene polymorphism with cardiovascular disease (CVD) incidence was examined in a population-based longitudinal study of the Japanese individuals. The incidence of CVD (stroke and coronary heart disease (CHD)) was assessed in a cohort population (n=1524) consisting of participants of the 2001-2005 Funagata study through March 2008. Cumulative incidences according to genotype were compared with the Kaplan-Meier product-limit method. During the follow-up, 78 subjects experienced a CVD event (stroke: n=54; CHD: n=30; both consecutively: n=6). At the end of the follow-up (longest and median follow-up periods: 81 and 68 months, respectively), the cumulative incidence of CVD for the TT genotype was significantly higher than that of the C-carriers (0.077 vs 0.042, P=0.004). Blood pressures and the prevalence of hypertension were not different between the genotypes. Cox's proportional hazard analysis showed that the TT genotype is a significant risk factor for CVD (hazard ratio (HR)=1.82 (95% confidence interval (CI) 1.14-2.89); P=0.012) and stroke (HR=1.76 (95% CI: 1.01-3.07); P=0.048) incidences after adjustment for age, sex, hypertension, hyperlipidemia, diabetes, alcohol drinking and smoking at baseline. The TT genotype of the C825T GNB3 gene polymorphism was found to be a significant risk factor for the incidence of CVD and stroke independent of hypertension and other established CVD risk factors in a Japanese population.

4. Specific HLA types are associated with antiepileptic drug-induced Stevens-Johnson syndrome and toxic epidermal necrolysis in Japanese subjects.

We investigated genomic biomarkers for Stevens-Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN), related to three antiepileptic drugs, zonisamide, phenobarbital and phenytoin.
HLA class I and HLA-DRB1 loci were genotyped for Japanese patients with zonisamide-, phenobarbital- or phenytoin-induced SJS/TEN (n = 12, 8 and 9, respectively) and for healthy Japanese volunteers (n = 2878).
Carrier frequencies of HLA-A*02:07 in patients with zonisamide-induced SJS/TEN and in the general Japanese population were 41.7 and 6.81%, respectively. Carrier frequencies of HLA-B*51:01 in patients with phenobarbital- and phenytoin-induced SJS/TEN and in controls were 75.0, 55.6 and 15.2%, respectively. HLA-A*02:07 and HLA-B*51:01, in a dominant model, were significantly associated with zonisamide- and phenobarbital-induced SJS/TEN, respectively (Pc = 0.0176 and 0.0042, respectively).
Our data suggest that HLA-A*02:07 and HLA-B*51:01 are potential biomarkers for zonisamide- and phenobarbital-induced SJS/TEN, respectively, in Japanese individuals.

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Department of Biochemical Genetics

Professor Shigetaka Kitajima MD, PhD.
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Scope of research :

Transcriptional regulation 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 other protein factors, whose dysfunction is implicated in various human diseases. Our research interest is focused on the basic mechanism of transcription cycle and implication of early response transcription factors in determining cell fate in stress response. We are also studying on the mechanism of cell cycle arrest of terminally differentiated cardiac cells and its re-activation to provide novel regeneration therapy.

Key words

・To provide novel paradigm of transcriptional regulation
・To understand role of transcription factor in cell fate determination

Research 1 : Transcription

Transcription proceeds from initiation via elongation to termination, and eventually PolII is recycled for the next rounds of transcription when gene expression is activated. Among many protein factors that regulate transcription cycle, TFIIF and Elongin A function during elongation phase and the dysregulation of Elongin A may cause to develop cancer such as von Hippel-Lindau disease. FCP1, a TFIIF-associating CTD phosphatase, dephosphorylates CTD during the transcription cycle, and its deficiency causes a genetic disease CCFDN. We focus on these factors in order to understand the role in transcription cycle and their implication in human disease.

1-1 FCP1, a causing gene of CCFDN, disturbs transcriptional re-cycing and inhibits cell-cycle progression by activating p53 response

FCP1 catalyzes de-phosphorylation of Ser2 of PolII CTD, thereby accelerating its re-cycling upon gene activation. We carried out its gene knockdown in several cells and found it resulted in activation of p53-p21 to cause reversible cell cycle arrest. We are now further studying to find out novel biological function of FCP1.

1-2 Elongin A plays dual roles in stress response (Highlight 1)

Elongin (Elongin ABC complex) has dual functions, one is activation of transcriptional elongation by RNA polymerase II (PolII), and another is the degradation of Rpb1 subunit of PolII. By scanning stress response genes by chip analysis, Elongin A is recruited to the HSP70 and ATF3 gene from their promoter through 3'-downstream region, showing it associates with the transcribing PolII. At the same time, Elongin A formed E3-ligase complex that target Pol II into ubiquitin-mediated degradation upon DNA damage. This is a novel finding and is the first report to assign Elongin A as DNA-damage-inducible Pol II degradation gene. By deletion mutation analysis, Elongin A is shown to exhibit these two activities via mutual exclusive domains of the molecule. Elongin A may function as one of safety net mechanism of gene transcription in mammalian cells.

Research 2: Cell fate determination by activating transcription factor (ATF) 3

Cells determine their life or death in response to environmental stress. Activating transcription factor (ATF) 3 is an early response gene and plays role in cell death, survival and proliferation. Our aim of ATF3 research is to understand dual role of ATF3 in oncogenesis, anticancer therapy, and various stress response, and to find novel clinical applicability to the control of cell fate.

2-1 System biology approach to elucidate biological role of ATF3 in stress response

ATF3 functions as both oncogene and tumor suppressor. In prostate and mammary cancers and Hodgkin disease, ATF3 is positively correlated with cell proliferation and enhanced metastasis. Conversely, ATF3 is a target gene of p53 and inhibits p53 degradation to stabilize its expression level. By combined genome-wide ChIP-chip and expression profile analysis, we showed ATF3 does regulate approximately 40% of p53 target genes, demonstrating that ATF3 functions as co-regulator of p53. Further, we generated genetically engineered mouse model of p53 and ATF3 gene knockout to unravel genetic codes of p53-ATF3 axis regulation. The genome-wide analysis of these mice is now revealing intriguing regulatory networks between these two transcription factors in cancer and stress response.

2-2 Role of Wnt-ATF3 regulatory axis in cell growth, invasion, and metastasis in human colorectal cancers

Our system biology study showed ATF3 could be regulated by Wnt pathway in human colorectal cancers. This prompted us to further look at HCT116 cells that are heterozygous for b-catenin. Using in vitro knocked-out HCT116 cells, we found ATF3 is direct target gene of Wnt canonical pathway. Its role in regulating cancer growth and metastasis is now under investigation.

2-3 ATF3 activates TAIL-based cancer cell killing through DR5 induction by natural products and HDAC inhibitors via ER stress pathway (Highlight 2)

We previously showed the cell death by TRAIL/CPT combination in human colon cancer cells is dependent on ATF3, partly because ATF3 co-operates with p53 to induce the DR5 transcription on the chromatin in cancers with wild type p53. In cancers with p53 mutation, we now revealed ATF3 is also an activator of TRAIL-based apoptosis through DR5 induction via ER stress/UPR pathway by zerumbone, celecoxib, and HDAC inhibitors. We propose that ATF3 plays role in both p53-dependent and -independent cancer cell killing, and might be useful as a biomarker of efficient anti-cancer therapeutics to overcome resistance to TRAIL.

Research 3: H3K36-sepcific histone methyltransferase ASH1.

Core histones that constitute nucleosomes together with DNA are reversibly modified by a large number of nuclear enzymes. Combinations of such modifications generate highly dynamic histone codes and play important roles in regulation of gene activities. In our laboratory, we have cloned one of mammalian histone lysine methyltransferases called ASH1 (absent, small, or homeotic discs-1) and shown that ASH1 specifically methylates histone H3 lysine 36. ASH1 synergizes strongly with MLL (mixed lineage leukaemia) in Hox gene expression and also plays a crucial role in activation of retrogenes in patients with facioscapulohumeral muscular dystrophy. Thus, our studies will help develop novel strategies to fight against human diseases such as leukaemia and muscular dystrophy.

Highlight 1

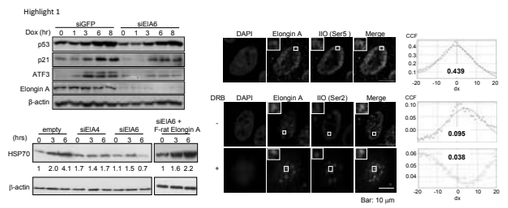

Fig 1 . Knockdown of Elongin A inhibits DNA damage-induced expression of p21, ATF3 and HSP70A.

Fig. 2. Elongin A co-localizes with transcriptionally active PolII, which is abolished by treatment with DRB, an inhibitor of transcriptional elongation.

Highlight 2

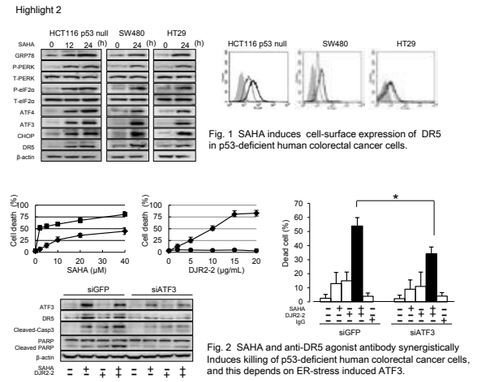

Fig. 1 SAHA induces cell-surface expression of DR5 in p53-deficient human colorectal cancer cells.

Fig. 2 SAHA and anti-DR5 agonist antibody synergistically Induces killing of p53-deficient human colorectal cancer cells, and this depends on ER-stress induced ATF3.

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Department of Genomic Pathology

Professor Shumpei Ishikawa
Asistant Professor Takayuki Isagawa
Asistant Professor Hiroto Katoh

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

Tumor-Stromal Interactions

Tumor tissue is composed of various kinds of cells including cancer cells, endothelial cells, fibroblasts and immune cells which are depending on each other for their survivals and growths. These cells excluding tumor cells are called as stromal cells, and forms tumor microenvironment. The role for the tumor microenvironment in the malignant transformation was unclear, but recent studies revealed that inflammatory immune cells (lymphocytes and macrophages) and fibroblast cells contribute to the tumor invasion and metastasis (Fig 1). Additionally, it is also known that the formation of the tumor stroma makes it difficult for anti-cancer drugs to be delivered and work effectively. Therefore, tumor stroma attracts the attention as a new druggable target against tumors.

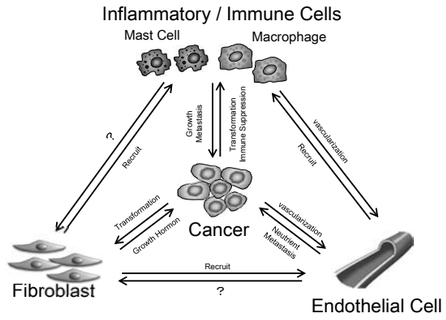

Fig 1. Cancer-Stromal Interaction

1. Genomic approach for the Tumor-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 2). 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.

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. 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.

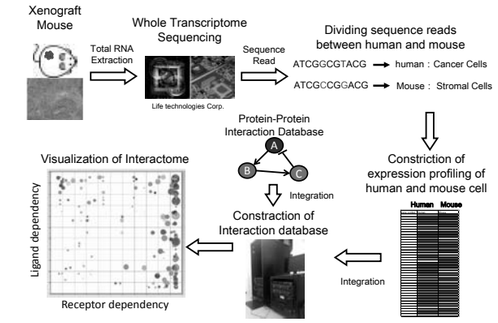

Fig 2. Genomic approach for cancer - stromal interaction

2. Functional Genomics Screening

In the department of genomic pathology, we developed a method for the comprehensive characterizations of fibroblast clones in the tumor stroma by combining a whole-genomic shRNA library and massively-parallel sequencing. Fig 3 shows that we could identify growth promoted or suppressed shRNA clones of fibroblasts in vivo by analyzing mouse tumors in which the shRNA-fibroblasts and cancer cells were co-injected. We are comprehensively revealing what kinds of gene knockdown in fibroblasts causes what kinds of responses in tumor tissues in vivo. We are exploring new druggable molecules targeting the disruption of the tumor stroma by elucidating molecular mechanisms of tumor stroma formation.

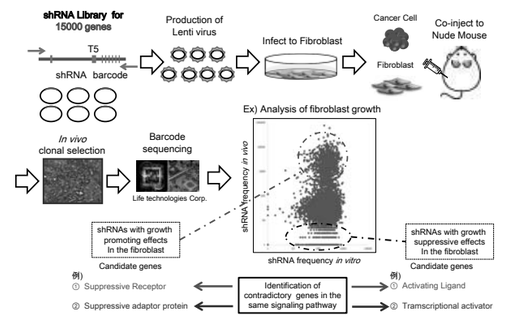

Fig 3. Functional Genomic Screening for elucidating the mechanism of Tumor stromal formation

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 a new gene harboring somatic mutations with a high frequency of more than 20% of clinical samples. This gene is involved in the regulations of cytoskeletal formations, cell motilities and various cell signaling pathways, and we think that this gene is an important driver gene for the diffuse gastric cancer. In the department of genomic pathology, we will continue to investigate molecular mechanisms of this newly identified gene in the diffuse gastric cancer. We are also going to further investigate various clinical disease samples by using genomics approach.

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Department of Epigenetics

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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 preimplantation 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 the 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 line provides us a new tool 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 distinguishing 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

Active DNA demethylation is required for complete imprint erasure in primordial germ cells.

Genome-wide DNA demethylation occurs in early embryonic development and in primordial germ cells (PGCs) (Figure 1). In the latter, DNA demethylation is indispensible for parental imprint erasure, which is a reprogramming process essential for normal developmental potential. We have recently demonstrated that DNA replication-independent active DNA demethylation is involved in imprint erasure in PGCs using an *in vivo* small molecule inhibitor assay. The data also suggest that active DNA demethylation plays a significant role in the complete erasure of paternal imprinting in the female germ line (Figure 2).

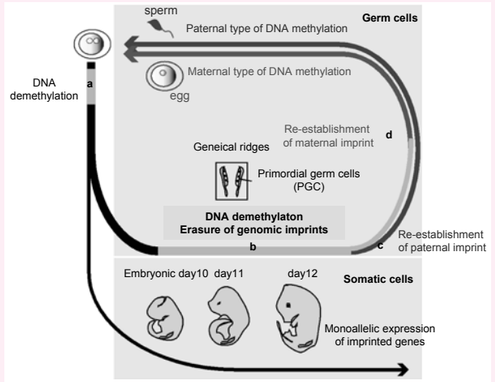

Fig 1. **Genomic imprinting in mammalian life cycle**
Sperms and eggs have paternal and maternal genomic imprints in their genomes, respectively (blue and red lines, c and d). Both paternal and maternal genomic imprints are resistant to the first global DNA demethylation in early embryonic development (a), therefore, PGC have the same imprints to somatic cells (black line) but erasure of genomic imprints occurs just around the time they enter into genital ridge, future testis and ovary (b) by the DNA replication-independent active demethylation mechanism.

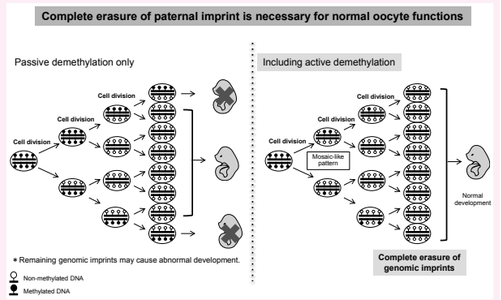

Fig 2. **Biological meaning of active DNA demethylation in imprint erasure**
Why active demethylation is essential in the erasure of genome imprints in PGCs? Female germ cells usually stop cell division on E12.5 and enter into meiosis on E13.5, therefore, it is expected that they can at most divide 3-4 times from the time of the initiation of DNA demethylation to the entry into meiosis. If no active demethylation pathway exists, the female PGCs would theoretically retain 1/8 - 1/16 of the DNA methylation levels, even when meiosis started after fertilization. Incomplete erasure (i.e. DNA demethylation) of the paternally imprinted regions in the oocytes would lead to abnormal imprinted gene expression in these regions, ultimately resulting in abnormal embryonic development.

Publications (Original papers)

1. Kohda T and Ishino F. Embryo manipulation via assisted reproductive technology and epigenetic asymmetry in mammalian early development. (Review) **Phillos Trans R Soc Lond B Biol Sci,** 368(1609):20120353 (2013).
2. Wakayama S, Kohda T, Obokata H, Tokoro M, Li C, Terashita Y, Mizutani E, Nguyen VT, Kishigami S, Ishino F and Wakayama T. Successful serial recloning in the mouse over multiple generations. **Cell Stem Cell** 12(3), 293-297 (2013).
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4. Iwasaki S, Suzuki S, Clark H, Ono R, Shaw G, Renfree MB, Kaneko-Ishino T and Ishino F. Identification of novel *PNMA-MS1* in marsupials suggests LTR retrotransposon-derived *PNMA* genes differently expanded in marsupials and eutherians. **DNA Res** 20(5), 425-436 (2013).
5. Nishimoto M, Katano M, Yamagishi T, Hishida T, Kamon M, Nabeshima Y, Nabeshima Y, Katsura Y, Satta Y, Deakin JE, Graves JAM, Kuroki Y, Ono R, Ishino, F, Okazaki Y, Kato H and Okuda A. *In vivo* function and evolution of the eutherian-specific pluripotency marker *UTF1*. **PLoS One** 8(7):e68119 (2013).
6. Kobayashi S, Totoki Y, Soma M, Matsumoto K, Fujihara Y, Toyoda A, Sakaki Y, Okabe M and Ishino F. Identification of an imprinted gene cluster in the X-inactivation center. **PLoS One** 8(8):e71222 (2013).
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Department of Bioinformatics

Professor Hiroshi Tanaka
Assistant Professor Kaoru Mogushi, 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.

1. Protein interaction network analysis of Alzheimer's disease

Alzheimer's disease (AD) is the most common cause of dementia. It is characterized by senile plaques (deposits of amyloid-beta) and neurofibrillary tangles (NFTs; accumulations of hyperphosphorylated tau proteins). To date, many experimental drugs for AD have been developed based on amyloid-beta hypothesis. However, they do not show any clear effects, suggesting the presence of cross talks among biological molecules including known proteins. In addition, it is considered that the relationships among various molecules dynamically change with the progression of AD. In order to elucidate how protein interaction networks (PINs) change across Braak NFT stage known to be associated with expansion of NFTs across brain regions, we integrated the human protein interaction datasets and the gene expression profiles of three distinct brain regions (entorhinal cortex, hippocampus and superior frontal gyrus) dissected from postmortem brains of AD patients in each Braak NFT stage. Consequently, we found that the PIN in entorhinal cortex, which is affected in the early stage of AD, significantly collapsed with Braak NFT stage compared to that of normal aging. Furthermore, we identified deubiquitinating enzyme UCHL5 as one of responsible proteins provoking the network perturbation.

2. Analysis of disease mechanism using omics-based approaches

Recent advances in analysis techniques in molecular biology have led to the investigation of genome-wide data such as genome, transcriptome and proteome. In order to reveal the underlying biological mechanisms from such a large amount of "omics" data, integration of biomedical knowledge with multivariate statistical analyses or machine learning methods is one of the most crucial tasks for bioinformatics research. We performed collaborative researches with our university hospital and other institutes mainly based on genome-wide analysis techniques such as DNA microarray and next generation sequencing. Our research activities focus on the following topics: 1) identification of diagnosis markers for prognosis prediction in hepatocellular carcinoma patients, 2) development of predictive markers for metastatic relapse in colorectal cancer, and 3) drug repositioning research in chronic obstructive pulmonary disease.

3. A network-guided module identification approach towards investigation of potential drug targets

A useful characteristic for identification of drug targets is modular structure in the human protein-protein interaction network. In networks with modular structure, interactions between proteins are much denser within a module than between modules. Proteins in a module have closely related functions with each other. If a module contains target proteins for a disease, proteins and interactions in the module could play important roles in disease mechanisms and may be potential candidate targets for the disease. In order to investigate potential drug targets, we analyzed modules in the human protein-protein interaction networks and found that drugs for different diseases target different modules in the network. For example, target modules for anti-Parkinson's are different from those for cancerous diseases. The listing of proteins and interactions in the modules for a given disease may help us to search more efficiently for drug action mechanisms and novel targets for the disease.

Publications

[Original Articles]

1. Kikuchi, M., Ogishima, S., Miyamoto, T., Miyashita, A., Kuwano, R., Nakaya, R., Tanaka, H. (2013). Identification of Unstable Network Modules Reveals Disease Modules Associated with the Progression of Alzheimer's Disease. PLoS ONE, DOI: 10.1371/journal.pone.0076162.
2. Tanaka, Y., Nogata, H., Tanaka, H. (2013). Effect of Music upon Awakening from Nap. Biomedical Soft Computing and Human Sciences, 18(2):29-37.
3. Tanaka, K., … , Mogushi, K., Tanaka, H., Sato, K., Suzuki, H., Mizushima, T. (2013). Mepenzolate bromide displays beneficial effects in a mouse model of chronic obstructive pulmonary disease. Nature Communications, 4:2686.
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7. Takahashi, M., Obayashi, M., Ishiguro, T., Sato, N., Niimi, Y., Ozaki, K., Mogushi, K., Mahmut, Y., Tanaka, H., …, Ishikawa, K. (2013). Cytoplasmic Location of alpha1A Voltage-Gated Calcium Channel C-Terminal Fragment (Cav2.1-CTF) Aggregate Is Sufficient to Cause Cell Death. PLoS One, 8(3):e50121.
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13. Obulhasim, G., Yasen, M., Kajino, K., Mogushi, K., Tanaka, S., Mizushima, H., Tanaka, H., Arii, S., Hino, O. (2013). Up-regulation of dbpA mRNA in hepatocellular carcinoma associated with metabolic syndrome. Hepatology International, 7:215-25.
14. Kudo, A., Mogushi, K., Takayama, T., … , Tanaka, H., Anzai, N., Sakamoto, M., Tanaka, S., Arii, S. (2013). Mitochondrial metabolism in the noncancerous liver determine the occurrence of hepatocellular carcinoma: a prospective study. Journal of Gastroenterology, Doi: 10.1007/s00535-013-0791-4.
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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. Detection of the import of procaspase-9 into the mitochondrial intermembrane space

The intermembrane space of mitochondria was found to contain plenty of cytosolic proteins by recent proteomic studies. We previously detected procaspase-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-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 $\Delta\psi$m-disrupted MT. The protein import was significantly facilitated by glutathione. Furthermore, the Procasp-9-Flag import was blocked by KD of Mia40gene. 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.

2. A novel mechanism of Wnt/β-catenin activation by p63

p63 (TP63, p51) is expressed in the TA and ΔN isoforms and plays positive and negative regulatory functions for various arrays of target genes. p63 RNA silencing and global gene expression analysis with squamous cell carcinoma cell lines indicated p63-mediated significant activation of Wnt/β-catenin target genes including CCND2, SNAI2 and DKK3. Luc reporter gene expression analyses indicated that DeltaNp63a, when transfected with TCF-4 and β-catenin, strongly activate Wnt responsive element (WRE)-dependent gene expression. Furthermore, as reported previously (Drewelus I, Cell Cycle 9: 580, 2010), DeltaNp63a was co-immunoprecipitated with flag-TCF-4, but not with flag-β-catenin, from DNA-free soluble nuclear extracts. However, ChIP experiments showed that p63 was not included in the LEF/TCF-β-catenin complex at the Wnt responsive elements (WREs) on the chromosomal DNA. Thus, p63 may facilitate Wnt/β-catenin signaling in the nucleus by interacting with the soluble form of TCF/LEFs, most possibly through blockage of the TCF/LEF-suppressors such as Groucho/TLE.

Publications

1. Khin M, Hnin S, Mitaka C, Kurata S, Tomita Inhibition of poly (adenosine diphosphate-ribose) polymerase attenuates lung-kidneycrosstalk induced by intratracheal lipopolysaccharide instillation in rats Respiratory research.2013 14 126-134
2. Miniwan T, Mitaka C Kurata S, Tomita M. Atrial natriuretic peptide attenuates kidney-lung crosstalk in kidney injury. Journal of surgical research 2013 186 217-225
3. Katoh I and Kurata S. Association of endogenous retroviruses and long terminal repeats with human disorders. Frontiers in oncology 11(3) 234-237

Frontier Research Unit
Laboratory of Gene Expression

Associate Professor Hidehito KUROYANAGI
Project Assistant Professor Mariko KIMURA (-Mar, 2013)

Recent whole genome sequence analyses revealed that a high degree of proteomic complexity is achieved with a limited number of genes. This surprising finding underscores the importance of alternative pre-mRNA splicing, through which a single gene can generate structurally and functionally distinct protein 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* (Figure). 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). Our reporter system will further elucidate expression profiles and regulation mechanisms of alternative splicing *in vivo*.

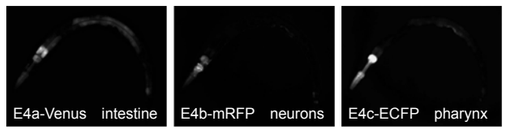


Figure A tri-chromatic fluorescence alternative splicing reporter worm for exons 4a/4b/4c of the *unc*-32 gene. Modified from PLoS Genet, 2013.

2. Global Search for Target Events of Tissue-Specific Splicing Factors *in Vivo*.

Through genetic analyses describe above, we obtained mutants of a variety of splicing regulators. To further decipher splicing codes *in vivo*, we are searching for alternative splicing events that are affected in the splicing factor mutants through transcriptome analyses by utilizing a next generation sequencer. We found new target events for a neuron-specific splicing factor UNC-75 and identified its *cis*-elements through bioinformatic and reporter analyses (Nucleic Acids Res, 2013). Further systematic analyses of the splicing factor mutants will lead to understanding of combinatorial regulation of alternative splicing events by multiple factors *in vivo*.

3 Regulation of Cardiac Muscle-Specific 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 are trying to elucidate the regulation mechanism of the cardiac muscle-specific alternative splicing of the *TTN* gene in vertebrates.

Publications

Original Research Articles
1. Kuroyanagi H, Watanabe Y, Hagiwara M. CELF family RNA-binding protein UNC-75 regulates two sets of mutually exclusive exons of the *unc-32* gene in neuron-specific manners in *Caenorhabditis elegans*. **PLoS Genetics**. 9: e1003337, 2013.
2. Kuroyanagi H, Watanabe Y, Suzuki Y, Hagiwara M. Position-dependent and neuron-specific splicing regulation by the CELF family RNA-binding protein UNC-75 in *Caenorhabditis elegans*. **Nucleic Acids Research**. 41: 4015-4025, 2013.
3. Iwasa H, Maimaiti S, Kuroyanagi H, Kawano S, Inami T, Ikeda M, Nakagawa K, Hata Y. Yes-associated protein homolog, YAP-1, is involved in the thermotolerance and aging in the nematode *Caenorhabditis elegans*. **Experimental Cell Research**. 319: 931-945, 2013.
4. Iwasa H, Kuroyanagi H, Maimaiti S, Ikeda M, Nakagawa K, Hata Y. Characterization of RSF-1, the *Caenorhabditis elegans* homolog of the Ras-association domain family protein 1. **Experimental Cell Research**. 319: 1-11, 2013.

Commentary
1. Hidehito Kuroyanagi. Switch-like regulation of tissue-specific alternative pre-mRNA processing patterns revealed by customized fluorescence reporters. **Worm** 2: e23834, 2013.

Project Research Unit
Affiliated Institutes

Project Research Unit

Pathophysiology

Associate Professor　Saburo Horikawa

Ischemia/reperfusion (I/R) injury can occur in several pathophysiological situations and is a major cause of tissue injury during transplantation and ablative surgery. I/R is an unavoidable process in these surgical operations. I/R injury is considered to be related to the generation of reactive oxygen species. The aim of our study is to understand the molecular mechanisms underlying I/R injury. Our research projects are: 1) acute lung injury induced by intestinal I/R; 2) hepatic I/R injury; 3) liver regeneration after partial hepatectomy; 4) portal vein stenosis; 5) fatty liver; 6) aquaporin-2 trafficking.

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.

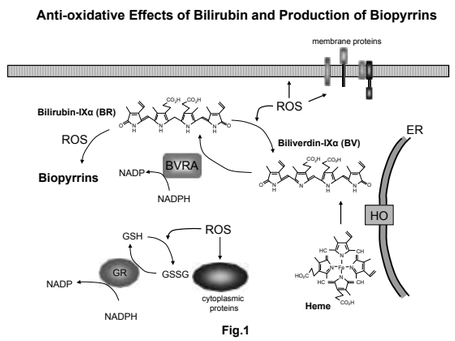


Fig.1

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. 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.
3. 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.
4. 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

Functional differences between the right and left primary auditory cortex of guinea pigs were investigated using optical imaging with a voltage-sensitive dye (RH795). Frequency-modulated (FM) sounds were applied at different sweep rates (0.04-1 kHz/ms). When upward FM sounds were applied at lower sweep rates, initial neural activities appeared at dorsal regions in the isofrequency bands corresponding to the start frequency of the FM sounds and then the secondary active spots appeared and moved across the isofrequency bands with the same rates as the FM sweep rates. When upward FM sounds were applied at higher sweep rates, initial activities appeared also at dorsal regions. However, the regions corresponded to higher frequency bands than the start frequency of the FM sounds.

These activity patterns were often observed in the left auditory cortex.

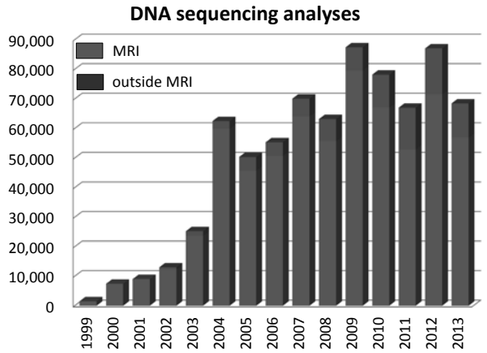
Publications

Hosokawa Y, Kubota M, Sugimoto S, Horikawa J. Neural activities to frequency-modulated sounds in the left and right primary auditory cortex of guinea pigs observed by optical recording. J Physiol Sci, Vol. 63, Suppl. 1, S164 (2013).

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 2013.



1. Sequencing analyses

A total of 68,470 samples from 3,371 researchers were sequenced in the year of 2013. Among them 11,493 (16.8%) 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 84 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.

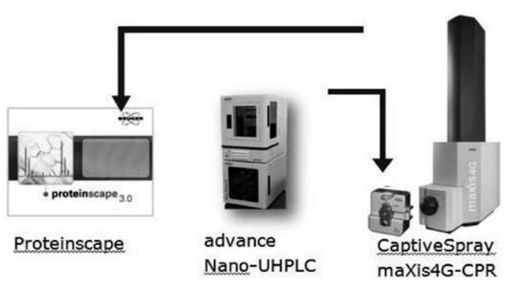
3. Introductory seminars

Introductory seminars were done for use of instruments (5 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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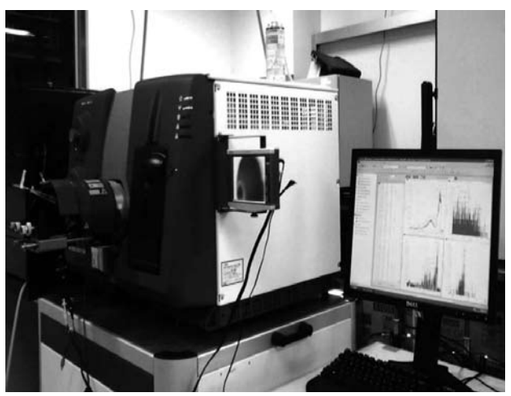
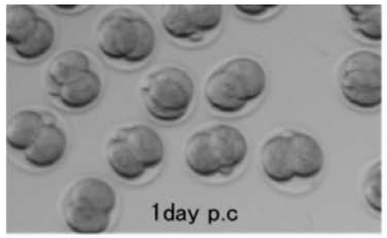


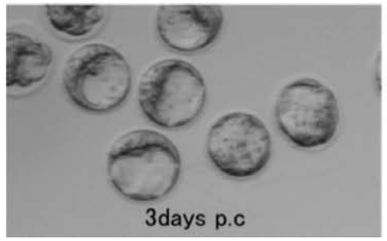
Qtrap5500 ABSCIEX

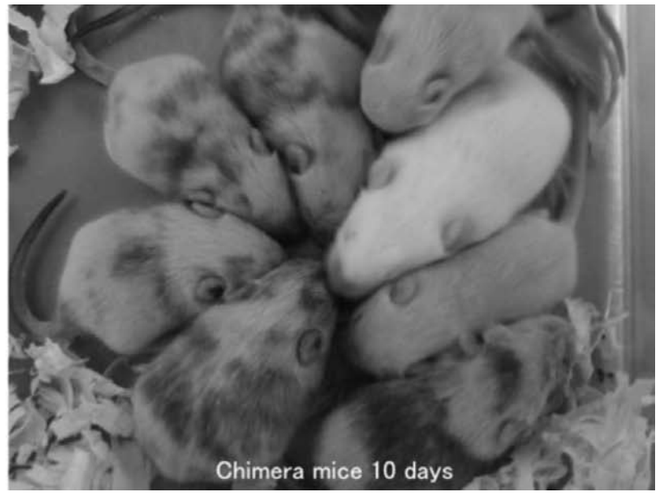
Laboratory of Recombinant Animals

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 to guide postgraduates in techniques for sampling of bioresources and 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.

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.

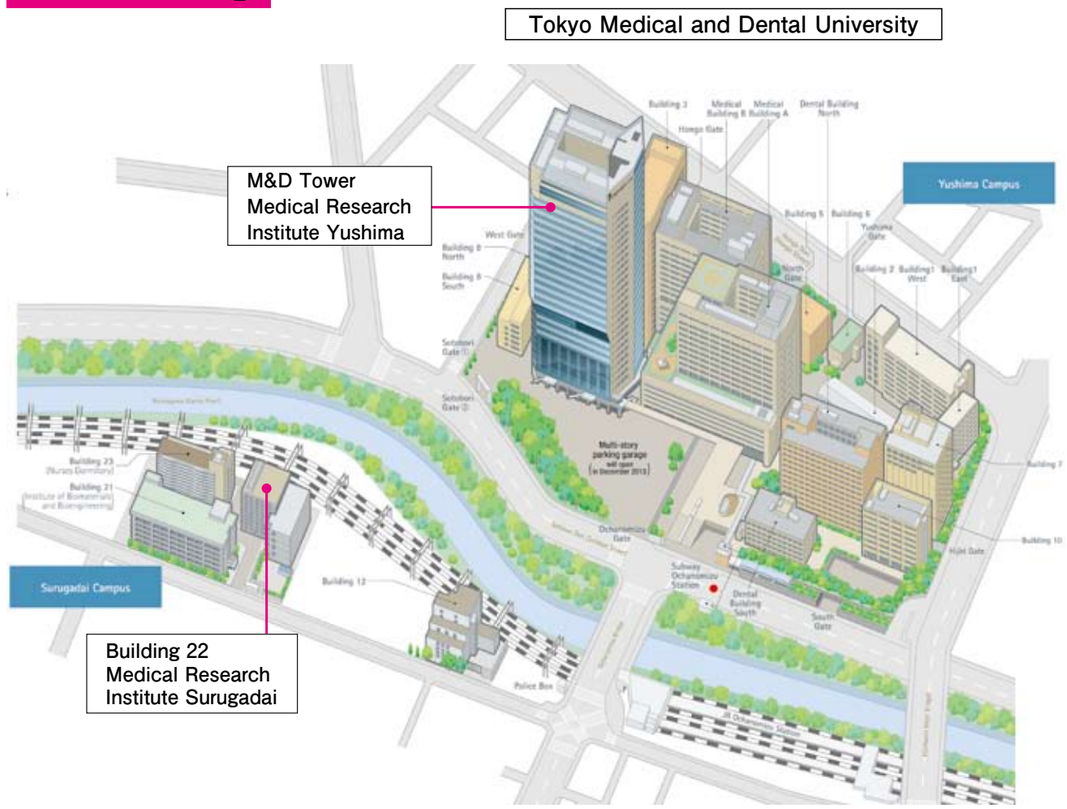
This Laboratory is managed by the Operating Committee composed of five Professors and three Associate Professors in the Institute, and the services are provided by one Technical Staff and one Technical Assistant.

From August 1, 2013, the use of the equipments and services is also opened to researchers in other departments within the University and those outside. Moreover, sorting service has started.

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	Emeritus Professor Open Innovation Center for Drug Discovery The University of Tokyo
NISHIKAWA Shin-ichi	Advisor JT Biohistory Research Hall

Access Map



Nearest Stations

JR Chuo line : Ochanomizu station
JR Sobu line : Ochanomizu station
Marunouchi line : Ochanomizu station
Chiyoda line : Shin-ochanomizu station

ANNUAL REPORT 2014

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