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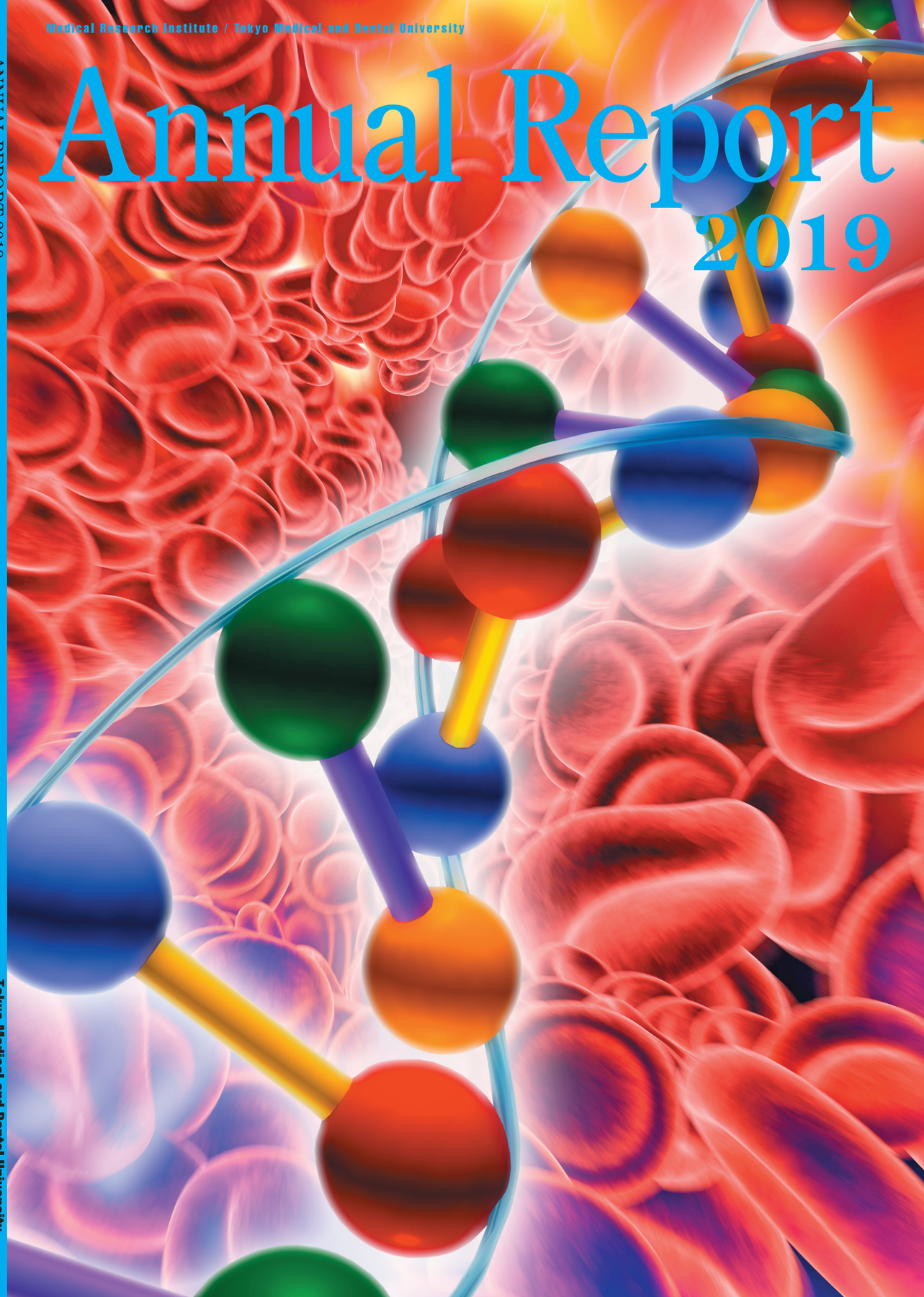
ANNUAL REPORT 2019

Tokyo Medical and Dental University

Medical Research Institute / Tokyo Medical and Dental University

Annual Report 2019



2019

Annual Report

Medical Research Institute

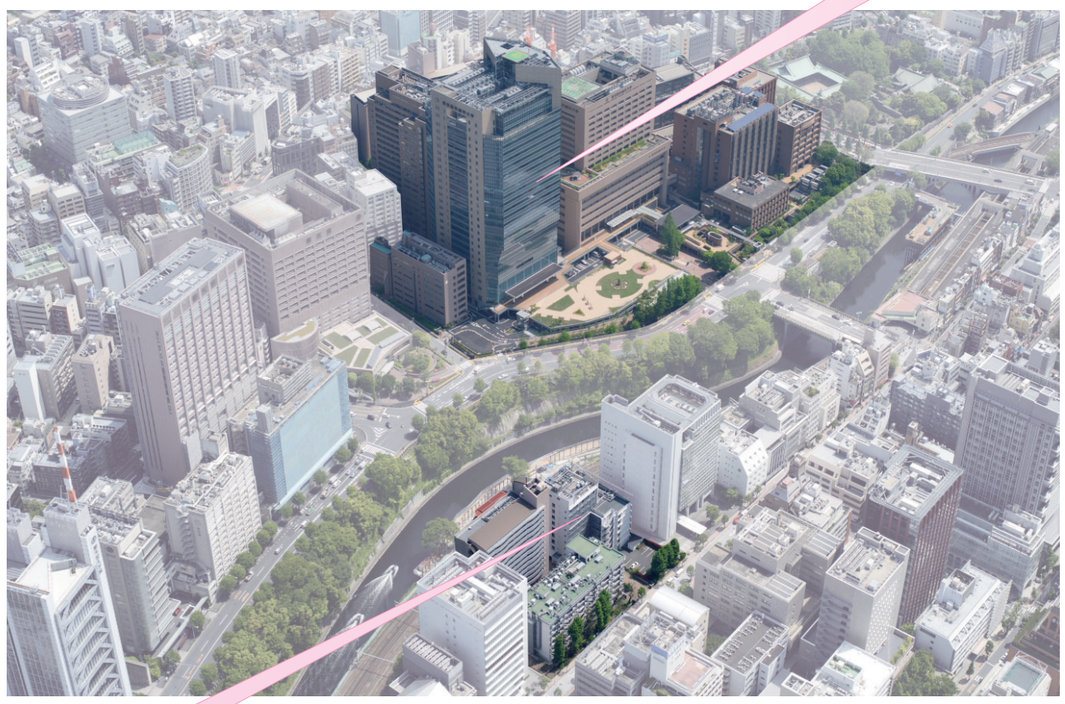
Tokyo Medical and Dental University

Contents

Yushima Area

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

Medical Research Institute
Department of Molecular Neuroscience, Department of Pathological Cell Biology, Department of Developmental and Regenerative Biology, Department of Immunology, Department of Molecular Cytogenetics, Department of Molecular Genetics, Department of Medical Science Mathematics, Department of Molecular Cell Biology, Department of Bio-informational Pharmacology, Department of Molecular Pathogenesis, Department of Epigenetics, Department of Stem Cell Regulation, Department of Neuropathology, Department of Structual Biology, Department of Biodefense Research, Department of Stem Cell Biology, Department of Genomic Pathology, Department of Molecular Epidemiology, Frontier Research Unit Laboratory of Gene Expression, Frontier Research Unit Redox Response Cell Biology, Frontier Research Unit Laboratory of Oxygen Biology, Tenure Track Research Unit Department of Cellular and Molecular Medicine, Administrative Office

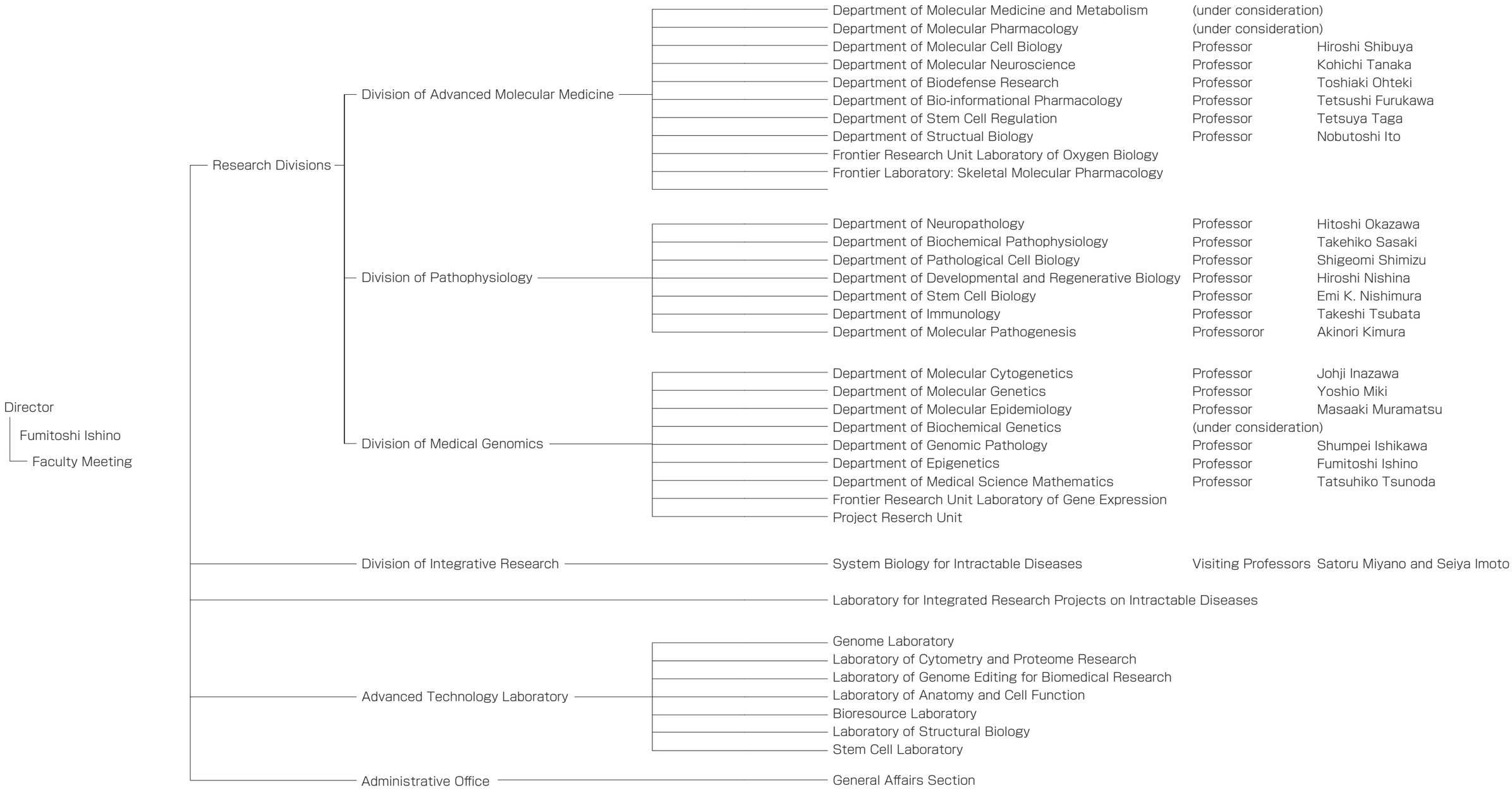


Surugadai Area

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

Medical Research Institute
Laboratory of Genome Editing for Biomedical Research

Medical Research Institute



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 Cell Biology】

- WNK signaling pathway is involved in neural development via GSK3β.
- WDR26 plays a negative role in β-catenin degradation in the Wnt signaling pathway.

【Molecular Neuroscience】

- Region-specific deletions of the glutamate transporter GLT1 differentially affect seizure activity.
- Region-specific deletions of the glutamate transporter GLT1 differentially affect nerve injury-induced neuropathic pain.

【Biodefense Research】

- Development of therapeutic agents targeting human common monocyte progenitor (cMoP) and monocyte lineage.
- Identification of novel microglia super-enhancers.
- Establishment of human tongue cancer organoid culture system.

【Bio-informational Pharmacology】

- We identified circulating biomarkers related to the onset and advancement of atrial fibrillation.
- We identified a key factor for differentiation of early mesodermal cells to cardiac mesodermal cells.
- We discovered epigenetic transcription regulation of inflammation-related genes in vascular endothelial cells.

【Stem Cell Regulation】

- The Sox17-Notch1-Hes1 pathway was demonstrated to be critical for maintaining the undifferentiated state of hematopoietic stem cell containing intra-aortic hematopoietic cell clusters in midgestation mouse embryos.
- Necrotic C6 glioma stem cells (GSCs) were found to facilitate their own niche formation involving tumor-associated macrophages (TAMs).

【Structural Biology】

- The crystal structure of nuclear receptors such as VDR and RXR with various small molecules were determined.
- A strong evidence against so-called “cistauosis” hypothesis in tau aggregation was discovered.

Department of Molecular Cell Biology

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

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

WNK (with no lysine (K)) kinase family has been recently identified serine/threonin protein kinase family, and is 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). WNK1 is also a causative gene of hereditary sensory and autonomic neuropathy type 2A (HSAN2A). Previously, we identified that WNKs phosphorylated and activated SPAK/OSR1 kinases, which in turn regulated various ion co-transporters. We also presented that the malfunction of this regulation caused the similar phenotypes of PHAII in mouse. However, the pathogenesis of HSAS2A is still unknown. Therefore, we started to look for new interacting factor(s) of WNK using model animals, and identified GSK3 β as a candidate.

1. Shaggy is a novel candidate effector of WNK signaling pathway in *Drosophila*

From the screening, we identified *shaggy* (*sgg*) gene as a candidate, which encoded the *Drosophila* homologue of mammalian GSK3 β. In *Drosophila* wing, the overexpression of *WNK* caused ectopic veins. Under *sgg* heterozygous mutant background, the formation of these extra veins by overexpression of *WNK* was suppressed. Furthermore, *WNK* mutant clones led to abdominal developmental defects. Sgg ectopic expression in *WNK* mutant clones rescued the abdominal phenotypes. These results suggest that *sgg* is a novel effector of the WNK signaling pathway.

2. GSK3 β functions as a positive effector downstream of WNK signaling pathway

The expression of GSK3 β induced the expression of *Lhx8* in cultured cells, which is the downstream transcription factor of WNK signaling pathway. Therefore, we examined the epistatic interaction between WNK1 and GSK3 β. The induction of *Lhx8* by overexpression of WNK was suppressed by the knockdown of *GSK3* β. However, *Lhx8* induction by GSK3 β was not suppressed by the knockdown of both *Wnk1* and *Wnk4*. Furthermore, similar results were obtained in the case of OSR1. These data suggest that the WNK–OSR1–GSK3 β pathway is conserved not only in flies but also in mammals, and that

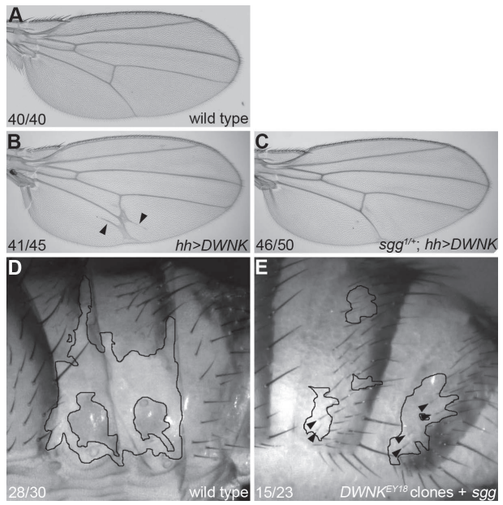


Fig.1 Shaggy is a novel candidate effector of WNK signaling pathway in *Drosophila*

GSK3 β functions as a positive effector downstream of the WNK signaling pathway.

3. GSK3 β is involved in the neural specification

WNK plays an important role in neurite elongation and neural specification through the regulation of *Lhx8* expression. In Neuro2A cells, knockdown of *GSK3* β caused the shortening of neurites after retinoic acid (RA) stimulation. Knockdown of *GSK3* β also decreased the expression of *Lhx8* and the choline acetyltransferase gene (*ChAT*; a marker for cholinergic neuron). However, the gene expression of glutamic acid decarboxylase 1 (*Gad1*; a marker for GABAergic neurons) increased. These results were similar to that induced by the knockdown of both *Wnk1* and *Wnk4*. Furthermore, under conditions of both *Wnk1* and *Wnk4* knockdown, the expression of *GSK3* β partially rescued the elongation of neurites, and *Lhx8* expression; this in turn increased *ChAT* expression and decreased *Gad1* expression. These results suggest that GSK3 β is involved in neural development and functions downstream of the WNK signaling pathway.

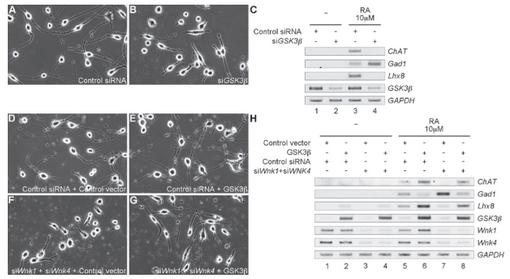


Fig.2 GSK3β functions as a positive effector downstream of WNK signaling pathway in the neural specification

Publications

Sato, A. and Shibuya, H. (2018). Glycogen synthase kinase 3 β functions as a positive effector in the WNK signaling pathway. **PLoS One** 13, e0193204.

Department of Molecular Neuroscience

Professor	Kohichi Tanaka
Associate Professor	Tomomi Aida
Assistant Professor	Saeko Ishida
Assistant Professor	Yuichi Hiraoka

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.

Recent human genetic studies have suggested that de novo mutations in GLT1 (EAAT2) cause early-onset epilepsy with multiple seizure types. Consistent with these findings, global GLT1 null mice show lethal spontaneous seizures. The consequences of GLT1 dysfunction vary between different brain regions, suggesting that the role of GLT1 dysfunction in epilepsy may also vary with brain regions. In this study, we generated region-specific GLT1 knockout mice by crossing floxed-GLT1 mice with mice that express the Cre recombinase in a particular domain of the ventricular zone. Selective deletion of GLT1 in the diencephalon, brainstem and spinal cord is sufficient to reproduce the phenotypes of the global GLT1 null mice. By contrast, dorsal forebrain-specific GLT1 knockout mice showed nonlethal complex seizures including myoclonic jerks, hyperkinetic running, spasm and tonic-clonic convulsion via the activation of NR2A-containing NMDA receptors during a limited period from P12 to P14 and selective neuronal death in cortical layer II/III and the hippocampus. Thus, GLT1 dysfunction in the dorsal forebrain is involved in the pathogenesis of infantile epilepsy and GLT1 in the diencephalon, brainstem and spinal cord may play a critical role in preventing seizure-induced sudden death.

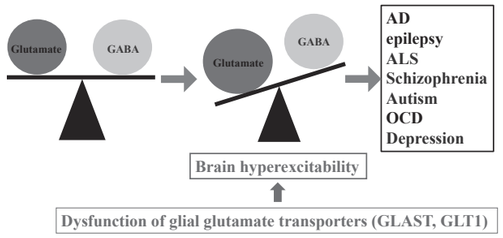


Fig1. Glutamate transporter dysfunction leads to neuropsychiatric diseases

Among glutamate signaling components, accumulating evidence suggests that the glial glutamate transporter GLT1 plays a critical role in neuropathic pain. Here, we generated periaqueductal gray (PAG)-specific and spinal cord-specific GLT1 knockout mice. Nerve injury-induced neuropathic pain was enhanced in spinal cord-specific GLT1 knockout mice but alleviated in PAG-specific GLT1 knockout mice. In addition, ceftriaxone upregulated GLT1 expression in the spinal cord, but not the PAG, of control mice and attenuated tactile hypersensitivity in nerve-injured control mice but not in nerve-injured spinal cord-specific GLT1 knockout mice. Based on these results, the anti-neuropathic pain effect of ceftriaxone is mediated by the upregulation of GLT1 expression in the spinal cord .

2. Role of DEPDC5 in the pathogenesis of epilepsy and psychiatric disorder

Epilepsy is one of the most frequent (~1%) neurological disorders characterized by spontaneous and recurrent seizures. However, pharmacoresistance occurs in 30% of the patients. Recently, a role for genetic factors in idiopathic epilepsies, with no identified structural lesion or metabolic cause, is becoming clear. DEP (Dishevelled, Egl-10 and Pleckstrin) domain containing protein 5 (DEPDC5) is a newly identified causative gene for epilepsy (Ishida et al., 2013). DEPDC5 has no transmembrane domain and no homology with known epilepsy genes encode ion channel or transmitter receptor subunits. Its role in epileptogenesis likely differs from the mechanisms known so far. In addition, some individuals also have psychiatric disorder, like autistic features and schizophrenia. This suggests that *DEPDC5* is a new key to clarify the common mechanism of refractory epilepsy and psychosis.

So far, we revealed that knockout Depdc5 in rats or mice results in embryonic lethal (Marsan and Ishida et al., 2016), and knock down it in Zebrafish leads hyperactive behavior (de Calbiac at al., 2018). We also clarified that Depdc5 heterozygous KO knockout mice show abnormal behaviors. We strongly promote our research to understand the pathogenesis. Research of DEPDC5 is likely to give new insight into epilepsy and psychosis research.

Publications

[Original papers]

1. Nakade, S., Mochida, K., Kunii, A., Nakamae, K., Aida, T., Tanaka, K., Sakamoto, N., Sakuma, T., Yamamoto, T. Biased genome editing using the local accumulation of DSB repair molecules system. *Nature Commun* 16. 3270, 2018.
2. Perkins, EM., Clarkson, YL., Suminaite, D., van Slageren, T., Rothstein, JD., Tanaka, K., Lyndon, AR., Skehel, P., Wyllie, DJA., Jackson, M. Loss of cerebellar glutamate transporters EAAT4 and GLAST differentially affects the spontaneous firing pattern and survival of Purkinje cells. *Hum Mol Genet* 27. 2614-2627, 2018.
3. Zhao, Z., Hiraoka, Y., Ogawa, H., Tanaka, K. Region-specific deletions of the glutamate transporter GLT1 differentially affect injury-induced neuropathic pain in mice. *Glia* 66. 1988-1998, 2018.
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5. Sugiyama, K, Tanaka, K. Spinal cord-specific deletion of the glutamate transporter GLT1 causes motor neuron death in mice. *Biochem. Biophys. Res. Commun*. 497. 689-693, 2018.
6. Sugimoto, J., Tanaka, M., Sugiyama, K., Ito, Y., Aizawa, H., Soma, M., Shimizu, T., Mitani, A., Tanaka, K. Region-specific deletions of the glutamate transporter GLT1 differentially affect seizure activity and neurodegeneration in mice. *Glia* 66. 777-788, 2018.
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10. de Calbiac H, Dabacan A, Marsan E, Tostivint H, Devienne G, Ishida S, Leguern E, Baulac S, Muresan RC, Kabashi E, Ciura S. Depdc5 knockdown causes mTOR-dependent motor hyperactivity in zebrafish. *Ann Clin Transl Neurol* 5. 510-523, 2018.

Department of Biodefense Research

Professor	Toshiaki Ohteki, DDS, Ph.D.
Junior Associate Professor	Taku Sato, Ph.D.
Assistant Professor	Masashi Kanayama, Ph.D.
Project Assistant Professor	Mihoko Kajita, Ph.D.
Adjunct Lecturer	Nobuyuki Onai, Ph.D.
Research Technician	Shoko Kuroda
Research Technician	Kisho Shiseki
Secretarial Assistant	Hisako Kamioka

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

1. Research on myeloid cells

1) Identification of novel sources of mononuclear phagocytes

Mononuclear phagocytes contain monocytes, macrophages and dendritic cells (DCs). In a recent decade, it has been continuing epoch-making discoveries in the field of mononuclear phagocytes and their functions are now beyond classical Immunology and extend to broad life phenomenon, e.g. tissue development/regeneration, wound-healing, and establishment of tumor environments and various inflammatory diseases.

DCs consist of conventional DCs (cDCs) and plasmacytoid DCs (pDCs), both of which play critical regulatory roles in the immune system. cDCs exhibit prominent antigen-presenting ability, whereas pDCs are characterized by their capacity to produce large amounts of type I interferons (IFNs). We have discovered the DC progenitors in the mouse bone marrow, and named common DC progenitors (CDPs) (***Immunity*** 2013; ***Nat Immunol*** 2007). Interestingly, CDPs are divided into 2 subpopulations. One is M-CSF receptor (R)$^{+}$ CDPs mainly producing cDCs, and the other M-CSFR^{-}CDPs producing a large number of pDCs. In addition to CDPs, common monocyte/macrophage progenitors, cMoP, identified in the mouse bone marrow and spleen by other group in 2013.

Based on these achievements in mouse, we have been trying to identify human progenitors of mononuclear phagocytes, and most recently succeeded to identify human cMoP (***Immunity*** 2017; ***Int Immunol*** 2018, **Fig. 1**). Human cMoP gives rise to only monocytes but not other hematopoietic cells including DCs. Given that monocytes and monocyte-derived macrophages cause a variety of inflammatory disorders, including metabolic syndromes and tumor development, our studies shed light on possible therapeutic applications for infectious diseases, cancers and autoimmune diseases. Collaborations with pharmaceutical company and Department of Hematology of TMDU toward the development of therapeutic agents targeting cMoP and monocyte lineage and with Department of Pediatrics of TMDU for the pathology clarification of congenital pulmonary alveolar proteinosis (PAP) are currently in progress.

2) Mechanism of brain function impairment by spatiotemporal transformation of microglial enhancer

The decline in tissue regeneration and homeostasis associated with life-stage progression is closely related to the functional alteration of macrophages. Microglia, a macrophage in the brain, is actively contributing to the brain development and maintenance during young age (regenerative microglia). However, with age, microglial inflammatory trait becomes prominent with impaired phagocytosis and brain-derived neurotrophic factor (BDNF) production etc (inflammatory microglia). As a result, functional neurons and synapses are decreased and destroyed. However, the overall picture and entire process of the microglial functional alteration and causative epigenomic transformation have not been clarified.

In this study, using a novel technology that can detect the active enhancer region and its activity with high sensitivity, we will identify the super enhancers (hereafter, SEs) responsible for the microglial transformation during life-stage progression, and elucidate the entire process of transformation dynamics. As SEs are activated in a cell-type specific manner, one can expect that it will lead to the development of novel technology to specifically control the age-related functional alteration of microglia. To date, we have identified 36,320 new microglial enhancers including 937 regions that become different with age (unpublished).

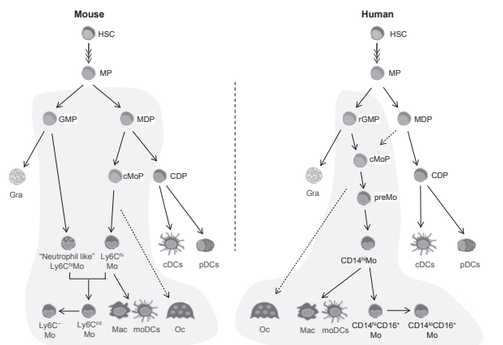

Fig.1 Monopoiesis in humans and mice
Reprinted from *Int Immunol* 30, 503-9 (2018). cDC, conventional DC; pDC, plasmacytoid DC; CDP, common DC progenitor; Gra, granulocyte; Mac, macrophage; Mo, monocyte; MP, myeloid progenitor; Oc, osteoclast; preMo, pre-monocyte

3) Mechanism of emergency myelopoiesis

Unlike steady-state hematopoiesis, hematopoiesis triggered at infection, irradiation and anti-cancer therapy is biased toward myeloid cell differentiation and production, that is "emergency myelopoiesis". However, due to the fluctuation of cell-surface marker(s) on hematopoietic stem progenitor cells (HSPCs), it has long been difficult to understand bona-fide emergency myelopoiesis. Recently, our laboratory succeeded in identifying a novel marker with less fluctuation during emergency myelopoiesis. Using this unique marker, we will elucidate the mechanism of emergency myelopoiesis.

2. Research on tissue stem cells

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

We found that type I IFNs induce proliferation and exhaustion in hematopoietic stem cells (HSCs), and that interferon regulatory factor-2 (IRF2), a transcriptional suppressor of type I IFN signaling, preserves the self-renewal and multi-lineage differentiation capacity of HSCs (***Nat Med*** 2009). Based on this finding, we show that type I IFN preconditioning, without irradiation or DNA alkylating agents, significantly enhanced the HSC engraftment efficiency in wild type (WT) recipient mice (***Blood*** 2013). Based on these achievements, we have further found that physiological levels of type I IFN signaling also affect other tissue stem cells (submitted).

2) Establishment of biobank for human tongue cancer

Oral cancer has an increasing trend of 270,000 new cases per year worldwide. Two-thirds of them are tongue cancers, and in advanced cases, they become refractory to treatment and have a poor prognosis, and causal genes have not been identified. Under these backgrounds, we succeeded in establishing a human tongue cancer organoid culture system. In the future, we aim to develop fundamental technologies that lead to personalized treatment.

Publications

1. Wang Z, Adachi S, Kong L, Watanabe D, Nakanishi Y, Ohteki T, Hoshi N and Kodama Y. Role of eosinophils in a murine model of inflammatory bowel disease. ***Biochem Biophys Res Commun*** doi:10.1016/j.bbrc.2019.02.056 [Epub ahead of print]
2. Nakanishi Y, Sato T and Ohteki T. IFN-γ-dependent epigenetic regulation instructs colitogenic monocyte/macrophage lineage differentiation in vivo. ***Mucosal Immunol*** 11, 871-80 (2018)

Personnel Changes
Moving out:
Asano J, Assistant Professor of Seirei Women's Junior College, Akita, Japan.

Department of Bio-informational Pharmacology

Professor	Tetsushi Furukawa, M.D., Ph.D.
Associate Professor	Jun Takeuchi, Ph. D.
Assistant Professor	Kensuke Ihara, M.D., Ph.D.
Post-doctoral Fellow	Hiroko Kushige, Ph. D.
	Masahiro Yamazoe, M.D., Ph.D.
	Yoshitake Higashijima, 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. Biological pacemaker utilizing in vivo genome editing

In the current clinical medicine, the only therapy for bradycardia is the electrical pacemaker, which has many serious problems such as battery longevity, device infection. As an alternative, biological pacemaker has been studied for decades. Previous attempts to make biological pacemaker by gene delivery had a limitation that the effect was transient. Application of genome editing by CRISPR/Cas9 is expected to generate permanent gene knockout. We found that in vivo genome editing can generate pacemaker activity, and are now optimizing this technique for better efficacy.

2. Systemic inflammation accompanied with atrial fibrillation

Various inflammatory markers and mediators such as c-reactive protein (CRP) and pro-inflammatory cytokines have been reported to be linked with the presence and outcome of atrial fibrillation (AF). These systemic inflammatory responses could cause and worsen not only AF related systemic dysfunctions including coagulation activity and endothelial dysfunctions, but also AF pathogenesis through atrial electrical and structural remodeling. However, the underling mechanism why AF is accompanied with systemic inflammation remains to be elucidated.

We focused the nucleic acid circulating in blood as potential contributor for systemic inflammation accompanied with AF. First, we found that AF patients had higher nucleic acid levels in plasma compared with non-AF subjects. We also confirmed this finding in AF mimicking models; rapid tachycardia stimulation for cultured atrial cardiomyocytes, and murine right atrium. Second, we observed that nucleic acid promoted IL-1β and IL-6 expression in macrophages and clarified its signal pathway. Additionally, we identified the essential characters of nucleic acids in inducing pro-inflammatory cytokines in macrophages. Taken together, we revealed the potential contributor for systemic inflammation accompanied with AF, suggesting the circulating nucleic acid might be the novel biomarker for AF occurence and development.

3. Understanding the mechanisms of heart induction and its compartment:

The mammalian heart has systematic four-chamber structures including the left-right/atria-ventricular with functional differences. These complicated morphologies in mammals create difficult questions in addressing the mechanisms of human heart diseases. To answer these questions, many scientists initially generated knockout mice, before establishing the methods for *in vitro* CM induction from embryonic stem cells (ESCs) or induced pluripotency stem cells (iPSCs). However, the general methods for cardiac cell induction are insufficient to completely produce the chamber-like heart structures with regional identities like the embryonic heart. Furthermore, no information has been reported about the differences of the heart structures, physiological functions and transcriptional regulations between in human and in mice. To address these questions, we performed two projects; 1. the cardiac induction and 2. the heart chamber formation.

4. Epigenetic transcription regulation of inflammation-related genes in vascular endothelial cells

The spatial organization of the genome is functionally linked to gene expression programs. Pro-inflammatory stimuli elicit rapid transcriptional responses via transduced signals to master transcription factors, but it is largely unknown how the genome and epigenome spatially control gene expression during early inflammation. Here, we performed Hi-C in combination with chromatin interaction analysis by paired-end tag sequencing (ChIA-PET) using anti-active RNA polymerase II antibody and found that inflammatory gene expression in human endothelial cells (ECs) is controlled by newly formed chromatin interactions between tumor necrosis factor alpha (TNF-α)-induced super-enhancers (SEs). Importantly, these SE-SE loops (approximately 200 to 500 kb length) are formed within 1 hour after TNF-α-treatment although megabase-size toplogically associating domains (long interactions) are unchanged. We also found that lysine demthylase 7A (KDM7A) and 6A (UTX) are rapidly mobilized to TNF-α-induced SEs where nuclear factor kappa-B are highly occupied, and demethylate their H3K9me2 and H3K27me3 marks, respectively, and are responsible for rapid formation of SE-SE loops. Collectively, these findings suggest that erasing of repressive histone marks by KDM7A and UTX within NFκB-related elements might functionally associate with formation of SE-SE loops and could be a cue signal during inflammatory responses in human endothelial cells.

Highlight

Electrophysiological Assessment of Murine heart with High-Resolution Optical Mapping (Figure)
Conventional optical mapping of murine heart, especially of its atria, has some critical problems due to its small size. To overcome them, we developed the novel electrophysiological assessment method for elucidating the underlying mechanism of arrhythmogenesis using murine heart by combining high spatial and temporal resolution optical mapping system and precise electrophysiological study (*J. Vis. Exp* 2018). This novel method will contribute to assessing the onset and maintenance mechanism of arrhythmias precisely in various mouse models.

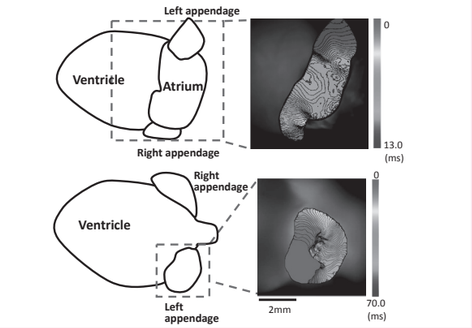

Figure. High-resolution optical mapping of mouse atrium
Upper: During normal sinus rhythm (lower surface of the heart)
Lower: During re-entrant atrial tachycardia (upper surface of the heart)

Publications

[original articles]

1. Natusme Y, Oaku K, Takahashi K, Nakamura W, Oono A, Hamada S, Yamazoe M, Ihara K, Sasaki T, Goya M, Hirao K, Furukawa T, Sasano T. Combined analysis of human and experimental murine samples identified novel circulating microRNAs as biomarker for atrial fibrillation. *Circ. J.*, 2018;82:965-973.
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Department of Stem Cell Regulation

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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 2018 are mainly on two subjects: 1. Characterization of fetal hematopoiesis, and 2. Characterization of cancer stem cells and their niche.

Research Projects

1. Maintenance of hematopoietic stem and progenitor cells in fetal intra-aortic hematopoietic cell clusters in the AGM region as a site of definitive hematopoiesis by the Sox17-Notch1-Hes1 axis.

The aorta-gonad-mesonephros (AGM) region, from which definitive hematopoiesis firstly arises in midgestation mouse embryo, has intra-aortic hematopoietic cell clusters (IAHCs) containing hematopoietic stem cells (HSCs) and hematopoietic progenitor cells (HPCs). We have previously reported that a transcription factor Sox17 is expressed in the IAHCs and that overexpression of Sox17 in CD45^{low}c-Kit^{high} cells comprising IAHCs maintained the formation of such cell clusters and multipotency in vitro during several passages. Recent reports by another group showed that Sox17 induces the *Notch1* promoter activity, but the underlying mechanisms and the role remained to be elucidated. We thus examined the importance of Notch1 in IAHC maintenance of the HSC/HPC phenotype. We showd that Notch1 expression is positively regulated by Sox17 via direct binding to its gene promoter. Sox17 and Notch1 were both found to be expressed in vivo in cells of IAHCs by whole mount immunostaining. When CD45^{low}c-Kit^{high} cells comprising IAHCs were transduced with the active form of Notch1 (NICD), they maintained the multipotent colony-forming activity in semisolid media during at least three passages. Moreover, cells stimulated by Notch1 ligand, Jagged1, or Delta-like protein 1, had the capacity to form multilineage colonies. We also examined the function of Hes1, which is known as a target gene of Notch1, in the IACCs. Hes1-transduced cells had a capacity to form multilineage colonies and expressed marker genes for the undifferentiation state. In support of this, knockdown of Notch1 and Hes1 in Sox17-transduced cells led to a reduction of their multipotent colony-forming capacity. These results suggest that the Sox17-Notch1-Hes1 pathway is critical for maintaining the undifferentiated state of IAHCs. (Figure 1).

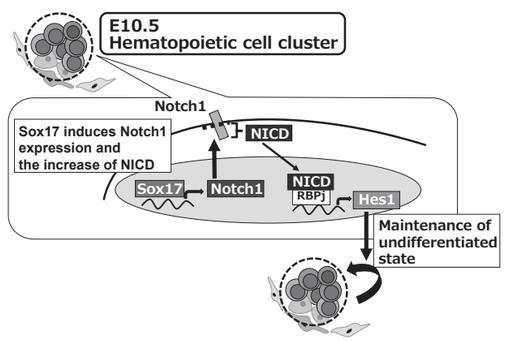

Figure 1: Model of the function of the Sox17-Notch1-Hes1 axis in intra-aortic hematopoietic cell clusters.

2. Elucidation of self-organized glioma stem cell niche

Glioblastoma, also known as GBM, is the most malignant glioma classified as WHO grade IV according to the defining histologies, necrotic foci and neovascularization. "Cancer stem cells" (CSCs), a small subset of tumor cells, are characterized by chemo/radio-resistance and have the ability to reconstitute original tumors. Therefore, CSCs are a key driver of tumor relapse and have been proposed as a promising target to eradicate cancers. As we published in 2004, 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. Recently we have reported that glioma CSCs (GSCs) have a self-expanding strategy that facilitate the development of CD204(+) and CD11c(+) tumor-associated macrophages (TAMs) through secreting a cytokine GM-CSF. In this year, we aimed to establish new therapeutic strategies especially by focusing on the roles of GSC-derived necrotic particles on the development of tumor-associated macrophages (TAMs). When incubated with GM-CSF-primed macrophages (Mϕs)/dendritic cells, SP-derived dead cell particles were specifically phagocytosed a minor subpopulations of CD204(+) and CD11c(+) Mϕs, and their absolute number was significantly increased. Interestingly, *Il12b* gene encoding a common subunit composing IL-12 and IL-23 cytokines was identified as a specific marker upregulated in dead cell-educated Mϕs, and IL-12 not IL-23 protein evidently enhanced the sphere-forming ability of GBM patient-derived cells. Finally, database analysis of gene expression in glioma patients demonstrated that the recurrent GBM patients with higher expression of *Il12b* gene exhibit significantly poorer survival than those with lower expression, suggesting that GSCs contribute to tumor progression by affecting TAM phenotypes through their own cell death. These findings could provide clues to understand the pathobiology of GSC-organized niche and to establish effective therapeutic strategies for cancer eradication (Figure 2).

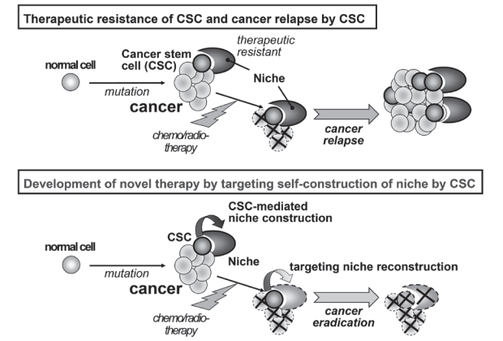

Figure 2: The concept of CSC-mediated niche construction and cancer eradication

Publications

[Original Article]

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2. Saito K, Nobuhisa I, Harada K, Takahashi S, Anani M, Lickert H, Kanai-Azuma M, Kanai Y, Taga T. Maintenance of hematopoietic stem and progenitor cells in fetal intra-aortic hematopoietic clusters by the Sox17-Notch1-Hes1 axis. Exp. Cell Res., 365:145-155, 2018

Department of Structural Biology

Professor Nobutoshi Ito
Associate Professor Teikichi Ikura
Assistant Professor Nobutaka Numoto

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. Crystal structure analysis of the complex of the vitamin D receptor and novel synthetic vitamin D_3 derivative compounds

Nuclear receptors bind lipophilic small molecules called hormones, and regulate transcription of genes. Vitamin D receptor (VDR) plays an essential role in regulating calcium and phosphate homeostasis in the body. It has been reported that active form of vitamin D_3 promotes bone anabolic activity by activating VDR, so that VDR is recognized as an effective target for the treatment of osteoporosis. Actually, derivatives of the activated vitamin D_3 are used as therapeutic agents for osteoporosis, but their use is limited due to hypercalcemia. Therefore, it is desired to further improvement in the molecular structure of the activated vitamin D_3 so as to improve its effect on treatment of osteoporosis while reducing undesirable side effects. We are continuously working on the three-dimensional structure analysis of complexes of the various derivatives of the activated vitamin D_3 and VDR. Recently, ADRO1, which activates VDR in a tissue selective manner has been developed. We have determined the crystal structure of VDR complexed with ADRO1 to elucidate the details of the ligand binding mode of ADRO1.

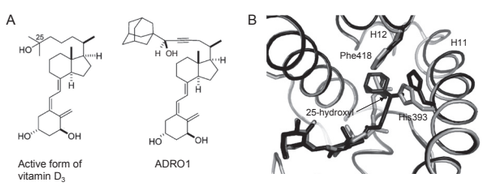

Fig.1 A) Structural formulas of active form of vitamin D_3 and ADRO1. B) Superposition of the structures of the VDR complexed with ADRO1 (black) and activated vitamin D_3 (grey).

ADRO1 aims to promote the structural change of the helix region (H11 and H12), which are important for activation of VDR, by modifying the methyl group of activated vitamin D_3 to a larger substituent, adamantyl group (Fig. 1A). It was also designed to enhance the rigidity of the molecule by introducing a triple bond. Evaluation of the affinity of ADRO1 to VDR exhibited slightly lower value than that of activated vitamin D_3, similar to the known partial agonists. However, the ability of ADRO1 to activate VDR assessed by the endogenous gene expression for the various tissue cells demonstrated significant selectivity. Particularly in kidney-derived cells, the activity was about 1.6 times higher than that of activated vitamin D_3. In contrast, the activity was markedly reduced in the intestine and skin derived cells.

The three-dimensional structure of the complex of ADRO1 and the ligand binding domain of rat VDR was solved by X-ray crystal structure analysis. Comparison between the obtained structures with that of activated vitamin D_3 binding complex reveals that the structures of H11 and H12, which are thought to be responsible for the activity of VDR, are changed as a result of introduction of a bulky adamantyl group in ADRO1. In particular, orientation of the side chain of His393 of H11 is largely rearranged and the position of the main chain Cα atom also moved (Fig. 1B), due to the induced fit for the bulky adamantyl group. In addition, Phe418 of H12 slightly changed the orientation. We have also clarified that these structural changes cause the difference of the electrostatic potential distribution on the molecular surface around the H11 region of VDR. It is strongly suggested that these structural changes would affect the tissue-selective VDR activation by ADRO1. We are conducting further studies to clarify the more detailed mechanism of the selective activity of the vitamin D_3 derivatives.

This work is performed under collaboration with Nihon University, University of Santiago de Compostela (Spain), and Rikkyo University.

2. Molecular mechanism of Alzheimer's disease

In Alzheimer's, the disease-related protein, Tau, is hyperphosphorylated and aggregates into neurofibrillary tangles. *Cis* isomer of the phosphorylated Thr231-Pro232 has been proposed as a precursor of aggregation ("Cistauosis") but this aggregation scheme is not yet completely accepted. In the present study, we synthesized peptides comprising a phosphorylated region including Thr231-Pro232 and an aggregation-core region R1 to investigate the isomer-specific aggregation of Tau. The phosphorylated peptide formed amyloid-like aggregation (Fig. 2). This aggregation was observed even in the presence of the catalytic domain of the peptidyl-prolyl-isomerase Pin1, which preferentially converts the *cis* isomer to the *trans* isomer, but decreased drastically in the presence of the WW domain of Pin1 selectively binding to the *trans* isomer. These results indicate that the *trans* isomer is aggregation-prone and that the WW domain of Pin1 effectively inhibits its aggregation. Consequently, the present experimental results obviously deny the "cistauosis" hypothesis in which the *trans* isomer is supposed not to aggregate. This work is performed in collaboration with several research groups at Hiroshima University, Nagoya University and Teikyo University.

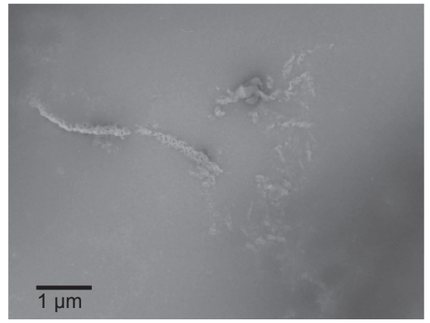

Fig.2 Electron microscopic image of amyloid-like aggregation of the phospho-Thr231-Pro232 peptide after 1-month incubation.

3. Improvement in Protein Data Bank

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

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3. Rocio Otero, Michiyasu Ishizawa, Nobutaka Numoto, Teikichi Ikura, Nobutoshi Ito, Hiroaki Tokiwa, Antonio Mouriño, Makoto Makishima, Sachiko Yamada. Synthesis, Tissue Selective Biological Activities, and X-ray Crystal Structural Analysis of Its Vitamin D Receptor Complex. J. Med. Chem. 2018. 61, 6658-6673.
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Frontier Research Unit
Laboratory of Oxygen Biology

Associate Professor Koh Nakayama, Ph.D.
Assistant Professor Ryo Yonashiro, Ph.D.

Aim of the Research

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

Research Projects

1. Molecular mechanism of oxygen sensing

Hypoxia-Inducible Factor (HIF)-α is a transcription factor which plays a central role during hypoxic response. HIF-α is actively degraded during normoxic condition, whereas it is stabilized and activated under hypoxic conditions. PHD hydroxylates and regulates the expression of HIF-α. There are three PHDs in mammals; namely PHD1, 2, and 3. We have previously identified that PHD3 forms a complex under hypoxic conditions (Figure). This complex serves to stabilize HIF-α by inhibiting the access of PHD3 to HIF-α.

Moreover, we recently identified that pyruvate dehydrogenase (PDH) is included in this complex (Figure). PDH is an enzyme which converts pyruvate into acetyl-CoA, and plays a key role in energy metabolism. We have demonstrated that PDH interacts with PHD3 under hypoxic condition. In PHD3$^{-/-}$ cells, PDH activity is significantly decreased, suggesting that PHD3 positively regulates PDH activity by directly interacting with it. Cancer cells are known to often exhibit glycolytic metabolism; however, the molecular mechanism of how such metabolism is formed has not been elucidated yet. We aim to understand the mechanism by focusing on the PHD3-PDH interaction mediated by the hypoxia complex.

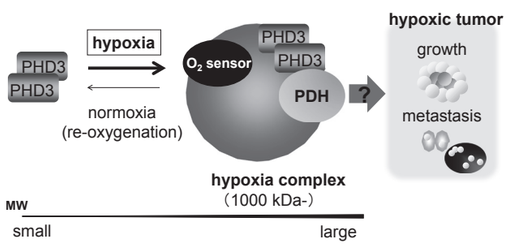


Figure Oxygen Sensing mediated by Hypoxia Complex

2. Pyruvate dehydrogenase PDH: an important regulator of cancer metabolism

We further analyzed the role of PDH in hypoxia and identified that PDH-E1β subunit is downregulated during chronic hypoxia. This downregulation is sustained upon reoxygenation, therefore causing glycolytic metabolism in breast cancer cells even under normoxic condition. Furthermore, knockdown (KD) of PDH-E1β in cancer cells caused a Warburg effect-like metabolism, which also points to the importance of PDH-E1β downregulation on inducing glycolytic metabolism in cancer cells. Finally, PDH-E1β-KD cells formed smaller tumor than the control cells in nude mice, indicating that sustained glycolytic metabolism is not sufficient to promote tumor growth, and implies a possibility of cancer cells also utilizing oxidative phosphorylation for their efficient energy production.

Publications

1. Yonashiro R., Eguchi K., Wake M., Takeda N., and Nakayama K. Pyruvate dehydrogenase PDH-E1β is downregulated under prolonged hypoxic conditions and controls tumor progression by altering the metabolic status of cancer cells. ***Cancer Res***. 78:1592-1603, (**2018**)

Frontier Laboratory: Skeletal Molecular Pharmacology

Associate Professor Yoichi Ezura, MD, PhD

Summary

This laboratory aims to elucidate the molecular regulation of the cellular responses in organs and tissues involved in calcium regulatory system. We focus on obtaining knowledge contributing to establish the methods of prevention and treatment of skeletal rare diseases and related common diseases including osteoporosis.

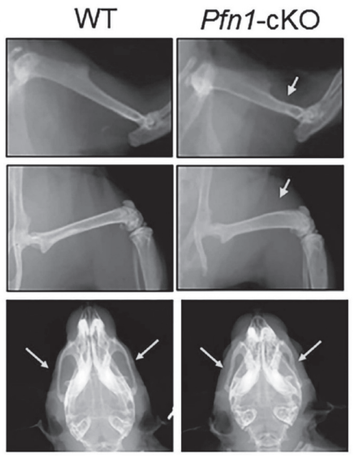
Research Project

1. Annexin A5 suppresses the enthesis bony overgrowth

(Collaboration with Dr. Akira Nifuji at Tsurumi University)

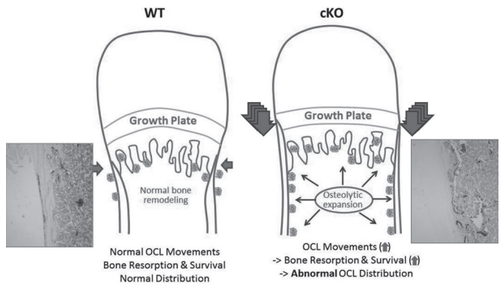
2. Dok-3 supresses exceeded mutual fusion of osteoclast precursors that is required for osteoclastogenesis

(Collaboration with Dr. Yuji Yamanashi at The Institute of Medical Science, The University of Tokyo)



3. Profilin1 in osteoclasts play important suppressive role in their movements and bone resorption

Profilin1 (Pfn1), one of the actin filament polymerizing regulators assists elongation of filopodia. We reported that skeletal precursor cells, osteocytes and chondrocytes depend on Pfn1 for their locomotive function or elongation of their characteristic processes. However, here we reported that Pfn1 deficiency in osteoclasts resulted in enhanced locomotion and bone resorption associated with abnormal distribution of osteoclasts in deformed long bones and facial bones during postnatal growth. Our findings are consistent with recent theory revealing a structural dependency of the actin filament elongation by Pfn1. Our mutant line would provide an useful model for congenital osteolysis of human disorders.



Publications

[Original articles]
1. Shimada A, Ideno H, Arai Y, Komatsu K, Wada S, Yamashita T, Amizuka N, Pöschl E, Brachvogel B, Nakamura Y, Nakashima K, Mizukami H, Ezura Y, Nifuji A*. Annexin A5 involvement in bone overgrowth at the enthesis. J Bone Miner Res. 2018 33(8):1532-1543
2: Kajikawa S, Taguchi Y, Hayata T, Ezura Y, Ueta R, Arimura S, Inoue JI, Noda M, Yamanashi Y*. Dok-3 and Dok-1/-2 adaptors play distinctive roles in cell fusion and proliferation during osteoclastogenesis and cooperatively protect mice from osteopenia. Biochem Biophys Res Commun. 2018, 498(4):967-974.
3: Hayata T, Chiga M, Ezura Y, Asashima M, Katabuchi H, Nishinakamura R, Noda M*. Dullard deficiency causes hemorrhage in the adult ovarian follicles. Genes Cells. 2018, 23(5):345-356.
4: Shirakawa J, Kajikawa S, Bottcher RT, Costell M, Izu Y, Hayata T, Noda M, Ezura Y. Profilin 1 negatively regulates osteoclast migration in postnatal skeletal growth, remodeling and homeostasis in mice JBMR-Plus (accepted Oct. 2018).

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】

- Development of gene therapy against Alzheimer's disease
- Discovery of involvement of an intellectual disability gene PQBP1 in Alzheimer's disease

【Biochemical Pathophysiology】

- Lipidomic signature for cancer stratification
- Pathophysiology of phosphoinositide-related diseases

【Pathological Cell Biology】

- Discovery of essential function of Beclin 1 in skin development.
- Discovery of novel caspase substrate that regulates mitophagy.

【Developmental and Regenerative Biology】

- Shedding light on zebrafish daily rhythms: Clock gene functions revealed

【Stem Cell Biology】

- Elucidation of the mechanisms of epidermal aging

【Immunology】

- Elucidation of the crucial role of NADPH oxidase (NOX)3 in prolonged ROS production crucial for B cell activation.
- Development of a synthetic CD22 ligand that regulates antibody production.

【Molecular Pathogenesis】

- ROCK inhibitor could prevent cardiomyopathy but not arrhythmia developed in M21-overexpressing mice
- Phylogenetic analyses revealed a unique feature of ULBP genes in primates, including duplication in the lineage of Old World monkey and appearance of ULBP6 only in humans

Department of Neuropathology

Professor	Hitoshi Okazawa
Practical professor	Kazuhiko Tagawa
Project Lecturer/Part-time Lecturer	Haruhisa Inoue, Masaki Sone, Toshiki Uchihara
Assistant professor	Kyota Fujita
Project Assistant professor	Hidenori Homma, Emiko Yamanishi

Outline

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

1) Investigation of molecular pathologies of neurodegenerative disease.
2) Development of new therapies for neurodegeneration.
3) Development of new seed drug for mental retardation.
4) Investigation of molecular function of Oct3/4.

This year's progress

1. Early changes to synapse gene regulation may cause Alzheimer's disease (Tanaka H. et al., Molecular Psychiatry. 2018)

Alzheimer's disease (AD) is the most common form of dementia, involving memory loss and a reduction in cognitive abilities. Patients with AD develop multiple abnormal protein structures in their brains that are thought to destroy or damage nerve cells (neurons). One of these structures, the senile plaque, is made up of clumps of beta-amyloid (Aβ) peptide which form in the spaces between neurons.

Many advanced clinical trials in patients with AD have attempted to slow down or reverse the disease by targeting these plaques for removal. However, despite the successful decrease in Aβ aggregation, these trials mostly have failed to improve memory or cognitive function in AD patients.

Before the formation of Aβ aggregates, studies revealed changes in the phosphorylation (a chemical modification) of certain proteins, including SRRM2. This protein was thought to be involved in a form of gene regulation known as splicing, but its exact function was unclear.

Now, we have examined levels of SRRM2 phosphorylation in a mouse model of AD, and found that they increased prior to Aβ aggregation. This ultimately prevented the nuclear transport of SRRM2 and led to reduced levels of PQBP1 protein, which has been linked with the neurodevelopmental and intellectual disorders.

We showed that the increased phosphorylation of SRRM2 prevented it from interacting with another protein which aids protein folding. In the absence of this interaction, SRRM2 remained unfolded so was not transported to the nucleus and was degraded in the cytoplasm. We next measured levels of SRRM2 and PQBP1 protein in the cerebral cortex of early-phase AD mice and human end-stage AD patients as well as in human AD iPS cells. Both proteins were greatly reduced compared with corresponding amounts in healthy controls.

To find out what effect a reduction in PQBP1 would have in vivo, we generated knockout mice in which the PQBP1 gene was disrupted. We observed cognitive decline and changes in the morphology of their synapses, which are junctions between neurons that allow electrical and chemical communication. These changes were caused by disrupted patterns of synapse gene splicing.

A viral vector containing PQBP1 was used to recover the synapse protein expression in these mice. Not only did this restore PQBP1 expression, but it also recovered the abnormal phenotypes. These findings offer a new insight into early changes that occur during AD pathology involving splicing proteins, suggesting possibilities for gene therapies by virus vectors.

2. Ser46-phosphorylated MARCKS is a marker of neurite degeneration at the pre-aggregation stage in PD/DLB pathology

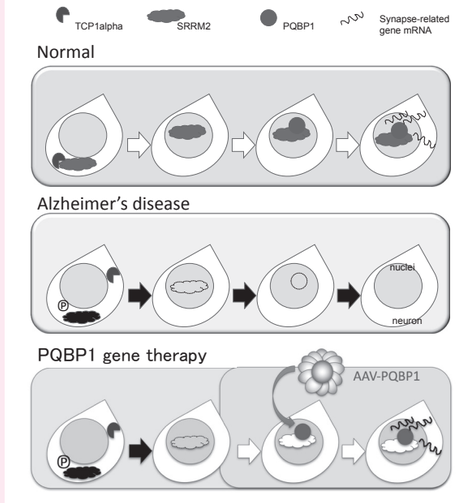
Phosphorylation of myristoylated alanine-rich C kinase substrate (MARCKS) reflects neurite degeneration at the early stage of Alzheimer's disease (AD), before extracellular Aβ aggregates are histologically detectable. In this study, we detected similar phosphorylation of MARCKS at Ser46 in BAC-Tg mice overexpressing human normal α-synuclein (α-Syn) in the glucocerebrosidase (GBA)-heterozygous-knockout (KO) background (human α-Syn-BAC-Tg/GBA-hetero-KO mice), as well as in human DLB patients. The increase in the level of pSer46-MARCKS began before α-synuclein aggregate formation, at a time when human α-Syn-BAC-Tg/GBA-hetero-KO mice exhibited no symptoms, and was sustained during aging, consistent with the pattern in human postmortem brains.

Next, we investigated the upstream kinases that phosphorylate MARCKS at Ser46. MARCKS is a representative substrate of PKC, as known from its name, myristoylated alanine-rich C kinase substrate. However, our previous experiments revealed that Erk1/Erk2 (MAPK3/MAPK1) instead of PKC phosphorylates MARCKS at Ser46. In this study, we revealed abnormal increase of Erk1/2 phosphorylation and its age-dependent enhancement in cortical neuron under the PD/DLB pathology. These results strongly imply a common mechanism of pre-aggregation neurite degeneration in AD and DLB pathologies.

Highlight

pathomechanism of SRRM2 phosphorylation at Sert1068

To understand the molecular mechanism underlying the change of subcellular localization of SRRM2, proteins binding to wild-type or phosphorylation site mutants of SRRM2 were screened by analysis of co-precipitated proteins using mass spectrometry. We revealed that phosphorylated SRRM2 caused by activated ERK1/2 lost its interaction with TCP1alpha (T-complex protein 1 subunit alpha). Next, we found that loss of interaction of TCP1alpha disables nuclear transport of SRRM2, resulting the destabilization of PQBP1 (polyglutamine binding protein 1). Concurrent deficiency of SRRM2 and PQBP1 was confirmed in human AD brains. Reduction of SRRM2 and PQBP1 impaired synapses via RNA splicing of synapse genes, which were revealed by comparison of RNA sequence analysis between 5xFAD (AD model mice) and PQBP1-cKO (neuron-specific knockout) mouse cerebral cortex. Therefore, restoration of PQBP1 gene by an adeno-associated virus (AAV) vector recovered synaptic structures and cognitive function in AD model mice.



Publications

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Department of Biochemical Pathophysiology

Professor Takehiko Sasaki, Ph.D.
Associate Professor Junko Sasaki, Ph.D.
Assistant Professor Junya Hasegawa, Ph.D.

The Department of Biochemical Pathophysiology, which began in 2018, is focused on the roles of cellular lipids in health and diseases. The unique strengths of our lab are a series of "knockout" mouse mutants for each lipid metabolizing enzyme and methods for lipid profiling based on LC-MS/MS, which help explore novel therapeutic targets and biomarkers for incurable diseases.

〈**Research Projects**〉

1. Developing predictive markers for cancer therapy by lipidomics

Phosphoinositide 3-kinases (PI3Ks) phosphorylate phosphatidylinositol 4,5-bisphosphate (PIP2) to produce phosphatidylinositol 3,4,5-trisphosphate (PIP3). Conversely, PTEN is the phosphatase that dephosphorylates the same position of the hydroxyl where PI3K phosphorylates, and converts PIP3 back to PIP2. These two enzymes or reactions play key roles in tumorigenesis and metastasis. High frequencies of gain-of-function mutations and amplification of *PIK3CA* encoding PI3K α as well as loss-of-function mutations and deletion of *PTEN* are found in various types of tumors. Given that PIP2 and PIP3 can regulate proteins involved in tumor cell proliferation, death, motility and invasion, PI3Ks are considered as potential therapeutic targets for cancers. A number of PI3K inhibitors have been developed and entered in clinical trials; however, they have so far had limited clinical success. Most studies have shown poor associations between drug responses and genetic alterations of *PI3KCA*, *PTEN* or other driver genes such as *RAS* and *HER2* encoding the upstream activators of PI3Ks. We have recently examined the fatty acyl profiles of phosphoinositides and cell death responses to a series of anti-cancer agents in lymphoma cell lines. Multivariate analyses revealed significant association of the PIP2 profiles with susceptibility to PI3K inhibitors. Our results demonstrate that PIP2 acyl signatures would be useful for distinct stratification of lymphomas from the ordinary classification that predicts clinical benefits to PI3K inhibitors.

2. Phosphoinositide metabolism to maintain femaleness

Mammalian sex is determined by the Y-linked Sry (Sex-determining region Y) gene. The supporting cell precursors of the fetal gonad differentiate into testicular Sertoli cells in the presence of Sry, while the cells differentiate into ovarian granulosa cells in the absence of Sry. These supporting cells commit to sexual differentiation of somatic and germ cells. Recent studies in mice have provided evidence that granulosa vs Sertoli cell fate decision is not necessarily permanent in postnatal life. In the adult ovary, Foxl2 (forkhead box L2) and estrogen receptors are required for maintaining granulosa cell fate by repressing male promoting signals, while in the adult testis, Dmrt1 (doublesex and mab-3 related transcription factor 1) and Sox9 (SRY-box 9) are required for maintaining Sertoli cell fate by repressing female promoting signals. Abolishing female promoting signals leads to loss of granulosa cell fate markers and up-regulation of Sertoli fate markers such as Sox9 and Dmrt1.

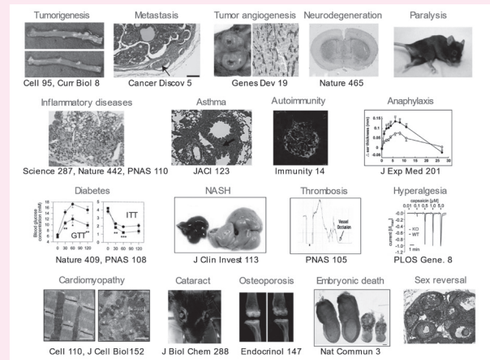
We found that conditional knockout mouse mutants lacking phosphatidylinositol 3,4,5-trisphosphate (PIP_3) phosphatases exhibited female infertility. Histological examination of the mutant ovaries revealed that Sertoli-like cells emerged in the follicles. The female mutant mice had significantly higher levels of testosterone in the serum. Our results demonstrate that PIP_3 metabolism plays a key role in cell fate determination towards granulosa cells in the ovaries, and propose a possible etiology of DSD, disorders of sex development.

3. Lysosomal signaling properties of phosphoinositides

Endosomes and autophagosomes fuse with lysosomes to degrade the contents including lipids and proteins for reuse. Importantly, recent studies show that lysosome functions as a platform for intracellular signaling pathways including mechanistic target of rapamycin complex 1 (mTORC1), a master regulator of autophagy and growth signals. mTORC1 can be activated upon translocation from the cytoplasm to the surface of lysosomes, where mTORC1 phosphorylates the downstream substrates such as p70S6K and 4E-BP1 to promote transcription of several growth-related genes, also phosphorylates ULK1 to inhibit autophagy. We have found that translocation of mTORC1 to lysosomes is abolished in cells deficient in a lysosomal phosphoinositide phosphatidylinositol 3,5-bisphosphate. Our findings demonstrate a novel mechanism of the regulation of mTORC1. Lipid species consisting of lysosomal membrane have been unclear. By virtue of our original lipidomic technologies, we are currently analyzing lipid species consisting of lysosomal membrane to understand the signaling properties of the organelle in detail.

Highlight

We have recently devised a new method for quantification and identification of phosphoinositides that have different fatty-acyl compositions. In the traditional method, phosphoinositides are radioactively labelled in cultured cells. Labelled lipids are isolated from cells by Bligh-Dyer extraction, deacylated using methylamine, and the resulting glycerophosphoinositol phosphates are then analyzed by anion-exchange chromatography. This existing method is very effective for the relative quantification of the 8 differentially phosphorylated forms of phosphoinositides in a biological sample; however, it is not applicable to clinical samples or cannot provide any structural information on the fatty acids.
In the study of lipidomics or metabolomics for lipids, electrospray ionization mass spectrometry is becoming increasingly popular. However, analysis of phosphoinositide is more difficult than that of other phospholipid classes, because they have multiple phosphate moiety and they are present at very low abundance in cellular lipid extracts. We devised techniques to overcome the difficulties brought about by these natures including the greater risk of losing phosphoinositides through adherence to the surfaces of tubes and tips during LC-MS, ion suppression of phosphoinositides in the presence of other major phospholipid classes, and the lower ionization/detection efficiencies of phosphoinositides as compared with other phospholipids. Comprehensive measurement of phosphoinositides were achieved by combining these techniques and "selected reaction monitoring" by a HPLC/triple quadrupole mass spectrometer. The lower limits of detection and quantification were 100 amol and 1 fmol for phosphatidylinositol, and 1 fmol and 5 fmol for phosphatidylinositol 3,4,5-trisphosphate (PIP3), respectively. By virtue of this highly sensitive and non-RI method for the measurement at molecular species level, we are currently trying to identify phosphoinositide "finger prints" that can be used as biomarkers for diagnosis, prognosis and stratification of incurable diseases such as cancers.



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5. Kimura H, Matsuyama Y, Araki S, Koizumi A, Kariya Y, Takasuga S, Eguchi S, Nakanishi H, Sasaki J, Sasaki T. The effect and possible clinical efficacy of in vivo inhibition of neutrophil extracellular traps by blockade of PI3K-gamma on the pathogenesis of microscopic polyangiitis. Mod Rheumatol. 28(3):530-541, 2018

Department of Pathological Cell Biology

Professor — Shigeomi SHIMIZU
Junior Associate Professor — Satoko ARAKAWA
Project Junior Associate Professor — Masatsune TSUJIOKA, Satoru TORII
Assistant professor — Shinya HONDA
Project Assistant Professor — Hirofumi YAMAGUCHI , Michiko MUROHASHI, Min Kyong SHIN, Hajime SAKURAI

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, Analysis of Atg5/Atg7-independent alternative macroautophagy.

Atg5 and Atg7 are considered to be essential molecules for the induction of autophagy. However, we found that cells lacking Atg5 or Atg7 can still form autophagosomes/autolysosomes and perform autophagic protein degradation when subjected to certain types of stress. Unlike conventional autophagy, autophagosomes appeared to be generated in a Rab9-dependent manner by the fusion of the phagophores with vesicles derived from the *trans*-Golgi and late endosomes. Therefore, mammalian autophagy can occur via at least two different pathways; the Atg5/Atg7-dependent conventional pathway and an Atg5/Atg7-independent alternative pathway.

In this year, we established keratinocyte-specific Beclin 1-knockout mice. Beclin 1 is a key regulator of multiple trafficking pathways, including autophagy and endocytosis. These mutant mice died within a day after birth owing to severe impairment of their epidermal barrier. Beclin 1 plays a role in autophagy in cooperation with Atg14, and in the endocytic pathway in cooperation with UVRAG, and $Atg14^{flox/flox}$/K5-cre mice do not show any abnormal phenotypes, suggesting that Beclin 1 plays a role in skin development via the endocytic pathway. Furthermore, we found that Beclin 1 deficiency causes mislocalization of integrins via a defect of recycling endosome, abnormal cell detachment of basal cells and their immature differentiation, and abnormal skin developmental. These phenotypes are reproduced in Beclin 1-silenced cultured keratinocytes. These results are the first genetic evidence showing the roles of Beclin 1 in recycling endosome and skin development.

2, Molecular mechanisms of programmed cell death

Programmed cell death, which is required for the development and homeostasis of metazoans, includes mechanisms such as apoptosis, autophagic cell death, and necrotic death. Apoptosis is carried out by the caspase activation and following substrates digestion. In this year, we discovered that PARL, a mitochondrial intramembrane serine protease, was directly cleaved by caspases during apoptosis, despite it is supposed to be protected from cytosolic caspases due to its localization. Mitochondria-mediated apoptosis is mediated by mitochondrial outer membrane permeabilization (MOMP). However, PARL was also cleaved in MOMP-independent apoptosis, excluding the possibility that caspases enter into mitochondria thorough MOMP and cleave PARL. The PT pore, another type of mitochondrial pore, was not involved in PARL cleavage as well. Instead, it was suggested that the mitochondrial inner membrane containing PARL was exposed from mitochondria when apoptosis was executed. Because full-length, but not caspase-cleaved, PARL could process Pink1, and cleaved PARL still has an serine protease activity, cleaved PARL should fail to recognize its substrates.

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.

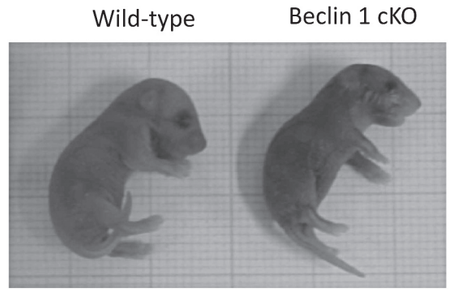

Figure1. Gross appearance of Beclin 1 cKO neonatal mice is shown.

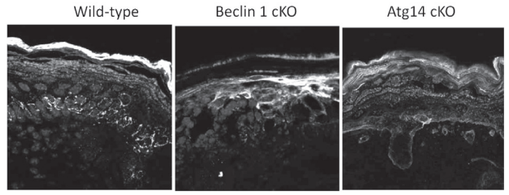

Figure2. Aberrant epidermal differentiation in Beclin 1 cKO mice, but not Atg14 cKO mice.
Immunostaining of dorsal skin of E18.5 embryos. Frozen sections were immunostained with anti-K5 (green) and anti-filaggrin (red) .

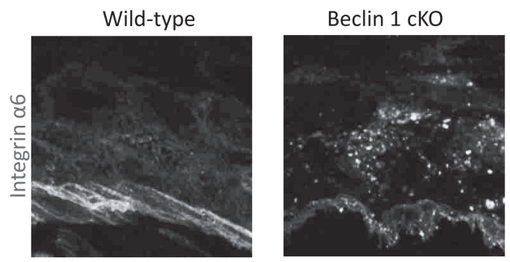

Figure3. Mislocalization of integrin a6 in the epidermis of Beclin 1 cKO mice.

List of Publications

[Original paper]

1. Dram1 regulates DNA damage-induced alternative autophagy. *M. Nagata*, *S. Arakawa*, *H. Yamaguchi*, *S. Torii*, *H.Endo*, *M. Tsujioka*, *S. Honda*, *Y. Nishida*, *A. Konishi*, *S. Shimizu*. **Cell Stress** 2: 55 - 65, 2018
2. Prediction of intracellular targets of a small compound by analyzing peptides presented on MHC class I. *Y. Sugimoto*, *M. Murohashi*, *S. Arakawa*, *S. Honda*, *S. Shimizu*. **BBRC** 508: 480-486, 2019
3. Role of Cyclooxygenase-2/Prostaglandin E2/Prostaglandin E Receptor 4 Signaling in Cardiac Reprogramming. N. Muraoka, K. Nara, F. Tamura, H. Kojima, H. Yamakawa, T. Sadahiro, K. Miyamoto, M. Isomi, S. Haginiwa, H. Tani, S. Kurotsu, R. Osakabe, S. Torii, S. Shimizu, H. Okano, Y. Sugimoto, K. Fukuda, M. Ieda . **Nature Commun**. 10: article No. 674, 2019
4. Beclin 1 regulates recycling endosome and is required for skin development in mice. *S. Noguchi*, *S. Honda*, *T. Saitoh*, *H. Matsumura*, *E. Nishimura*, *S. Akira*. *S. Shimizu*. **Communications Biology** *inpress* 2019

Department of Developmental and Regenerative Biology

Professor	Hiroshi Nishina, Ph.D.
Lecturer	Kengo Honma, Ph. D.
Assistant Professor	Erika Ishihara, Ph.D.
Research Assistant Professor	Yukari Mori, M.D., 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 he development of new rational therapy for the diseases.

1. SAPK/JNK Signaling Pathway

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

2. Hippo Signaling Pathway

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

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

3. Circadian Clock

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

Highlight

The presence of senescent, transformed or damaged cells can impair tissue function or lead to tumorigenesis; therefore, organisms have evolved quality control mechanisms to eliminate them. Here, we show that YAP activation induced by inactivation of the Hippo pathway specifically in damaged hepatocytes promotes their selective elimination by using *in vivo* mosaic analysis in mouse liver. These damaged hepatocytes migrate into the hepatic sinusoids, undergo apoptosis and are engulfed by Kupffer cells.

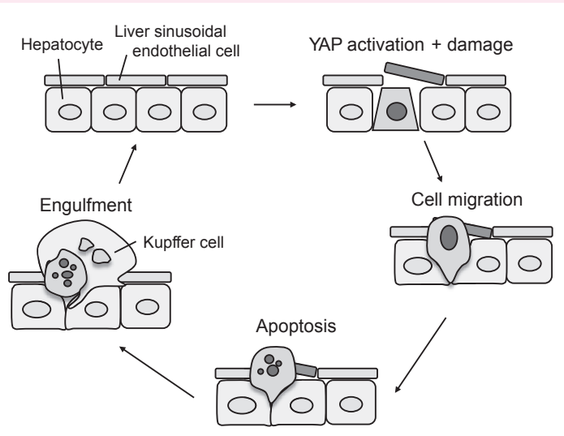

Fig.1.Model of YAP-induced damaged hepatocyte elimination.

Publications

1. Erika Ishihara and Hiroshi Nishina (2018) The Hippo-YAP pathway regulates 3D organ formation and homeostasis. ***Cancers*** 2018, 10(4), 122.
2. Hiroki Goto, Miki Nishio, Yoko To, Tatsuya Oishi, Yosuke Miyachi, Tomohiko Maehama, Hiroshi Nishina, Haruhiko Akiyama, Tak Wah Mak, Yuma Makii, Taku Saito, Akihiro Yasoda, Noriyuki Tsumaki, and Akira Suzuki (2018) Loss of *Mob1a/b* in mice results in chondrodysplasia due to YAP1/TAZ-TEADs-dependent repression of SOX9. ***Development*** 145, dev159244.
3. Asami Kawasaki, Masayasu Okada, Atsushi Tamada, Shujiro Okuda, Motohiro Nozumi, Yasuyuki Ito, Nozomu Yoshioka, Daiki Kobayashi, Manabu Abe, Tokiwa Yamasaki, Ryo Yokoyama, Takeshi Shibata, Yutaka Yoshida, Yukihiko Fujii, Kenji Sakimura, Hiroshi Nishina, Kosei Takeuchi, and Michihiro Igarashi Growth cone phosphoproteomics reveals that GAP-43 phosphorylated by JNK is a marker of axon growth and regeneration. ***iScience*** 4, 190-203.
4. Junichi Maruyama, Kazutoshi Inami, Fumiyoshi Michishita, Xinliang Jiang, Hiroaki Iwasa, Kentaro Nakagawa, Mari Ishigami-Yuasa, Hiroyuki Kagechika, Norio Miyamura, Jun Hirayama, Hiroshi Nishina, Daichi Nogawa, Kouhei Yamamoto, and Yutaka Hata (2018) Novel YAP1 Activator, Identified by Transcription-based Functional Screen, Limits Multiple Myeloma Growth. ***Molecular Cancer Research*** 16 (2), 197-211.
5. Yoko Shinagawa-Kobayashi, Kenya Kamimura, Ryo Goto, Kohei Ogawa, Ryosuke Inoue, Takeshi Yokoo, Norihiro Sakai, Takuro Nagoya, Akira Sakamaki, Satoshi Abe, Soichi Sugitani, Masahiko Yanagi, Koichi Fujisawa, Yoshizu Nozawa, Naoto Koyama, Hiroshi Nishina, Makoto Furutani-Seiki, Isao Sakaida, Shuji Terai (2018) Effect of histidine on sorafenib-induced vascular damage: Analysis using novel medaka fish model. ***Biochem. Biophys. Res. Commun***. 496, 556-561.
6. Ryosuke Inoue, Kenya Kamimura, Takuro Nagoya, Norihiro Sakai, Takeshi Yokoo, Ryo Goto, Kohei Ogawa, Yoko Shinagawa-Kobayashi, Yukari Watanabe-Mori, Akira Sakamaki, Satoshi Abe, Hiroteru Kamimura, Norio Miyamura, Hiroshi Nishina and Shuji Terai (2018) Effect of Neural Relay on Liver Regeneration in Mice: Activation of Serotonin Release from Gastrointestinal Tract. ***FEBS Open Bio*** 8, 449-460.
7. Norio Miyamura and Hiroshi Nishina (2018) [review] YAP regulates liver size and function. ***Cell Cycle*** 17(3), 267-268.
8. Norio Miyamrua and Hiroshi Nishina (2018) [book] Molecular Mechanisms of Liver Development: Lessons from Animal Models. ***Stem Cells and Cancer in Hepatology*** (Yun-Wen Zheng, eds) pp1-20, Academic Press, London.

Department of Stem Cell Biology

Professor	Emi K. Nishimura, M.D., Ph. D.
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Assistant Professor	Hiroyuki Matsumura, Ph. D.
Project Assistant Professor	Yasuaki Mohri, Ph. D., Hironobu Morinaga, Ph. D., Kyosuke Asakawa, Ph. D.

Stem cell systems play fundamental roles in sustaining tissue turnover and homeostasis. Our goal is to understand the mechanisms of tissue homeostasis driven by stem cell systems in mammals and to apply that knowledge to better understand the mechanisms underlying tissue/organ aging, cancer development and other diseases associated with aging. We further aim to apply this knowledge to drug discovery, regenerative medicine and the prevention and treatment of age-associated diseases.

1) Identification of stem cells in the skin

The skin is the largest organ in the body. Hair follicles are mini-organs located in the skin that 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 in each hair cycle. We previously identified the source of those melanocytes, "melanocyte stem cells" (McSCs), which are located in the hair follicle bulge and supply mature melanocytes required for hair and skin pigmentation (Nishimura EK et al. Nature, 2002). Subsequently, we identified similar McSCs in non-hair-bearing skin areas (Okamoto N et al. PCMR, 2014). Further, we recently succeeded in identifying epidermal stem cells with sufficient self-renewing potential by using genetic tracing of stem cell clones (Liu N et al. Nature, *in press*).

2) Mechanisms of stem cell maintenance

The underlying mechanisms of stem cell maintenance are a fundamental issue in stem cell biology and medicine. We previously found that the niche microenvironment plays a dominant role in the fate determination of McSCs (Nishimura EK et al. Nature, 2002). That finding prompted us to further study the mechanisms involved and led us to demonstrate that hair follicle stem cells (HFSCs), which reside in the hair follicle bulge, serve as a functional niche for the maintenance of McSCs (Nishimura EK et al. Cell Stem Cell, 2010) (Tanimura S et al. Cell Stem Cell, 2011). The niche functions of HFSCs are mediated by extrinsic niche factors, including transforming growth factor β (TGF-β), that are secreted from HFSCs to maintain McSCs in a quiescent and immature state. Meanwhile, intrinsic defects in stem cells, such as those caused by *Mitf* or *Bcl2* deficiencies in mice, also induce the depletion of McSCs, which leads to the progressive expression of the hair graying phenotype. Therefore, we concluded that the incomplete maintenance of McSCs either by defective signaling from the stem cell niche or by intrinsic defects in stem cells, results in an insufficient supply of mature melanocytes for hair pigmentation in mice expressing the progressive hair graying phenotype.

3) A stemness checkpoint underlies the quality maintenance of tissues

Physiological hair graying and hair thinning are typical outward signs of aging in mammals, yet the mechanisms underlying those phenotypes had been largely unclear. We found that the incomplete maintenance of McSCs during the course of aging causes hair graying (Nishimura EK et al. Science, 2004). We then showed that genotoxic stress triggers/accelerates the aging process and abrogates the self-renewal of McSCs by triggering their differentiation without inducing cellular senescence. Further study of aged wild-type mice and progeroid mouse models, including *ATM*-deficient mice, revealed that a "stemness checkpoint", which determines whether stem cells are qualified to self-renew or rather are forced to differentiate, maintains the quality of the stem cell pool and eliminates stressed/damaged stem cells from tissues (Inomata K et al. Cell, 2009). Similar checkpoint mechanisms have been found in HFSCs (Matsumura H et al. Science, 2016) and in epidermal stem cells (Liu N et al. Nature, 2019 *in press*) by us and also in other somatic stem cells by other groups. We are currently studying the underlying molecular mechanism.

4) Dynamic elimination of aged stem cells causes hair follicle aging

To study the fate and dynamics of aged somatic stem cells, we performed *in vivo* fate tracing analysis of HFSCs and demonstrated that the dynamic elimination of HFSCs through their epidermal differentiation causes the stepwise miniaturization of hair follicles and eventual hair loss in mice. The DNA damage response in HFSCs causes proteolysis of Type XVII Collagen (COL17A1/BP180), a critical molecule for HFSC maintenance, to trigger HFSC aging that is characterized by the loss of stemness signatures and epidermal differentiation. Aged HFSCs are thus cyclically eliminated from the skin through their epidermal differentiation-mediated shedding from the skin surface, thereby causing hair follicle miniaturization. The aging process can be recapitulated by *Col17a1*-deficiency and prevented by the forced maintenance of COL17A1 in HFSCs, demonstrating that COL17A1 in HFSCs orchestrates the stem cell-centric aging program of the epithelial mini-organ (Matsumura H et al. Science, 2016).

5) Stem cell competition in the epidermis underlies skin homeostasis and aging

The skin protects living organisms from the outside world by acting as a barrier throughout the life-span, suggesting that the skin has more robust and flexible anti-aging mechanisms than mini-organs such as hair follicles. We have performed *in vivo* clonal analysis in mice by focusing on the expression of the hemidesmosomal protein COL17A1 by epidermal stem cells. Those studies revealed that the expression of COL17A1 fluctuates physiologically through genomic/oxidative stress-induced proteolysis, and that the resulting differential expression of COL17A1 in individual stem cells generates a driving force for cell competition (Figure 1). Clones that express high levels of COL17A1 divide symmetrically and outcompete/eliminate adjacent stressed clones that express low levels of COL17A1 and divide asymmetrically. Stem cells with higher potential or quality are thus selected for homeostasis, but their eventual loss of COL17A1 limits their competition, thereby causing aging. The resulting hemidesmosome fragility and stem cell delamination depletes adjacent melanocytes and fibroblasts to promote skin aging. Conversely, the forced maintenance of COL17A1 rescues skin organ aging, thereby indicating potential new approaches for anti-aging therapeutic intervention.

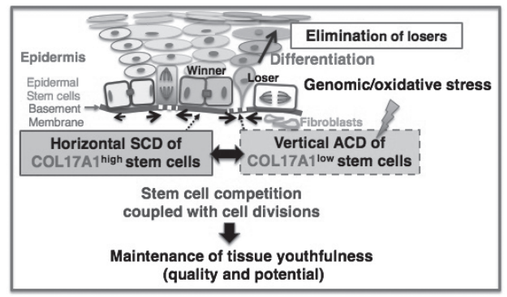

Figure 1: Stem cell competition orchestrates skin homeostasis and aging. SCD : symmetric cell division; ACD : asymmetric cell division.

Annual publications

Liu N, Matsumura H, Kato T, Ichinose S, Takada A, Namiki T, Asakawa K, Morinaga H, Mohri Y, De Arcangelis, Georges-Labouesse E, Nanba D, Nishimura EK
Stem cell competition orchestrates skin homeostasis and ageing.
Nature, doi: 10.1038/s41586-019-1085-7. **2019** *in press*

Noguchi S, Honda S, Saitoh T, Matsumura H, Nishimura E, Akira S, Shimizu S.
Beclin 1 regulates recycling endosome and is required for skin development in mice.
Commun Biol. 2:37. **2019** doi: 10.1038/s42003-018-0279-0.

Sasaki M, Shinozaki S, Morinaga H, Kaneki M, Nishimura EK, Shimokado K
iNOS inhibits hair regeneration in obese diabetic (ob/ob) mice.
Biochem and Biophys Res Commun., 501(4):893-897, 2018

Invited lecture/presentation at international meetings

Emi K. Nishimura: Epidermal stem cell competition coupled with stem cell divisions in mammalian epidermis:2019 Keystone Symposia Conference-Cell Competition in Development and Disease-: (Tahoe City, USA) February 24-28th, 2019

Emi K. Nishimura: Melanocyte stem cells and melanoma: Montagna Symposium on the Biology of Skin: (Oregon, USA) October 18-20th, 2018

Emi K. Nishimura: Stem cell and niche dynamics in aging skin: Gordon Research Conferences-Issue niches and resident stem cells in adult epithelia-: (New Hampshire, USA) August 19-24th, 2018

Emi K. Nishimura: Stem cell-centric Mechanisms of hair follicle aging: Gordon Research Conference-Cornea and Ocular Surface Biology and Pathology-: (California, USA) February 18-23, 2018

Emi K. Nishimura: Stem cells orchestrate hair follicle aging program: International Meeting on RECQ Helicases and Related Disease 2018: (Kazusa Akademia Hall: Chiba) February 16-18, 2018

Emi K. Nishimura: Stem cell orchestrate hair follicle aging: JSPS & NUS Joint 2nd Symposium "New Horizons in Normal And Cancer Stem Cell Research": (Kumamoto : 熊本大学山崎記念館) January 18-20, 2018

Department of Immunology

Professor	Takeshi Tsubata, M.D., Ph.D.
Associate Professor	Takahiro Adachi, Ph.D.
Assistant Professor	Chizuru Akatsu, Ph.D.
Project Assistant Professor	Shuichi Kinpara, Ph.D.
Researcher	Nazim Medhzidov, Ph.D.
Lecturer	Ji-Yang Wang, Ph.D.

Immune responses to non-protein antigens play crucial roles in host defense against pathogens, and autoimmune diseases. The mechanisms for immune responses to non-protien antigens are distinct from those to protein antigens, but are largely unknown. The aims of our research are to elucidate the mechanisms for antibody responses to non-protein antigens, and to develop novel drugs for autoimmune diseases and cancer immunotherapy by regulating antibody responses. Followings are our research subjects.

1) Elucidation of the mechanisms for humoral immune responses to polysaccharide antigens.
2) Elucidation of the mechanisms for autoantibody production in lupus and immuno-neurological disorders.
3) Elucidation of the role of glycan signals in the regulation of humoral immune responses, and development of glycomimetics for therapy.
4) Elucidation of the Role of reactive oxygen species (ROS) and membrane traffic in B cell activation
5) Development of novel drugs for autoimmune diseases by regulating regulatory B cells.
6) Development of therapeutic vaccines that substitute for therapeutic antibodies

1. Mechanisms for the production of reactive oxygen species (ROS) required for B cell activation

Although ROS is generated by various stress and is toxic to the cells, ROS can function as a signaling molecule. Major mechanisms for ROS production are mitochondrial respiratory chain and NADPH oxidases (NOXes). ROS play an essential role in B cell activation and proliferation after ligation of B cell antigen receptor (BCR) because treatment with ROS scavengers abrogate proliferation of BCR-ligated B cells. We could not detect ROS from mitochondria, but ROS production was suppressed by NOX inhibitors in BCR-ligated B cells, suggesting that NOXes are involved in BCR ligation-induced ROS production. Using mice and B cell lines deficient in various NOXes, we demonstrated that NOX3 plays a crucial role in BCR ligation-induced ROS production (Feng et al. 2019). Because NOX3 produces ROS at 2 hours after BCR ligation or later, further study on NOX3 will elucidate the role of late phase signaling in B cell activation (see Highlight).

2. Role of the endogenous ligands of inhibitory B cell co-receptors.

B cells express various different inhibitory B cell co-receptors including CD22, Siglec-G and CD72. These inhibitory co-receptors activate phosphatases such as SHP-1 and SHIP-1. Activated phosphatases then negatively regulate BCR signaling. Although most of the inhibitory co-receptors inhibit B cell activation by activating the tyrosine phosphatase SHP-1, inhibitory co-receptors show distinct functional properties. Siglec-G$^{-/-}$ mice show expansion of B-1 cells that constitute a B cell subset distinct from conventional B cells. CD22$^{-/-}$ mice show abnormal development of conventional B cells, and CD72$^{-/-}$ mice develop SLE-like autoimmune disease (Figure 1). Because these co-receptors use the same effector molecule, i.e., SHP-1, distinct functional properties of these inhibitory co-receptors appear to be due to the distinct ligand recognition. Previously, we demonstrated that CD72 recognizes the lupus self-antigen Sm/RNP, and inhibits activation of B cells reactive to Sm/RNP to produce anti-Sm/RNP autoantibody (Akatsu et al. JEM 2016), which plays a crucial role in development of SLE.

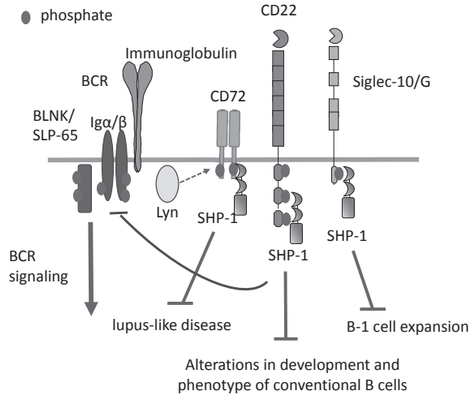

Figure1. Functional properties of inhibitory B cell co-receptors.
B cells express various inhibitory B cell co-receptors that inhibit BCR signaling. Many of these inhibitory co-receptors utilize SHP-1 as an effector molecule. CD22, Siglec-G/10 and CD72 show different functional properties by recognizing distinct ligands.

BCR transmits low level signaling in the absence of interaction with antigens. This antigen-independent low level BCR signaling called tonic signaling regulates survival and differentiation of B cells. Because CD22$^{-/-}$ mice show abnormal B cell development characteristic for high tonic signaling, we addressed whether ligand recognition of CD22 regulates tonic BCR signaling and B cell differentiation. We demonstrated that CD22 associates with BCR by recognizing glycans on BCR as a ligand (Alborzian Deh Sheikh et al. 2018). We further demonstrated that interaction of CD22 with endogenous ligand reduces basal signaling level of B cells, and that mice deficient in CD22 ligand show abnormal B cell development indicative of high tonic signaling as is the case for CD22$^{-/-}$ mice. These results suggest that CD22 down-regulates tonic signaling by constitutively associating with BCR through recognition of the glycans on BCR as a ligand. Taken together, ligand recognition determines functional properties of inhibitory B cell co-receptors (Tsubata, Front Immunol. 2018)

Highlight

NADPH oxidase (NOX) 3 is involved in B cell activation through prolonged production of reactive oxygen species (ROS)

Although ROS is generated by various stress and is toxic to the cells, ROS can function as a signaling molecule. After BCR ligation, B cells produce ROS in two phases. ROS production starts immediately after BCR ligation, and persists within one hour. ROS is then produced again from 2 to 8 hours after BCR ligation. ROS production at the early phase is mediated by NOX2, but is not required for B cell activation. In contrast, we demonstrated that production of ROS at the late phase enhances prolonged activation of NF-κB and PI-3K pathways, and is indispensable for proliferation of BCR-ligated B cells. We further demonstrated that deletion of NOX3 but not NOX1 by CRISPR/Cas9 system markedly reduces ROS production at the late phase in the B cell line BAL17. There findings clearly indicate that NOX3 is activated at 2 hours after BCR ligation or later, and that ROS derived from NOX3 enhances prolonged BCR signaling thereby augmenting activation and proliferation of B cells (Figure 2). These results clearly show the crucial role of NOX-derived ROS in BCR signaling, and also the crucial role of prolonged BCR signaling in B cell activation. ROS is known to augment signaling by inactivating phosphatases. Future studies will determine the phosphatase that is inactivated by NOX3-derived ROS, and elucidate the role of ROS in in vivo B cell activation during immune responses.

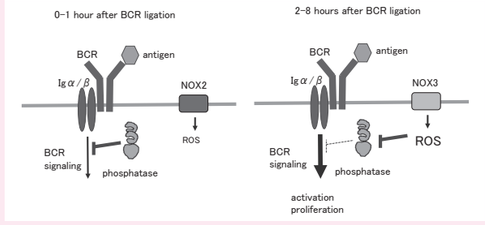

Figure2. Role of NOX-mediated ROS production in B cell activation
When BCR is ligated, ROS production at the early phase (0-1 hour) mediated by NOX2 is not involved in B cell activation. At the late phase (2-8 hours), prolonged ROS production mediated by NOX3 enhances BCR signaling probably by inactivating phosphatases thereby inducing activation and proliferation of B cells.

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Department of Molecular Pathogenesis

Professor	Akinori Kimura, M.D., Ph.D.
Associate Professor	Takeharu Hayashi, M.D., Ph.D.
Assistant Professor	Jianbo An, PhD
Assistant Professor	Taeko Naruse, Ph.D.

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

1. Molecular mechanisms for hereditary cardiomyopathy

We generated transgenic mouse lines expressing human M21 in the heart (M21-Tg). M21 is an inhibitory molecule of myosin light chain phosphatase regulating calcium sensitivity of cardiac muscle contraction. We found that calcium sensitivity of cardiac muscle contraction was increased in M21-Tg and M21-Tg exhibited cardiomyocytes hypertrophy, myocardial disarrays and later development of cardiac failure, similar as those found in patients with hypertrophic cardiomyopathy (HCM). M21-Tg will be a useful model for development of therapeutics for HCM.

2. Molecular mechanisms commonly found for atherosclerosis and inflammatory bowel disease

We generated a transgenic mouse line expressing coronary atherosclerosis-associated *MKL1* under the CD68 promoter (MKL1-Tg) and found that these mice exhibited abnormality in function of macrophages. In addition, MKL1-Tg crossbred to ApoE-KO. We found aortic atherosclerosis was exacerbated in the overexpression of *MKL1* in ApoE-KO background, suggesting a pathological contribution of abnormal development of macrophages in atherosclerosis.

3. Molecular mechanisms for Takayasu aortitis

In a collaboration study, we identified six novel susceptibility genes for Takayasu aortitis. A pathway analysis suggested the involvement of NK cells in the pathogenesis.

4. Analysis of HLA in human and macaques

We have analyzed HLA class I genes and HIV in Vietnamese patients with HIV infection and identified a correlation of specific HLA-B alleles and escape mutation in HIV. In addition, we developed a next-generation sequencing (NGS)-based genotyping system for MHC class I genes in rhesus and cynomolgus macaques.

5. Genome diversity in MHC class I-like genes in macaques

We have investigated an MHC class I-like gene, *ULBP5*, in macaques. We found that *ULBP5* was duplicated and each *ULBP5* gene carried polymorphisms in the contact sites to a NK cell receptor, NKG2D, suggesting a polymorphic nature of NK cell function in macaques. In addition, a phylogenetic analysis revealed that *ULBP6* was duplicated from *ULBP3* after divergence from higher primates, because *ULBP6* was homologous to *ULBP3* and not found in gorillas and chimpanzees.

Highlight

We have generated transgenic mouse lines specifically expressing human M21 in the heart (M21-Tg). Two high expressing lines (lines 29 and 32) and one low expressing line (line 6) were obtained. Both high expressing lines showed cardiomyocytes hypertrophy with myofibrillar disarrays and later development of cardiac failure with dilatation of ventricles similar to dilated phase of hypertrophic cardiomyopathy in humans (Figure). In addition, these mice developed sinus bradycardia and atrioventricular conduction defect. However, phosphorylation of ventricular myosin light chain was not changed by the overexpression of M21. The contractile dysfunction but not arrhythmias were improved by treatment with the Rho kinase inhibitor fasudil. These findings suggested that the overexpression of M21 results in cardiac dysfunction and conduction disturbance via non-myosin light chain 2 phosphorylation-dependent regulation.

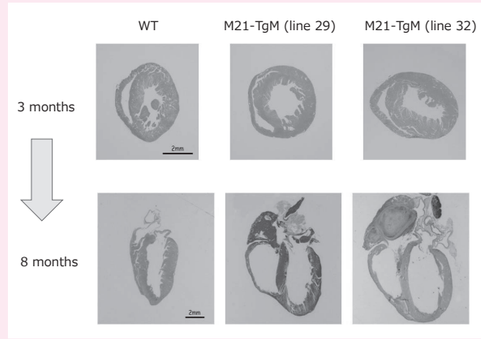

Figure Pathological findings in hearts from M21-Tg lines and control mice
Both high expressing M21-Tg (lines 29 and 32) developed severe systolic dysfunction.

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(#; equal contribution, *; corresponding author)

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10. Naruse TK, Akari H, Matano T, *Kimura A. Diversity of ULBP5 in the Old World monkey and divergence of ULBP gene family in primates. Proc Jpn Acad Ser B Phys Biol Sci. 2018; 94(10): 441-453.
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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 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 underlying the initiation and progression of human diseases, 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 research is to understand the molecular basis underlying cancer and genetic diseases including multiple congenital anomalies/intellectual disabilities (MCA/ID). We have contributed as follows;

1. Identification of novel genes including microRNAs responsible for cancer and development of miRNA-targeting therapeutics in cancer.
2. Understanding the pathogenesis of intractable cancers and genetic disorders based on integrative omics including systems biology.
3. Establishment of diagnostic devices for the implementation of precision medicine in cancer and genetic disorders.

【Molecular Genetics】

We aimed to analyze functions of breast cancer-related molecules including BRCAs and reveal the mechanism of breast carcinogenesis.

1. We sought novel synthetic lethal interactions between BRCA1/2 and chemical compounds provided by Chemical Biology Screening Center at TMDU.
2. We focused on breast cancer invasion, and analyzed the mechanism for disruption of the breast duct and basement membrane by estrogen.
3. We have focused on the exosome and found that the driver gene mutation of a primary tumor cell would cause organotropic adsorption of exosome into the tissue and construct pre-metastatic niche.

【Molecular Epidemiology】

1. We are studying the mechanism how metabolism of F1 is affected by intrauterine environment using DOHaD model mice.
2. We established mother and birth cohort, BC-GENIST, to study health related epigenome markers in collaboration with TMDU hospital.

【Genomic Pathology】

1. We performed immuno-genomics analysis of tumor infiltrating lymphocytes in gastric cancer and identified that Sulfated glycosaminoglycans are major and functional B cell antigens in cancers. In addition, we successfully created antibodies with antitumor activity by immune-genomics analysis.
2. We are trying to develope a Content-Based Image Retrieval system for histopathology images using deep neural networks.
3. We have developed an algorithm for the global profiling of cancer-stromal interactions by massively-parallel sequencing of cancer xenograft transcriptome. We are analyzing patient-derived xenograft (PDX) samples, where clinical cancer tissue is directly transplanted into immune-compromised mouse.

【Epigenetics】

1. We reported the existence of sushi-ichi-related retrotransposon homologue family of genes (SIRH family genes) and demonstrated that they play placental essential eutherian-specific functions, such as *Peg10*, *Peg11/Rtl1* and *Sirh7*, or important brain functions, such as *Sirh11*, *Sirh3* and *Sirh8*.
2. We have developed a new method for induce heart-like structure (heart organoid) from mouse ES cells. In this method, heart organoid develops mimicking normal heart development *in vivo*.
3. We have identified a new important factor, *Ftx*, for maintenance of X chromosome inactivation. It is a lncRNA but its deletion causes a microphthalmia-like phenotype in a female-specific manner.

【Medical Science Mathematics】

1. We proposed new methodologies that integrate GWAS with other omic data, which allows alternative ways of making new discoveries from GWAS data. One important result achieved using this approach combined human GWAS and mouse transcriptome data using our novel integration method to study Alzheimer's disease; we discovered new disease-causing genes and confirmed these findings in additional human hippocampus samples with eQTL analysis.
2. As one type of next-generation sequencing data analyses, we developed a new method that predicts intermediate-size insertions and deletions (indels) using BWA soft-clipped fragments and unmapped reads. Using these original methodologies, we have contributed to highly accurate WGS and target sequencing analyses of human genomes that also revealed phenotype-related variations.

Department of Molecular Cytogenetics

Professor	Johji Inazawa, M.D., Ph.D.
Lecturer	Jun Inoue, Ph.D.
Assistant Professor	Yasuyuki Gen, M.D., Ph.D.
Assistant Professor	Tomoki Muramatsu, Ph.D.
Research Assistant Professor	Daniela Tiaki Uehara, Ph.D.

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

I. Precision cancer medicine based on omics and functional research

1. Analysis of circulating tumor cells derived from pancreatic cancer cell line, Panc-1, for revealing the molecular mechanisms of metastasis.

Distant metastasis to several organs occurs by circulating tumor cells (CTC). We hypothesized that a subset of CTC had features that are more malignant than tumor cells at the primary site. We established a highly malignant cell line, Panc-1-CTC, derived from the human pancreatic cancer cell line Panc-1 using an in vivo selection method. Panc-1-CTC cells showed greater migratory and invasive abilities than its parent cell line in vitro. In addition, Panc-1-CTC cells had a higher tumor-forming ability than parent cells in vivo. To examine whether a difference in malignant phenotypes exists between Panc-1-CTC cells and parent cells, we carried out comprehensive gene expression array analysis. As a result, Panc-1-CTC significantly expressed transforming growth factor beta-induced (TGFBI), an extracellular matrix protein, more abundantly than did parent cells. TGFBI is considered to regulate cell adhesion, but its functions remain unclear. In the present study, knockdown of TGFBI reduced cell migration and invasion abilities, whereas overexpression of TGFBI increased both abilities. Moreover, elevated expression of TGFBI was associated with poor prognosis in patients with pancreatic cancer. (Sato et al. Cancer Sci. 2018).

2. Molecular basis for cell metabolism-based personalized cancer medicine

Intracellular metabolism is altered through the activation or inactivation by gene aberrations of metabolism-related genes in cancer cells. Therefore, we have attempted to develop novel therapeutic strategies for precision cancer medicine (PCM) by understanding the biological significance of metabolic characteristics in cancer cells. In the current year, we developed the molecular basis for microRNA (miRNA)-based cancer therapy by targeting autophagy, redox, and anti-apoptosis. Furthermore, we proposed a novel therapeutic strategy for ovarian cancer based on the requirement of amino acids as a nutrient source of cancer cells (Furusawa et al. Carcinogenesis 2018).

3. Exploration of novel tumor-suppressive miRNAs (TS-miRs)

MicroRNA (miRNA), endogenous small non-coding RNA, regulates multiple target gene expressions by partial complementation. In cancer, many miRNAs have been found as tumor suppressive miRNAs (TS-miRs) and such miRNA mimics could be applied to cancer therapy in nucleic acid medicine. We identified *miR-3140* as a novel tumor suppressive miRNA by function-based screening of a library containing 1090 miRNA mimics, directly suppressed *BRD4* by binding to its coding sequence (CDS). *miR-3140* concurrently downregulated *CDK2* and *EGFR* by binding to their 3' untranslated regions. *miR-3140* inhibited tumor cell growth *in vitro* in various cancer cell lines, including EGFR tyrosine kinase inhibitor-resistant cells. Interestingly, *miR-3140* downregulated the BRD4-NUT fusion protein and suppressed *in vitro* tumor cell growth in a NUT midline carcinoma cell line, Ty-82 cells. Furthermore, administration of *miR-3140* suppressed *in vivo* tumor growth in a xenograft mouse model. Taken together, *miR-3140* might be a candidate for the development of miRNA-based cancer therapeutics (Tonouchi et al. Sci Rep 2018).

II. Development of miRNA-based cancer therapy

Under the open innovation system, we are currently developing miRNA-based drugs for cancer therapy. This is being done through the examination of the anti-tumor effects by the DDS-based administration of miRNA-based drugs in xenograft mice, optimization of the chemical modification of synthesized miRNAs, and a safety test for the administration of miRNA-based drugs in dogs. This project aims to develop novel therapeutic strategies using miRNA-based cancer therapy.

III. Molecular investigation of congenital disorders

Intellectual disability (ID) is a lifelong and complex neurodevelopmental disorder affecting 2-3% of the population, often associated with multiple congenital anomalies (MCA). Due to the extensive genetic heterogeneity of ID, the diagnosis is challenging and remains unknown for a large subset of cases. Since 2005, we have been investigating the causes of ID/MCA of unknown etiology in 645 subjects recruited from 23 hospitals and medical institutes in Japan. First, we carried out an investigation of copy number variants (CNVs) by chromosomal microarray analyses using two BAC-arrays and a SNP-array, which detected a total of pathogenic CNVs in 24% of the cases (155/645) (Hayashi et al. J Hum Genet 2011; Uehara et al. J Hum Genet. 2016). Next, aiming at detecting disease-associated single nucleotide variants (SNVs), we applied next generation sequencing in 105 cases previously negative for pathogenic CNVs through targeted resequencing by a 75-gene custom panel. In total, pathogenic variants were identified in 19% of the cases (20/105). We have also carried out an investigation following the identification of the *CASK* gene as a cause of ID and microcephaly with pontine and cerebellar hypoplasia (MICPCH). We recruited 41 additional MICPCH patients and identified causative or candidate genomic aberrations in 37, then clarifying the etiology in 90% of the cases (Hayashi et al. PloS One 2017).

Articles

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Department of Molecular Genetics

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Associate Professor — Akira Nakanishi, Ph.D.
Assistant Professor — Shigeaki Sunada Ph.D.

Breast cancer is a typical hormone-dependent cancer, which is caused by estrogen-dependent cell growth, and utilizes the increase of survival signal due to DNA damage repair dysfunction and ultimately acquires estrogen-independent growth capacity. We investigated the mechanism of breast carcinogenesis by estrogen, the maintenance mechanism of genome stability by BRCA 1 · 2, and have tried developing a novel treatment for breast cancer targeting DNA damage response. Furthermore, we have analyzed the mechanism of DNA stability regulated by estrogen and BRCA2. Our goal is to reveal the functions of molecules associated with breast cancer and to develop novel treatments for breast cancer based on these information.

1. Analysis of disruption mechanism of the breast duct and basement membrane by estrogen

With the progress of breast cancer from Ductal carcinoma in situ (DCIS) to invasive ductal carcinoma (IDC), tumor cells induce disruption of the breast duct and basement membrane, and invade surrounding tissues, eventually leading to metastasis. Several studies have investigated the relationship between estrogen and breast carcinoma, and the evidence was provided that concentration of estrogen in the tissues of breast cancer patients increased more than 10 times in the blood. However, the molecular mechanisms underlying estrogen effects are not well understood. Here we report that the action of estrogen is involved in the collapse of breast duct formation. The estradiol (E2) increased the secretion of IL-1β and MMP-3 and also increased the expression levels of JNK, p38 and IκB phosphorylation and cleaved caspase 3. Furthermore, activation of MMP-3 was assessed after stimulation with E2. We used the most common MCF10A acini as 3D cell model for human breast glands and verified that E2 induced apoptosis in MCF10A cells and disrupted the basement membrane using immunofluorescence confocal microscopy (Fig. 1). Our results suggest that E2 promotes the secretion of IL1β and MMP-3 and induces apoptosis via the p38/JNK and NFκB pathways to disrupt the breast duct.

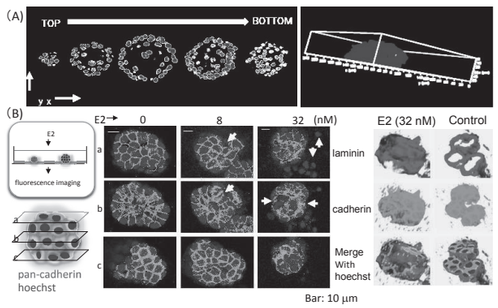

Figure 1. Confocal images of MCF-10A acini treated with estradiol (E2)
(A) MCF-10A acinus. Confocal z-stack images were obtained from MCF-10A 3D culture, and 3D images were reconstructed. (B) Estradiol (E2) effects on luminal structure. Confocal images of MCF-10A acini treated with E2 (8, 32 nM) or ethanol (control) through a Day 31. The luminal structure were partially collapsed by the treatment of E2 (white arrows: left panel). Confocal images 3D of MCF-10A acini treated with E2 (32 nM) or ethanol (control) (right panel). Blue; hoechst, green; pan-cadherin, and red; laminin staining.

2. The physiological roles of interactions between BRCA2 and the estrogen receptor

The onset and malignancy of BRCA2-related breast and ovarian cancers have been previously associated with estrogen. Complexes of estrogen (E2) and the estrogen receptor (ER) reportedly function as transcription factors, although the roles of BRCA2 in the transcriptional function of E2–ER complexes have not been clarified. Last year, we showed that upon overexpression in 293T cells, FLAG-ER interacts with BRCA2-FLAG via the helical domain corresponding with amino acids 2240 to 2940. Because these interactions were not those of direct protein-binding, we suggested that BRCA2 indirectly binds to ER via a transcriptional co-regulator. For identifying BRCA2-assoicated transcriptional coregulators of ER, we performed chromatin immunoprecipitation (ChIP) assays with an anti-BRCA2 antibody and analyzed the products using mass spectrometry (MS). After synchronizing MCF7 cells in the S phase with thymidine, DNA was fragmented using ultrasonication and only DNA–protein complexes were recovered using DNA binding beads. Subsequently, DNA was digested by adding DNase and was immunoprecipitated with an anti-BRCA2 antibody. BRCA2-bound proteins were then digested with trypsin. Our MS analyses identified NCOA1, NCOA5, NCOA6, CREB binding protein (CBP), CITED2, and DACH1 as BRCA2-associated partners. Moreover, luciferase assays of the representative transcriptional E2–ER target pS2 revealed that BRCA2 expressed in an E2-dependent manner suppresses the promoter activity of E2–ER. These results collectively suggest that BRCA2 regulates the transcriptional activity of E2-ER via a transcriptional co-regulator of ER. Further studies of the related mechanisms are in progress.

3. Development of novel DNA repair inhibitors

DNA repair inhibition has been expected to apply for cancer therapy because that can cause cell lethality combined with DNA damage agents such as radiation and chemicals. Then, we performed some screening tests with a chemical library to explore the novel DNA repair inhibition factors. We utilized known compounds from Chemical Biology Screening Center (TMDU) for drug-repositioning, and investigated the combined effects of them with DNA damage agents (PARP inhibitor or Etoposide). Some compounds have been extracted that show sensitizing effect with DNA damage agents by in vitro screening tests based on cellular toxicity and DNA double-strand break repair. We introduce the candidates from hit identification. Sensitizer A, identified as a sensitizer for PARP inhibitor, induced repair mistakes during homologous recombination (HR) repair. Sensitizer B, identified as a sensitizer for Etoposide, blocked non-homologous end joining (NHEJ) repair pathway (Fig. 2). These compounds would affect DNA repair by different effects from their known mechanisms. In order to create lead compounds as novel DNA repair inhibitors, we will investigate detailed molecular mechanisms and validate the evidence in animal tests.

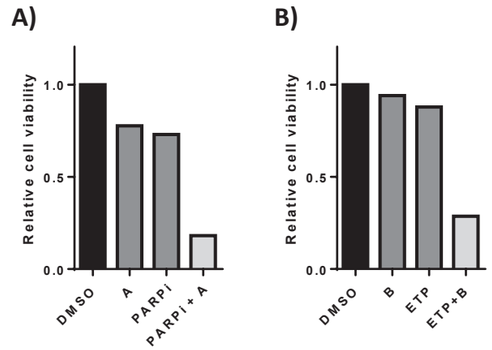

Figure 2. Anti-tumor effects of new combination of DNA damage agents with compounds
A): PARP inhibitor and Compound A, B): Etoposide and Compound B

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Department of Molecular Epidemiology

Professor Masaaki Muramatsu, M.D. & Ph.D.
Associate Professor Noriko Sato M.D.& Ph.D.
Assistant Professor Chihiro Imai, Ph.D.

Many common chronic diseases are multifactorial in that they are caused by multiple genetic and environmental factors. By applying the 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 contribute to the development of these diseases. We also focus on the mechanism of the Developmental Origin of Health and Disease (DOHaD) hypothesis and study how epigenetics of the fetus and the mother regulates

1. Effectiveness of personal genomic testing for disease-prevention behavior when combined with careful consultation with a physician

There are many direct-to-consumer (DTC)-type personal genomic testing (PGT) services commercially available to the public, providing the specific disease susceptibilities of individuals. While these services do not appear to stimulate disease-prevention behavior, few studies have addressed the methods to do so. We investigated the effectiveness of combining a consultation with a physician with the delivery of test results from a DTC-type PGT, as a preliminary study to identify the effective genomic testing for disease-prevention (Fig 1.). A prepared physician disclosed the PGT results of twenty healthy subjects and provided a specific consultation on the high-risk diseases for each subject. The effects on the sense of health, understanding of possible future diseases, and preventive behaviors for each subject were examined pre-PGT, post-PGT, and 3, 6, and 12 months post-PGT. Significant increases between the pre- and post-PGT scores were observed for the awareness of lifestyle effects on developing those diseases ($P < 0.05$) and the awareness of the ability to influence disease onset ($P < 0.01$) (Fig 2.). The follow-up questionnaire results showed that over 60% of the subjects changed their lifestyles in favor of disease prevention. These results suggest that combining the DTC-PGT with a careful physician consultation may be effective at motivating people toward preventive behavior. This result highlights the importance of the physician's counseling to help participants' understanding the roles of genetic and environmental factors in developing common chronic diseases.

2. Metabolic and immunological shifts during mid-to-late gestation influence maternal blood methylation of CPT1A and SREBF1

Mid-to-late gestation is a unique period for women to experience dynamic changes in lipid metabolism. Although the recent intensive epigenome-wide association studies (EWAS) using peripheral leukocyte have revealed that the lipid-related traits alter DNA methylation, the influence of pregnancy-induced metabolic changes on methylation levels of these differentially methylated sites is not well known. In this study, we performed the prospective cohort study of pregnant women (n = 52) using the MassARRAY EpiTYPER assay and analyzed methylation levels of variably methylated sites, including CPT1A intron 1 and SREBF1 intron 1 CpGs, which were previously verified to

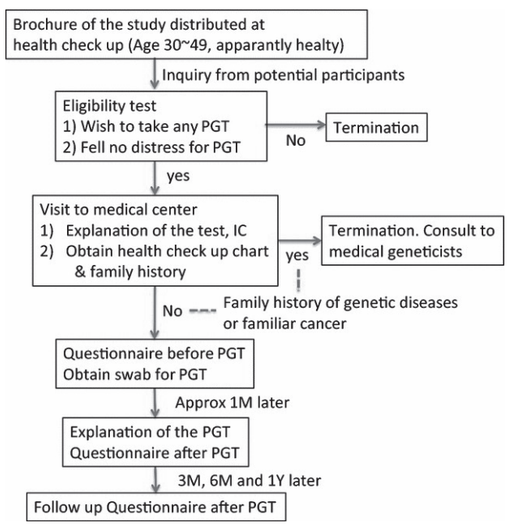

Fig 1

be robustly associated with adiposity traits. Although methylation of SREBF1 was associated with body mass index (BMI) and low-density lipoprotein cholesterol at mid-gestation, this association was attenuated at late-gestation, which was consistent with the metabolic switch from an anabolic to a catabolic state. Whereas, the BMI-association with CPT1A intron 1 methylation appeared to strengthen at late-gestation; this association was mediated by pre-pregnancy BMI-dependent change in leukocyte proportion during mid-to-late gestation. Thus, the methylation of adiposity-related differentially methylated regions was sensitive to metabolic and immunological changes during the mid-to-late gestation.

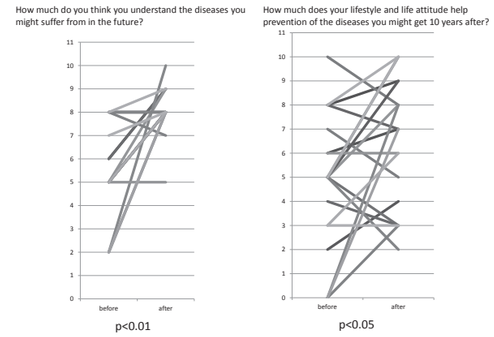

Fig 2

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[Presentation at international meetings]

Noriko Sato. "Perinatal Immunomethylomics towards DOHaD". 17th Surugadai International Symposium & Joint Usage/Research Program of Medical Research Institute International Symposium. 2018.11.19. Tokyo, Japan.

Department of Genomic Pathology

Professor Shumpei Ishikawa
Assistant Professor Hiroto Katoh
Assistant Professor Daisuke Komura

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.

Research introduction

1. Genomic approach for the cancer-stromal interaction

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). 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 (Fig. 1). 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.

2. Cancer Immunogenomics

Tumor infiltrating lymphocytes (TILs) seem to play important roles in cancer immunity, as suggested by the finding that the amount of TILs correlates with prognosis in various cancer. However, their functions have remained largely unknown. We try to uncover the functions of TILs in cancer environment by analyzing their antigen receptor sequences using massively-parallel sequencing technology.

3. Genomics Analysis for Clinical Disease Tissues

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.

4. Functional Genomics Screening

We are conducting various kinds of functional genomics screening by combining whole genomic shRNA lentivirus libraries and next-generation sequencing technologies. Our goal is to identify novel therapeutic molecular targets against cancers, and to this end we are exploring possible candidate genes by developing a couple of shRNA screening methods. An example of our screening strategies is a tumor implantation model in which various human cancer cell lines infected with whole-genomic shRNA lentivirus library are inoculated into mice. In this model, we can quantitatively characterize the populations of cancer cell clones with each shRNA before and after the tumor implantations. We are now identifying candidate genes which significantly suppress cancer cell growths in vivo. We performed numbers of functional genomics screenings targeting various human cancers, having identified some candidate therapeutic target genes.

5. Image analysis and machine learning in digital pathology

Various genetic abnormalities in cancer cells result in their cellular and architectural abnormalities. Historically, investigating these relationships have provided various insights into cancer biology and gene functions. However, manual investigation of all the relationships in all cancer types by human pathologists is infeasible. We apply deep learning algorithms, which exhibits superior performance in object recognition over conventional machine learning algorithms, to histopathological image analysis of various cancer types. Our aims include inference of clinically relevant somatic mutations from histopathological images, which can be a cost-effective diagnostic tool, and uncovering novel function of genes by investigating the effect of the mutation on the appearance of cancer tissues.

Highlight

1. Content-based Image Retrieval for histopathology images using deep texture representations

The technique of searching similar images using images queries from image databases is called CBIR (Contents Based Image Retrieval). CBIR is extremely useful in pathological diagnosis. Even experienced pathologists sometimes encounter cases they have never seen before. In such a case, they ask other pathologists for their opinions or they look for similar tissue images from the pathology image atlas, which is a time-consuming task. If it is possible to quickly and accurately retrieve similar cases from a database of pathological images, the probability and time to reach a correct diagnosis for a difficult case are greatly improved. In CBIR of a pathological image, it is important to quantify the histopathological similarity, unlike the usual general images. We have found that the texture information (deep texture) extracted from the deep neural network well expresses the features of the pathological tissue image of cancer and that the texture information is useful for similar image retrieval. Using the deep texture, we have developed a system called Luigi that searches similar cases of pathological tissue images (https://luigi-pathology.com/). The image database contains about 7300 cases from 32 cancer types in TCGA, and all genome abnormality information of cancer is linked to the images. When a registered user selects a similar case, genomic abnormality of the cancer is displayed. We plan to estimate cancer genomic abnormalities from pathological tissue images by using deep texture in the future.

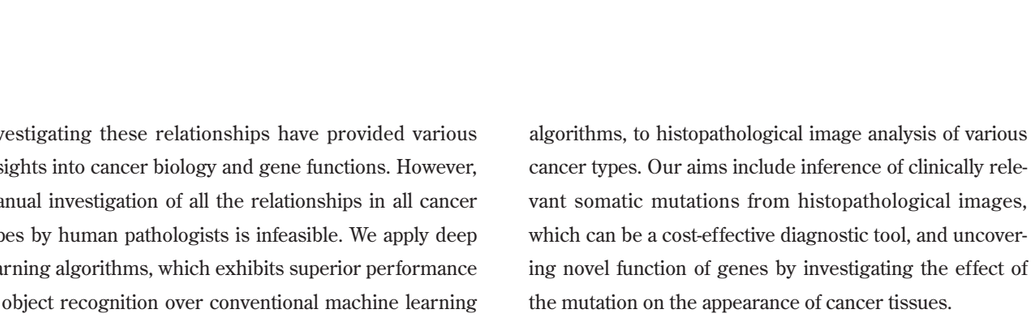

Fig. 1

Publications

Original Paper

1. Bándi P., Geessink O., Manson Q., Dijk M van., Balkenhol M., Hermsen M., Bejnordi B., Lee B., Paeng K., Zhong A., Li Q., Zanjani F.G., Zinger S., Fukuta K., **Komura D.**, Ovtcharov V., Cheng S., Zeng S., Thagaard J., Dahl A., Lin H., Chen H., Jacobsson L., Hedlund M., Cetin M., Halici E., Jackson H., Chen R., Both F., Franke J., From detection of individual metastases to classification of lymph node status at the patient level: the CAMELYON17 challenge. *IEEE Transactions on Medical Imaging* 2018:1–1.
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Department of Epigenetics

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Project Assistant Professor	Ayumi Matsuzawa
adjunct lecturer	Takashi KOHDA, Shin Kobayashi, Hirosuke Shiura

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'. Mammalian-specific LTR retrotransposon-derived genes are essential for mammalian development, such as placenta and brain functions. These studies show us how Epigenetics and Genetics are 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. Analysis of genomic imprinting diseases

Paternal duplication of human chromosome 14 syndrome (Kagami-Ogata syndrome) is a one of assigned intractable diseases in Japan. It is a severe genomic imprinting disease that leads bell-shaped thorax, neonatal lethality associated with respiratory problem. Maternal duplication of human chromosome 14 syndrome (Temple syndrome, respectively) is another genomic imprinting disease that leads to sever developmental delay and muscle abnormality. We have recently identified *PEG11/RTL1* is a major responsible gene for these two syndromes by analyses of two mouse models (submitted). We are now focusing on the development of new therapy for Kagami-Ogata syndrome by regulating expression of *PEG11/RTL1*.

2. A lncRNA Ftx is important for maintenance of X chromosome inactivation (Highlight)

In mammals, females have two X chromosomes but one of them is inactivated in somatic cells for gene compensation between males. Actually, embryos that have two active X chromosome die at an early developmental stage. We have demonstrated that a long non-coding RNA (lcnRNA) *Ftx* plays an important role in maintenance of X chromosome inactivation and its lack leads to microphthalmia-like phenotype in a female-specific manner.

3. Parental age and gene expression profiles in individual human blastocysts

Physiological environment of the process of gametogenesis, including parental age, may affect the epigenome of the embryo after fertilization. Thus, it is especially important to clarify the influence of parental age on gene expression in the embryo in terms of transgenerational epigenetics to improve the techniques used in assisted reproductive medicine. By performing single embryos RNA-seq analysis on human blastocysts fertilized by intracytoplasmic sperm injection, we identified a number of genes in which the expression levels are lowered with increasing maternal age: some are considered to be important for meiotic chromosomal segregation and others are cytokine important for embryo implantation, suggesting that epigenetic modification of oocyte genome, may change with parental age and be transmitted to the next generation (Sci Rep 2018).

4. Faithful DNA methylation of genomic imprinting in ES cells

ES and iPS cells are useful tools in genetic engineering and regenerative medicine. However, it is not well recognized that DNA methylation memories of genomic imprinting are fragile in these pluripotent cells. Even though they keep their pluripotency for long time but lose imprinting memories in most cases. Here we established a preferable *in vitro* condition for maintaining faithful DNA methylation imprinting in mouse embryonic stem cells (Genes Cells 2018).

Highlight

Why do only female mice lacking Ftx exhibit microphthalmia? (Hosoi et al. Nature Communication 2018)

This research is a collaboration research with Dr. Shin Kobayashi at National Institute of Advanced Industrial Science and Technology, providing a good example of non-Mendelian inheritance, therefore, of female-specific epigenetics. In mammals, females have two X chromosome but one of them is inactivated by expression of *Xist*, a master regulator of X chromosome inactivation (XCI). Dr. Koabayashi previously identifed an X-linked long non-coding RNA *Ftx* located within a *cis*-acting regulatory locus on the X chromosome, referred to as the "X-inactivation centre (Xic). *Ftx* starts its expression predominantly in female preimplantation embryos when XCI first occurs (PLoS One 2013). He carried out this study as a Medical Top Track (MTT) fellow in our Institute (MRI) and also a project lecturer after the MTT program.

Interestingly, *Ftx* is imprinted and expressed only from the paternally derived X chromosome (X^P), like *Xist* at preimplantation stage. It was proposed that the *Ftx* lncRNA is an upregulator of *Xist* by Chureau *et al.* using male embryonic stem (ES) cells. We found that targeted deletion of *Ftx* lncRNA causes eye abnormalities resembling human microphthalmia only in a subset of females (Figure 1). Importantly, the inheritance pattern cannot be explained by X-linked dominant or recessive inheritance, where males typically show a more severe phenotype than females. In *Ftx* KO mice, some X-linked genes, such as *Tmem29*, *Mecp2* and *Ogt*, remain active even on the inactive X, suggesting that defects in maintenance of XCI in somatic cells cause a female-specific phenotype (Figure 2). We also found that the expression level of *Xist* is decreased in females homozygous or heterozygous for *Ftx* deficiency. Therefore, we propose that loss-of-*Ftx* lncRNA abolishes gene silencing on the inactive X chromosome, leading to a female microphthalmia-like phenotype.

From these findings, we propose that partial failure in XCI can cause genetic diseases with female-specific or female-predominant inheritance even in humans. Although there is no reported human family showing microphthalmia to be inherited in a female-specific manner, there are some X-linked genetic diseases which show greater sensitivities in female than in male subjects, such as craniofrontonasal syndrome (OMIM 304110) and early infantile epileptic encephalopathy-9 (OMIM 300088). Thus, it is highly probable that they are caused by partial XCI failure because the *Ftx* lncRNA is conserved in humans. Therefore, mutational analysis of human *FTX* homologues might help us to understand this unusual class of X-linked human genetic diseases, for which women show more severe phenotypes than do men.

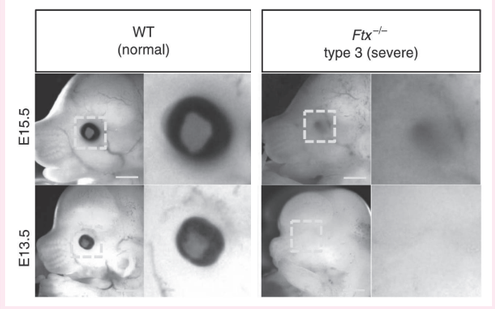

Figure 1. Microphthalmia observed in female embryos of *Ftx* KO mice
The eye abnormalities of *Ftx* KO mice closely resemble human microphthalmia. The yellow boxed areas are shown enlarged in the right panels. Abnormalities were classified into three types by their appearance, but only WT (left) and type 3 KO with no eye visible on the face (right) were shown. Scale bar = 1 mm.

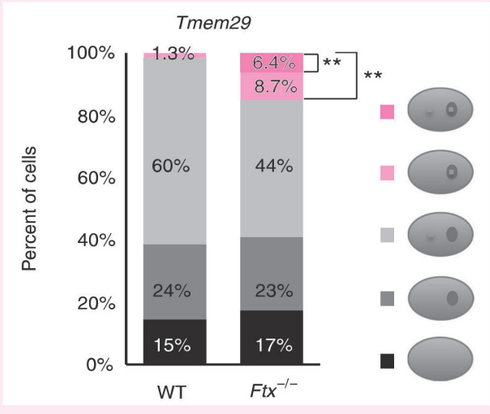

Figure 2. Abnormal gene expression of X-liked genes in female *Ftx* KO mice
Ftx KO mice showed partial failure of XCI. Frequency of ectopic X-linked *Tmem29* gene expression from inactive X chromosomes in eyes at E13.5. Approximately 100 nuclei in each eye were counted and classified based on the patterns of Xist clouds and nascent X-linked gene transcripts. Cells showing misexpression of *Tmem29* from the inactive X chromosome are shown as dark or light pink, indicating that XCI was partially compromised in the mutant female.

Publications

Original papers

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2. Kawai K, Harada T, Ishikawa T, Sugiyama R, Kawamura T, Yoshida A, Tsutsumi O, Ishino F, Kubota T and Kohda T. Parental age and gene expression profiles in individual human blastocysts. Sci Rep **8**(1):2380 (2018). doi: 10.1038/s41598-018-20614-8.
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Department of Medical Science Mathematics

Professor	Tatsuhiko Tsunoda
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Assistant Professor	Jo Nishino, Takashi Kamatani
Secretary	Yumi Nakamura

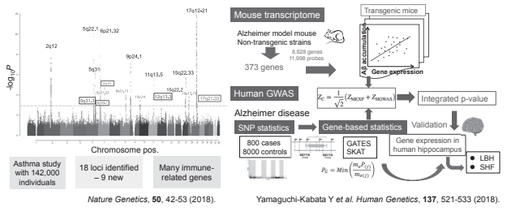
Research Summary

Effective utilization of rapidly developing omic profiling technologies and, in particular, the introduction of personalized/precision/preventive medicine have recently become major goals of medical research. This paradigm shift requires moving away from traditional approaches that do not adequately consider the individual characteristics of each patient. Our laboratory develops strategies to address these challenges by bringing the ideas and methods from mathematics and computational sciences to the medical domain. The first part of our approach is driven by integrative analysis of clinical and omic data and aims to explore the etiologies of intractable diseases such as cancer, common diseases, and neurodegenerative diseases. Next, we classify each disease into finer categories using molecular profiles and clarify underlying causal mechanisms with systems-based approaches. Lastly, we apply mathematical and machine learning techniques to infer optimal therapy for each patient to guide treatment decisions by their hospital or clinic. Similar approach can be used for disease prevention based on an individual's medical history.

Research Projects

1. New trend of GWAS

One success we have achieved by using such techniques is multi-ancestry GWAS, which revealed nine novel asthma-related loci **[1]**. In addition to this work, we have collaborated internationally, applied the meta-analysis on collected datasets, and identified disease-related genes and/or quantitative trait loci **[1]-[3]**. We have also developed a methodology to increase statistical power using a hierarchical mixture model and empirical Bayes, and applied it to estimate the sample size required for significant findings for major depressive disorder **[4]-[6]**. We proposed new methodologies that integrate GWAS with other omic data, which allows alternative ways of making new discoveries from GWAS data. One important result achieved using this approach combined human GWAS and mouse transcriptome data using our novel integration method to study Alzheimer's disease; we discovered new disease-causing genes and confirmed these findings in additional human hippocampus samples with eQTL analysis **[7]**.



Nature Genetics, **50**, 42-53 (2018). Yamaguchi-Kabata Y *et al. Human Genetics*, **137**, 521-533 (2018).

Figure 1 International collaboration revealed 9 new Asthma related-loci (left), and integrative omic-analysis applied with human GWAS and mouse transcriptome found new Alzheimer genes.

2. Whole genome sequence (WGS) and whole exome sequence (WES) analysis

We developed a new method that predicts intermediate-size insertions and deletions (indels) using BWA soft-clipped fragments and unmapped reads **[8]**. Using these original methodologies, we have contributed to highly accurate WGS and target sequencing analyses of human genomes that also revealed phenotype-related variations **[8]-[10]**. Another type of next-generation sequencing data analyses is WES analysis. This approach made particularly strong impact in the studies of Mendelian diseases using family data. We have identified many disease-causing genes of intractable diseases, e.g. neurodegenerative diseases, by using our original experimental methods and analysis pipeline that were developed to achieve both high coverage and accuracy **[11]-[15]**.

3. Big-data analysis of clinical and whole omics data in medicine

Recently we have developed the following original methodologies and applied them to real omic data: (1) Network topology analysis for identifying key molecules for disease and drug-response and constructing their prediction models **[16]**, (2) A highly-accurate model for predicting drug-toxicity by using machine learning to combine features from omic and physicochemical properties **[17]**, (3) Prediction models for post-translational amino-acid modifications and protein structures by using various physicochemical and sequence properties **[18]-[26]**, and (4) New scheme to analyze non-image data, e.g. gene-expression profiles, with deep neural network by converting the data into an image-like form (submitted). Furthermore, now we are collaborating with several clinical trial groups for cancer immunology to identify biomarkers and construct drug-efficacy prediction models. We have obtained the necessary clinical data and took omic profiles of cancer patients' tissues and analysis of these original datasets is currently underway.

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Frontier Research Unit
Laboratory of Gene Expression

Associate Professor **Hidehito KUROYANAGI**
Project Assistant Professor **Shotaro WANI (~March, 2018)**

Post-transcriptional regulation is an important layer for gene expression regulation. Based on recent transcriptome analysis, most of human multi-exon genes produce multiple mRNA isoforms through alternative pre-mRNA processing and hence multiple structurally and functionally distinct protein isoforms in cell-type-specific manners. We are trying to decipher so-called "cellular codes" that determine the cell-type specific pre-mRNA processing patterns and elucidate the mechanisms of pathogenesis of genetic diseases caused by defects in the post-transcriptional regulation of gene expression.

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

We have 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 alternative pre-mRNA processing events in living nematode worms *C. elegans* and identified *trans*-acting factors and *cis*-elements involved in the regulation (Mol Cell Biol, 2007; Genes Dev, 2008; PLoS Genet, 2012, 2013; NAR, 2013, 2016; Nat Struct Mol Biol, 2014; Nat Commun, 2016). We have recently reported that alternative splicing of a tropomyosin gene is differentially regulated in the head muscles and confers a specific function (Mol Biol Cell, 2018; Cytoskeleton, 2018). Through these studies, we now realize that molecular mechanisms for the post-transcriptional gene regulation are conserved throughout metazoan evolution (WIREs RNA, 2017).

Alternative Splicing Regulator RBM20 in Dilated Cardiomyopathy.

Dilated cardiomyopathy (DCM) is a disease in which the heart becomes enlarged and no longer pumps blood effectively. Autosomal-dominant familial DCM is linked to mutations in a variety of genes including a splicing regulator RBM20. We have recently reported for the first time that phosphorylation of the two serine residues in an RSRSP stretch, a hotspot of the DCM mutations in the *RBM20* gene, was essential for nuclear localization, which allows RBM20 to interact with its target pre-mRNAs (Sci Rep, 2018). We generated an *Rbm20*^{S637A} knock-in mouse, mimicking an un-phosphorylatable mutation found in a well-studied case of DCM, and confirmed that the residue is critical for the splicing regulation by RBM20 in the heart (Sci Rep, 2018) (Figure). Further study of the knock-in mouse will lead to better understanding of the DCM pathogenesis and developing therapeutics (Front Mol Biosci, 2018).

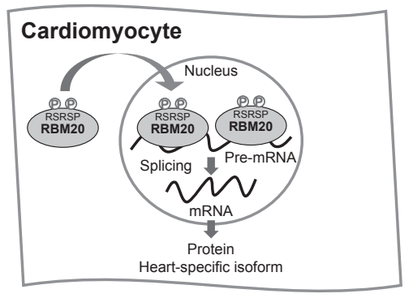

Figure. Phosphorylation of the RSRSP stretch is critical for nuclear localization and functions of the RBM20 protein

Publications

Original Articles

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Review Article

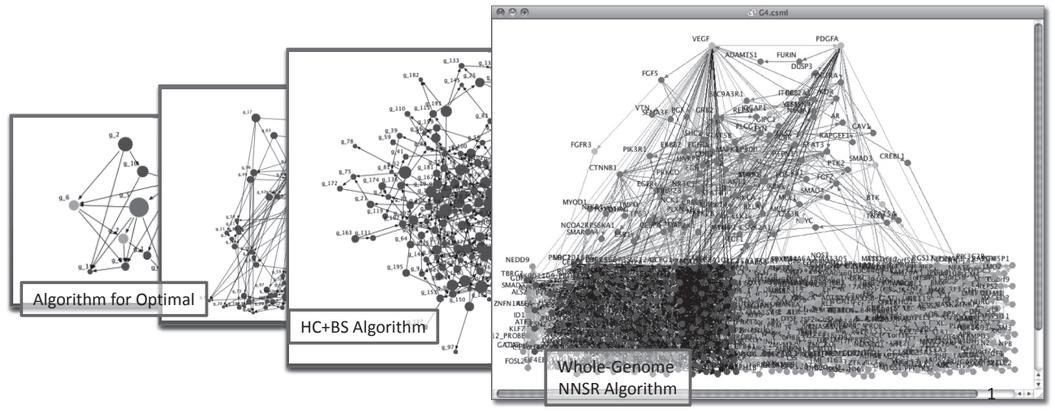
Takeshi Watanabe, Akinori Kimura, Hidehito KUROYANAGI. Alternative splicing regulator RBM20 and cardiomyopathy. Frontiers in Molecular Biosciences, section RNA, 5: 105, 2018.

Laboratory for Integrated Research Projects on Intractable Diseases
Advanced Technology Laboratories

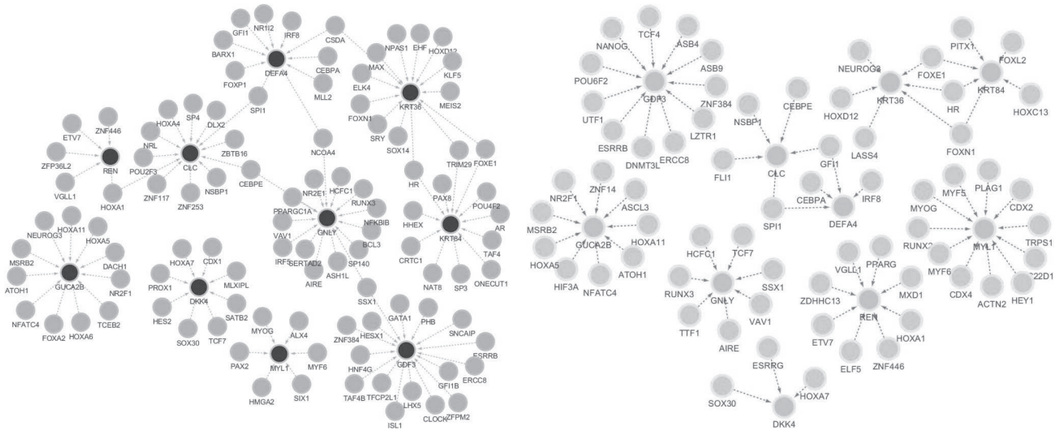
Systems Biology for Intractable Diseases

Affiliate Professor Satoru Miyano, Ph.D.
Affiliate Professor Seiya Imoto, Ph.D.

It is getting clearer that pathogenesis of intractable disease is a state that deviates from an integrated systems control in the abnormal situation where multiple genes are affecting one another intricately. The recent advances in biomedical research have been producing large-scale, ultra-high dimensional, ultra-heterogeneous omics data through the advanced technologies such as genome sequencing and proteome analysis. The aim of this section is to clarify the biological mechanisms and their failures in the system by applying computational strategy for systems biology and by analyzing these omics data using supercomputer. It is expected that key molecules of the diseases will be searched by the systems biology analysis of molecular pathways and networks related to the diseases which could not be analyzed in the traditional approaches. This section is collaborating with various laboratories in Medical Research Institute for understanding the pathogenesis of the diseases toward drug discovery and new therapy development.



We have developed a series of programs on supercomputers for mining gene networks of size from 30 (optimal) to 20,000 (genome-wide) including non-coding RNAs based on various mathematical models (Bayesian N, State Space Model, Structural EQ, etc.). These programs are open-access with source codes.



Gene network for the anticancer drug-resistant sample (left) and gene network for the anticancer drug-sensitive sample estimated from Sanger Genomic of Drug Sensitivity in Cancer data by the method of Park et al. J Comp Biol. 2019.

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Laboratory for Integrated Research Projects on Intractable Diseases

IBD project2 Laboratory for Integrated Research Projects on Intractable Diseases

Professor Shigeomi SHIMIZU
Akinori KIMURA
Toshiaki OHTEKI

Summary

Inflammatory bowel disease (IBD) primarily includes ulcerative colitis and Crohn's disease. Our goal is to understand the mechanism of IBD development and find the new therapies and treatments of the disease.

Research Outcome

1, Involvement of MKL1 in IBD pathogenesis: Because expression of Mkl1 was found to be increased in colonic lamina propria macrophages from DSS-treated mice, a transgenic mouse line (MKL1-Tg) overexpressing human MKL1 specifically in monocyte/macrophage cell lineage was established. MKL1-Tg spontaneously displayed IBD phenotypes and showed higher susceptibility to DSS-induced colitis, indicating that MKL1 crucially contributes to the development of colitis via regulating function of macrophages. In addition, we found that MKL1 overexpression exacerbated atherosclerosis in ApoE-KO mice, which might explain higher prevalence of coronary atherosclerosis in IBD patients.

2, Involvement of IFN-γ–Stat1 pathway in colitogenic macrophage differentiation in vivo: Colonic macrophages induce pathogenic inflammation against commensal bacteria, leading to inflammatory bowel disease (IBD). Although the ontogeny of colonic macrophages has been well studied in the past decade, how macrophages gain colitogenic properties during the development of colitis is unknown. Using a chemically induced colitis model, we showed that accumulated Ly6C$^+$ cells consisting of inflammatory monocytes and inflammatory macrophages strongly expressed representative colitogenic mediators such as TNF-α and iNOS. The IFN-γ–Stat1 pathway was required for generating colitogenic macrophages, given that *Stat1*$^{-/-}$ mice had less severe colitis and fewer colitogenic macrophages. Notably, IFN-γ induced histone acetylation at the promoter regions of the *Tnf* and *Nos2* loci in the monocyte and macrophage lineage, indicating that IFN-γ–dependent epigenetic regulation instructs the development of the colitogenic monocyte and macrophage lineage *in vivo*.

3, Involvement of autophagy in IBD: In order to elucidate the relationship between autophagy and IBD, we generated various types of intestine-specific autophagy knockout mice. We also searched for small compounds that improve IBD via autophagy induction. To this end, we first developed two types of fluorescent probes for monitoring autophagy that exhibit fluorescence upon being incorporated into autophagosome. Then, we performed high-throughput screening of a chemical library, and identified 120 small compounds and 31 natural products that induce autophagy. Among them, we identified natural products that improve IBD.

Publications

1, Nakanishi Y, Sato T, Takahashi K, and Ohteki T. IFN-γ–dependent epigenetic regulation instructs colitogenic monocyte/macrophage lineage differentiateon in vivo. **Mucosal Immunol** 2018; 11: 871-80.

2, Iwashita H, Tajima-Sakurai H, Nagahora N, Ishiyama M, Shioji K, Sasamoto k, Okuma K, Shimizu S and Ueno Y. Fluorescent small molecules for monitoring autophagic flux. **FEBS let**. 2018; 592: 559-567.

3, An J, Naruse TK, Hinohara K, Soejima Y, Sawabe M, Nakagawa Y, Kuwahara K, Kimura A: MRTF-A regulates proliferation and survival properties of pro-atherogenic macrophages. **J Mol Cell Cardiol**, In Press

Research Project on Striated Muscle Diseases

Project Leader, Molecular Pathogenesis Akinori KIMURA
Gene Expression Hidehito KUROYANAGI, Shotaro WANI
Molecular Pathogenesis Jianbo AN, Taeko K. NARUSE

Heart-Specific Splicing of the *TTN* Gene in Dilated Cardiomyopathy.

Dilated cardiomyopathy (DCM) is a heart disease characterized by left ventricular dilatation and systolic dysfunction. DCM can be caused by mutations in sarcomere protein genes including *TTN*. The *TTN* gene consists of 364 exons and its pre-mRNA splicing pattern is developmentally regulated and varies between cardiac and skeletal muscles. An RNA-binding protein RBM20 is a major regulator of the heart-specific alternative pre-mRNA splicing of *TTN*. Mutations in *RBM20* are known to cause autosomal-dominant familial DCM, where most of the *RBM20* missense mutations were mapped to an RSRSP stretch. We constructed a fluorescence reporter mini-gene to visualize the heart-specific splicing regulation of *TTN*. By utilizing this reporter, we have revealed that the two serine residues in the RSRSP stretch are critical for nuclear localization of RBM20. We also found that the residues are constitutively phosphorylated in cells. Furthermore, we identified an S634W missense mutation within the RSRSP stretch in a family of DCM patients, which also affected nuclear localization of RBM20. Rbm20^{S637A} knock-in mouse showed a remarkable effect on titin isoform expression. Our study unveiled the function of the RSRSP stretch as a critical part of a nuclear localization signal and offers the Rbm20^{S637A} mouse as a model for DCM (Sci Rep, 8: 8970, 2018; Front Mol Biosci, 5: 105, 2018).

Hypoxic Breast Cancer Project

Principal Researcher Koh Nakayama, Ph.D.
Collaborators Ryo Yonashiro, Ph.D.
Hiroshi Shibuya Ph.D.
Yoshio Miki, M.D., Ph.D.
Fumitoshi Ishino, Ph.D.

Research Subject

Tumor microenvironment is often hypoxic, and induces malignant transformation of cancer cells. Breast cancer is one of the major cancers in women worldwide. Thus, it is important to understand how hypoxic environment affects the character of breast cancer. We have been focusing on acute and chronic phases of hypoxic responses, and identified mechanisms to regulate transcription and metabolism under such conditions. In this project, we aim to understand the regulatory mechanism of gene expression in breast cancers which is mediated by epigenetic changes. We currently analyze DNA demethylating enzyme TETs. TETs mediate gene expression by altering the methylation status in cells. Hypoxic regions are known to be formed in breast tumors, and DNA methylation is also reported to be increased in breast cancers. However, it is not clear if hypoxia has anything to do with the methylation status in breast cancer. Thus, we try to address this question by combining approaches based on hypoxic and epigenetic studies (Figure). Our final goal is to establish a new technology to detect an early stage of breast cancer by using the molecules identified in this project.

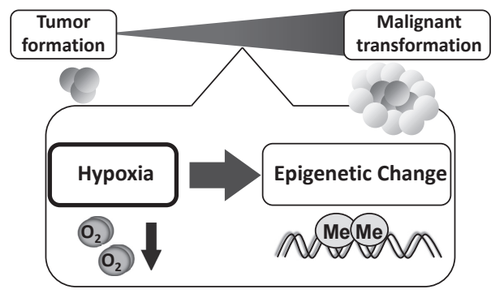


Figure. Scheme of Hypoxic Breast Cancer project

Core Research Project for the implementation of Precision Medicine of Head and Neck and Esophageal Squamous Cell Carcinoma

Department of Molecular Cytogenetics　Johji Inazawa

Lymph node metastasis (LNM) is well known to be an early event associated with poor prognosis in patients with head and neck squamous cell carcinoma (HNSCC), including oral SCC (OSCC) and esophageal squamous cell carcinoma (ESCC). In addition, surgical treatment against these types of cancer causes the deterioration in the quality of life due to the change in the facial appearance and difficulty in meal intake. Thus, we focused on cancer metastasis and tried to identify cancer metastasis-related genes and biomarkers for the prediction of LNM.

In this study, we have constructed a network for collecting and analyzing clinical samples through a cooperation between the medical and dental hospitals and the Bioresource Research Center (BRC) of TMDU. Over 600 clinical samples have been collected as of Oct. 2018 (HNSCC: 204 samples, ESCC: 420 samples).

Tumor-specific aberrant DNA methylation of CpG islands around the promoter regions of tumor-related genes has been investigated as a possible biomarker for use in early diagnosis and prediction of prognosis. Thus, we performed a genome-wide screening of DNA methylation status in a discovery cohort of 67 primary ESCC tissues and their paired normal esophageal tissues for identification of biomarkers as an LNM predictor. From the result of this screening and the analysis of validation cohort with 59 primary ESCC samples, we finally found that HOXB2 and SEPT9 may be useful epigenetic biomarkers for the prediction of the presence of LNM in ESCC.

Moreover, we identified the genomic factors associated with the diagnosis and prognosis of OSCC via next - generation sequencing (NGS). We evaluated data from 220 cases of OSCC. Genomic DNA was eluted using formalin - fixed, paraffin - embedded samples (FFPE), and targeted resequencing of 50 cancer - related genes was performed. In total, 311 somatic mutations were detected in 220 patients, consisting of 68 synonymous mutations and 243 non - synonymous mutations. Distant metastasis was noted in nine of 37 patients (24%) with receptor tyrosine kinase (RTK)-amplification, accounting for 43% of the 21 cases of distant metastasis. The cumulative 5 - year survival rate was 64.6% in the receptor tyrosine kinase RTK-amplification group vs 85.2% in the no RTK-amplification group. Moreover, we identified significantly poorer prognosis in the TP53 mutation/ RTK-amplification group, for which the cumulative 5 - year survival rate was 41.6%. In conclusion, the results of this study demonstrated that RTK-amplification is a prognostic factor for distant metastasis of OSCC, indicating the necessity of using NGS in clinical sequencing.

DOHaD research towards preventive and pre-emptive approach against chronic intractable disease

Principal researcher　Noriko Sato
Comprehensive Reproductive Medicine　Naoyuki Miyasaka
Molecular Epidemiology　Chihiro Imai
Univ. Yamanashi　Takashi Kohda
Tokyo Med. Univ.　Katsuko Sudo

Summary

Developmental Origin of Health and Disease (DOHaD) is the concept that the process through which the environment encountered before birth and/or infancy shapes the long-lasting bodily function and physiology. Epidemiological studies have shown that inadequate conditions in early stage of life can predispose us to lifelong diseases, which are developed later. It is crucial to accumulate genomic and epigenomic epidemiological data of prospective birth cohorts, and to analyze how environment interacts with fetal genome and modulates its phenotype, which is still a burgeoning field of research in Japan. Our Birth Cohort – Gene and ENvironment Interaction Study of TMDU (BC-GENIST) will sort out the current environmental conditions possibly threatening mother and child health. We will identify the interindividual epigenetic differences caused by the interaction between the genetic polymorphisms and environmental variables. In order to elucidate the molecular mechanisms how the prenatal conditions form the future disease phenotype, animal models are being investigated. Particularly, we are currently studying the effects of parental ageing on embryo development and the effects of periodontal disease on pregnancy and metabolism.

Research Project

It is not known how pregnancy-induced weight gain or hyperlipidemia influences the methylation levels of obesity-related differentially methylated CpG sites in blood cells. We performed the prospective cohort study of pregnant women (n = 52) using the MassARRAY EpiTYPER assay, and analyzed methylation levels of *CPT1A* and *SREBF1*, which were previously verified to be robustly associated with adiposity traits. The methylation-based estimation of leukocyte proportion was also conducted using controls whose age, sex and BMI were similar to those of the pregnant women. The association of methylation levels of *CPT1A* and *SREBF1* with BMI and LDL-C was mostly reproduced as EWAS reports only at mid-gestation. Consistent with the metabolic shift from an anabolic to a catabolic state during mid-to-late gestation, those association were weakened at late gestation. However, the BMI-association with *CPT1A* intron 1 methylation was strengthened at late-gestation, which was mediated by BMI-dependent change in lymphocyte proportion. By tracing the same individuals and simultaneously assessing the leukocyte composition, we have successfully shown that the blood methylation of adiposity-related differentially methylated CpGs during mid-to-late gestation followed the individual metabolic and immunological alteration.

Publications

[Original Paper]
Pavethynath S, Imai C, JIN X, Hichiwa N, Takimoto H, Okamitsu M, Tarui I, Aoyama T, Yago S, Fudono A, Muramatsu M, Miyasaka N and Sato N, "Metabolic and immunological shifts during mid-to-late gestation influence maternal blood methylation of CPT1A and SREBF1", Int. J. Mol. Sci., 2019, 20,1066.

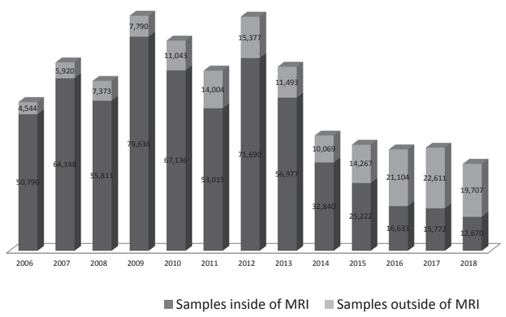
[Presentation at international meetings]
Noriko Sato. "Perinatal Immunomethylomics towards DOHaD". 17th Surugadai International Symposium & Joint Usage/Research Program of Medical Research Institute International Symposium. 2018.11.19. Tokyo, Japan.

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 2018.

We have cooperated with Research Core Center of the University.



1. Sequencing analyses

A total of 32,377 samples from 3,066 researchers were sequenced in the year of 2018. Among them 19,707 (60.9%) samples were requested by researchers outside the medical Research Institute (see below). This year the new next generation sequencer Ion S5 has been installed. Analysis by using next generation sequencing equipment (Ion PGM and Ion S5) has been started in 2013 and 20 runs were done in the year of 2018. Library preparation service for next generation sequencing has been started in 2015 and 118 samples were done in the year of 2018.

2. Equipment under the management of the Genome Laboratory.

DNA sequencer (ABI3130xl) × 2, Next generation sequencer (Ion PGM, Ion S5), 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

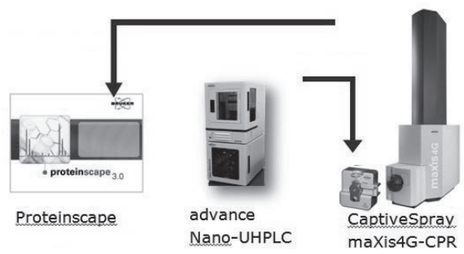
Introductory seminars were done for use of instruments (4 times).

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 installations written below.

For proteome analysis, we have a 2D-electrophoretic system, LC-MSMS system and Biacore system, MicrocalorimetryTC200, Eppendorf InjectMan NI2, Leica M165FC in this laboratory. We can accept the consignment analysis of proteins with 2D-electrophoresis and the mass spectrometry by request of researchers in this university. In addition, we can provide technical advices on cytometry and proteome researchers who wish to start their own research.

We belong to RCC (Research Core Center) and Nanken-Kyoten in TMDU.

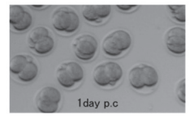


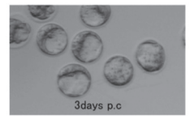
maxis-4G-CPRsis Bruker Daltoncs

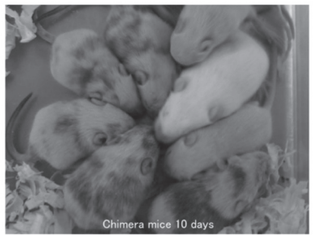
Laboratory of Genome Editing for Biomedical Research

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 this Laboratory, excellent technical staffs generate, clean up, maintain and store recombinant mice to facilitate the biomedical research in Medical Research Institute. In FY 2015, using genome editing technology, we have started a new support service to generate recombinant animals for TMDU researchers.

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.

Website: http://www.tmd.ac.jp/mri/lacf/index.html

<<Common equipment>>

- Confocal laser microscope
  … LSM710, LSM510META (Carl Zeiss)
- Cryostat … CM3050s (Leica)
- Rotary microtome … HM-325E, HM-335E (Microm)
- Vibrating microtome … PRO7 (D.S.K.)
- Automated Tissue Processor
  … RH-12DM (Sakura Finetek)
  Excelsior ES (Thermo Fisher Scientific)
- Tissue-embedding-station
  … Histostar (Thermo Scientific)
- Real-time PCR … 7500, 7900HT (Applied Biosystems)
- Laser microdissection … LMD7000 (Leica)
- Stereo microscope … SZX-16 (Olympus)

<<seminars>>

A user of Confocal laser microscope and Laser Capture Microdissection is required to attend a seminar for learn of the correct way to use.

In this fiscal year, seminars were held on the following dates.

Confocal laser microscope…5/29 and 10/30

Laser Capture Microdissection…5/16

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. Cooperation with a research core center of this university has started from the current year. Now the Laboratory is equipped with basic and state-of-the-art research instruments, including high-speed cell sorters (MoFlo Legacy and MoFlo XDP), confocal laser scanning microscopes (FV10i-W for time-lapse images, and FV10i-DOC for one shot images).

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

The use of the equipment and cell sorting service are available to researchers in other departments within the University and those outside since August 1st, 2013. The number of users is increasing every year.

The number of overall use cases was 457 in the year of 2018. We held 3 short courses for beginners to help them use the equipment.

Bioresource Laboratory

Bioresource Lab. of Medical Research Institute was established in 2004, to support researchers and help postgraduates in cell culture. The center safely supplies 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.

In the relevant year, we cooperated with the Research Center for Industry Alliances concerning the distribution of cell lines outside the university, and we prepared external documents to ask the provider to comply with the depositor's rights and safe use of cell lines. For the purpose of proper storage and maintenance of cell lines, we undertake with mycoplasma contamination test (see Fig. 1). In this fiscal year, as in the previous year, there was a high number of entrusted cases. 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. In that fiscal year, we accepted new requests from Tohoku University and others. Regarding biological sample preservation work that is accepted only within the university, we have requested storage of many specimens as before. A large liquid nitrogen tank (see Fig. 2) that can store samples without influence even at the time of power failure is effectively utilized by users.

In this department, there was a change in practical personnel at the beginning of the fiscal year. The operation work of this Laboratory continues to be successful. In the fiscal year, we revised the content of the support room operations and received approval from our Ethics Committee.

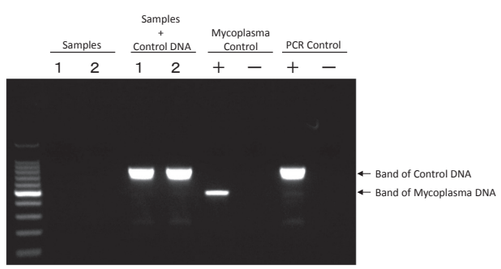


Fig. 1 Mycoplasma Test

Fig.2 Large liquid nitrogen tank G430-S (Taiyo Nippon Sanso Coporation)

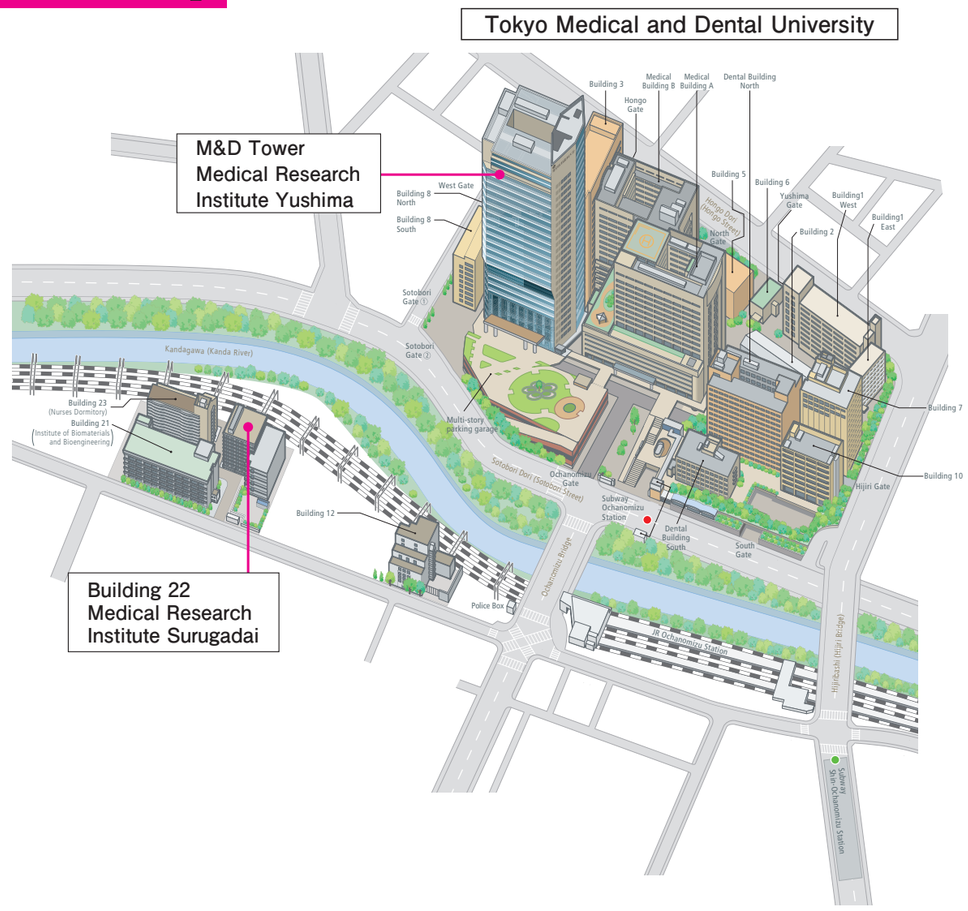
Laboratory for Structure Analysis

The Laboratory for Structure Analysis is equipped with a high-brilliancy X-ray generator (Rigaku MicroMax007HF) and an imaging plate X-ray detector (Rigaku R-AXIS VII) for the structure determination of biological macromolecules. The laboratory is also equipped with a dynamic light scattering (DLS) instrument (Malvern Zetasizer μV), enabling the measurements of the particle size (thus the oligomeric state and aggregation) of protein molecules. An introductory practical course was held for students of Graduate School. The laboratory joined the Joint Usage/Research Program of the Institute and is open for users from the outside of the university.

Advisory Committee Members

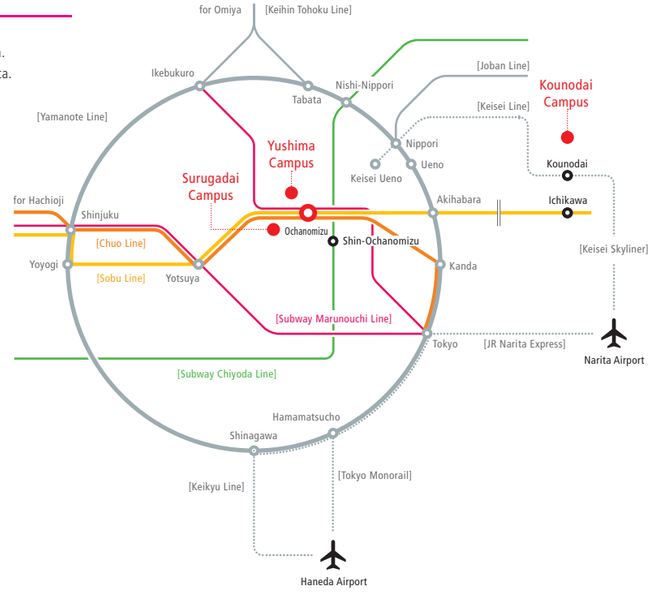
GO Mitiko	Trustee Nagoya University
SASAZUKI Takehiko	University Professor Kyushu University
TANAKA Takaharu	President Hoshi University
TANIGUCHI Masaru	Special Advisor RIKEN Center for Integrative Medical Sciences
NAGAI Ryozo	President Jichi Medical University
NAKAGAMA Hitoshi	Director National Cancer Center Research Institute
NAGANO Tetsuo	Vistiting/Emeritus Professor Drug Discovery Initiative The University of Tokyo
NISHIKAWA Shin-ichi	Advisor JT Biohistory Research Hall

Access Map



Nearest Stations

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



ANNUAL REPORT 2019

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