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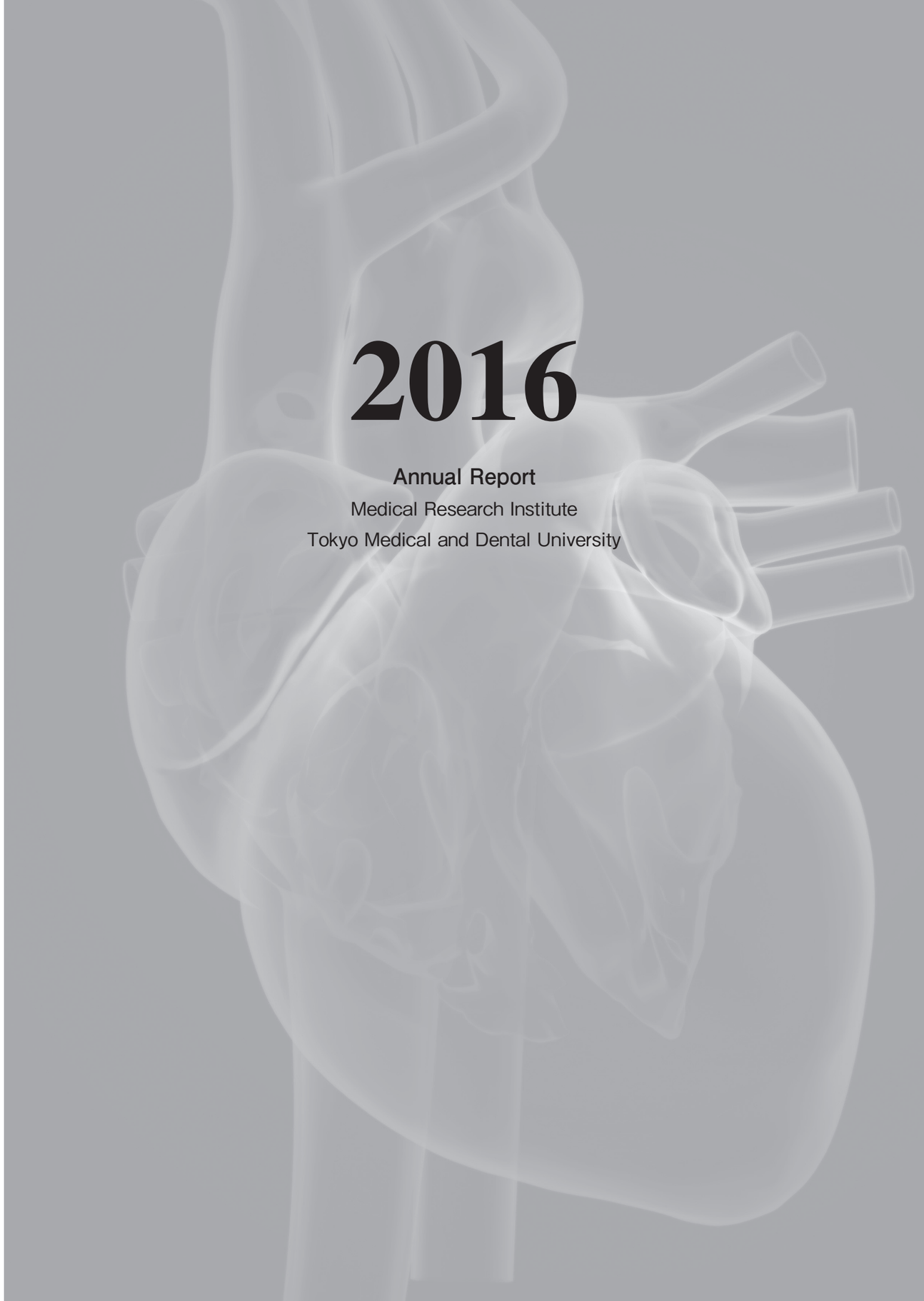
ANNUAL REPORT 2016

Tokyo Medical and Dental University

Medical Research Institute / Tokyo Medical and Dental University

Annual Report 2016



2016

Annual Report

Medical Research Institute

Tokyo Medical and Dental University

Contents

Medical Research Institute

Yushima Area

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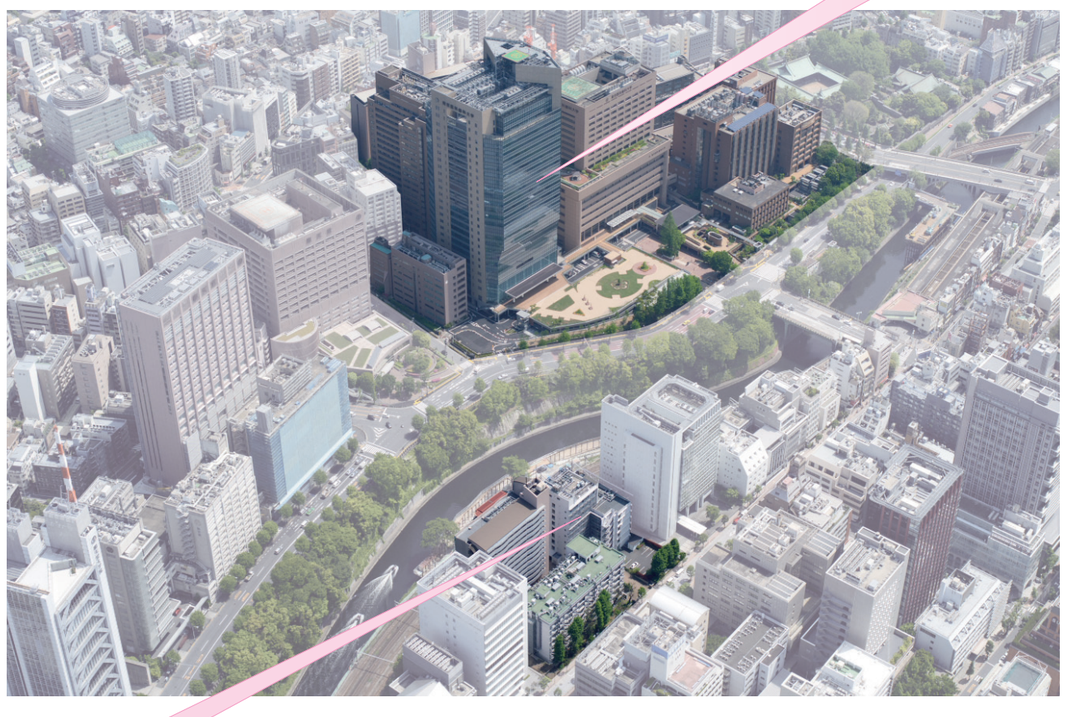
Medical Research Institute

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, Frontier Research Unit Redox Response Cell Biology, Frontier Research Unit Laboratory of Oxygen Biology, Tenure Track Research Unit Department of Cellular and Molecular Medicine, Project Research Unit, Administrative Office



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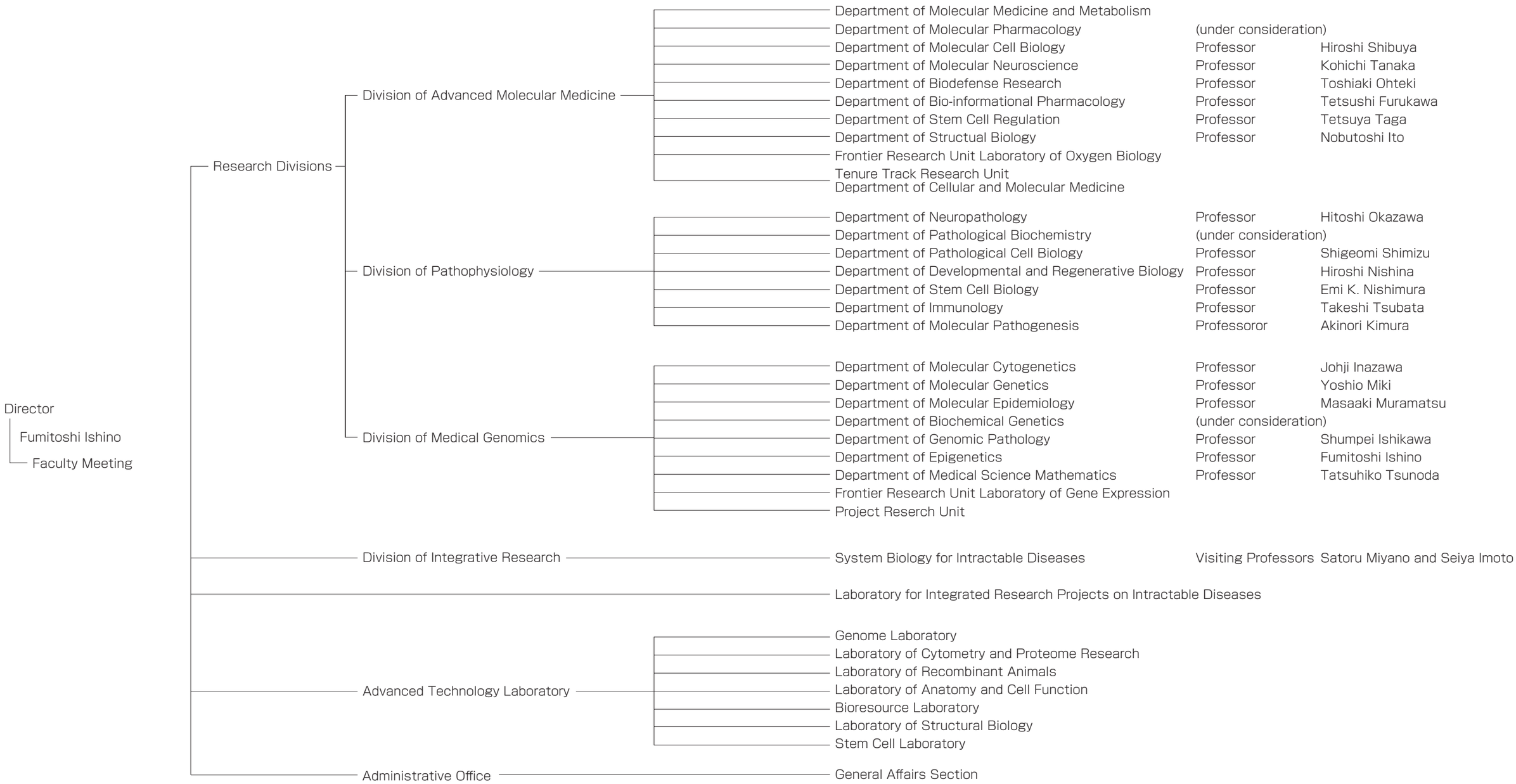
Medical Research Institute

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

Medical Research Institute



Highlight

Highly efficient CRISPR knock-in in mouse
Super convenient method for targeted long cassette insertion

Tokyo Medical and Dental University (TMDU)

CRISPR/Cas (clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas)) system, which is based on chemically synthesized small RNAs and commercially available Cas9 enzyme, enabled long gene-cassette knock-in in mice with highest efficiency ever reported.

Genome editing using CRISPR/Cas system has enabled direct modification of the mouse genome in fertilized mouse eggs, leading to rapid, convenient, and efficient one-step production of knockout mice without embryonic stem cells. In contrast to the ease of targeted gene deletion, the complementary application, called targeted gene cassette insertion or knock-in, in fertilized mouse eggs by CRISPR/Cas mediated genome editing still remains a tough challenge.

Professor Kohichi Tanaka and Dr. Tomomi Aida at Laboratory of Molecular Neuroscience, Medical Research Institute, TMDU has now overcome this issue by developing innovative highly efficient CRISPR/Cas system, which resulted in targeted insertion of long gene cassette including enhanced green fluorescent protein (EGFP) into mouse genome in fertilized eggs with efficiency up to approx. 50%. The team reproduced the natural state of CRISPR/Cas system, which consists of three components: Cas9 protein, CRISPR RNA (crRNA), and trans activating crRNA (crRNA), instead of commonly used two-component system which consists of Cas9 mRNA and single guide RNA (sgRNA), leading to extremely high efficiency. The cloning-free CRISPR/Cas system further provides highly convenient and accurate gene modification, and its successful transmission to the next generations.

The new work will be published in international scientific journal *Genome Biology* (http://genomebiology.com/) as an article entitled "Cloning-free CRISPR/Cas system facilitates functional cassette knock-in in mice" at 1:00 AM on April 29, 2015 (UK time zone).

This improved CRISPR/Cas system will be useful for a variety of applications, including creation of humanized mice for modeling of genetic diseases, drug metabolisms, immunity, and infectious diseases. Further, accurate targeted insertion will improve the safety of gene therapy in human patients in the future. The new system can be also applied to other purposes such as production of livestock, fishes, plants, and microorganisms carrying useful traits.

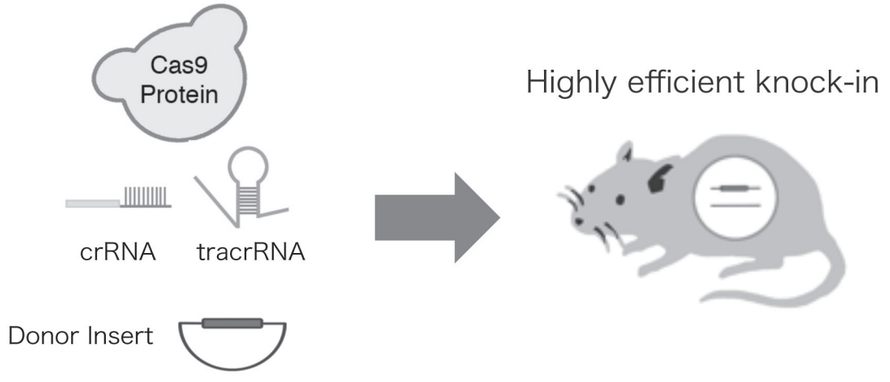
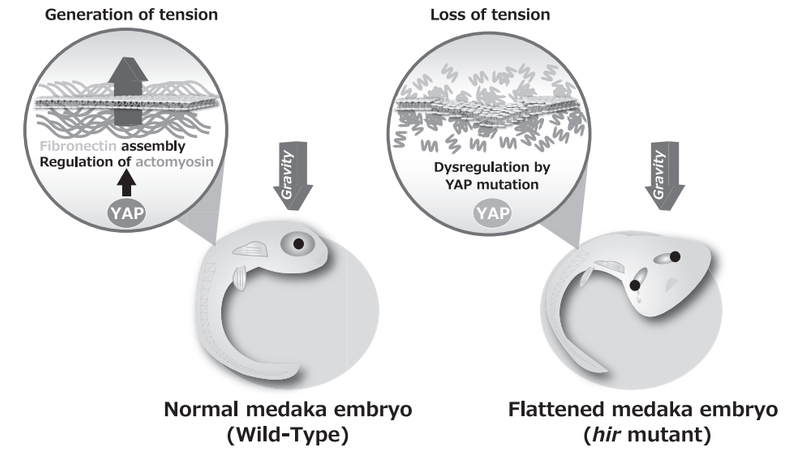


Image: This image shows schematic diagram of the highly efficiennt CRISPR/Cas system, which leads to super efficient targeted insertion (knock-in) of a long donor insert into mouse genome.

Discovery of flat medaka mutant solves a 100-year-old mystery of 3D body morphogenesis against gravity.

A century ago Sir D'Arcy Wentworth Thompson, a British mathematical biologist, predicted that terrestrial animal body shapes are conditioned by gravity. However, there has been no animal model directly demonstrating how the mechano-morphogenetic processes are coordinated to generate a body shape against gravity. A unique medaka mutant, *hirame* (*hir*), which means flatfish in Japanese was isolated by a phenotype-driven mutagenesis screen for mutations affecting organogenesis in medaka (*Oryzias latipes*) embryos. Due to mutation of the transcriptional co-activator YAP, *hir* embryos are sensitive to deformation by gravity and exhibit a markedly flattened body. Actomyosin-mediated tissue tension and fibronectin fibril formation are abnormal in *hir* embryos, leading to tissue flattening and tissue misalignment, both of which contribute to flattened body. YAP-knockdown human 3D spheroid also exhibits collapse upon exposure to external forces, which is reminiscent of the *hir* phenotype. Together, these findings reveal a novel mechanism of gravitational resistance in the 3D morphogenesis of a variety of animals including fish and humans. This was discovered in a multi-institutional research project spearheaded by TMDU Hiroshi Nishina's group, IST Austria Professor Carl-Philipp Heisenberg's team, and Makoto Furutani-Seiki's lab from the University of Bath in the UK,.

Nature, doi: 10.1038/nature14215



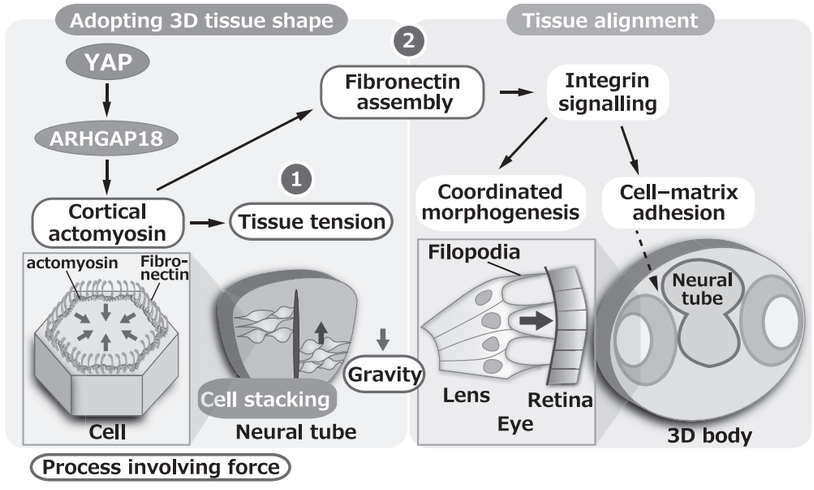


Fig. Schematic summarizing how YAP controls 3D tissue shape and alignment.

Division of Advanced Molecular Medicine

Aim and Scope

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

【Molecular Pharmacology】

- We demonstrate that Nck regulates preosteoblastic/osteoblastic migration and bone mass.
- We demonstrate that TGF-β regulates Ift88 gene expression at least in part via posttrascriptional manner.
- We discovered that sympathetic tone regulates osteoblastic migration in association with cell cycle transition by using FUCCI system.
- We show that Lgr4 gene is regulated by BMP and is required for BMP effects on osteoblastic differentiation.
- We found that Pfn1 is a novel target of BMP and suppresses BMP-induced differentiation of osteoblasts at least in part via transcriptional event.

【Molecular Cell Biology】

- WDR26 plays a negative role in β-catenin degradation in the Wnt signaling pathway.
- WNK signaling pathway is involved in neural development via Lhx8 gene expression.

【Molecular Neuroscience】

- Highly efficient CRISPR knock-in in mouse.
- A CDC42EP4/septin-based perisynaptic glial scaffold facilitates glutamate clearance.

【Biodefense Research】

- Discovery of human common monocyte progenitor (cMoP).
- Elucidation of induction mechanisms of inflammatory bowel disease by commensal bacteria.
- Bacterial c-di-GMP affects hematopoietic stem/progenitors and their niches through STING.

【Bio-informational Pharmacology】

- Genetic mutations of a His-Purkinje system transcription factor IRX3 cause exercise-related lethal arrhythmias.
- miR-27b causes atrial arrhythmia induced by high-fat diet.
- Cardiac safety screening system is established using a 3D-cardiac simulator.

【Stem Cell Regulation】

- Sox17 was found to contribute to the maintenance of hematopoietic cell clusters containing HSCs in the midgestation AGM region, via the Notch1-Hes1 pathway.
- Increases of GFAP-positive astrocytes were observed in the forebrain in such regions as cerebral cortex in histone demethylase gene mutant mice which show abnormal behavior.
- A polymer mimicing glioma stem cell niche was found to bind to transferrin. Involvement of iron-storing tumor-associated macrophages to cancer progression was suggested.

【Structural Biology】

- The crystal structure of a mutant of the Dengue virus envelope protein.
- The complex structures of a protein kinase involved in macular degeneration with its specific inhibitor various was determined.
- Molecular mechanism of inhibition of the aggregation of the Alzheimer's disease-related tau protein by Pin1 PPIase was investigated.

Department of Molecular Pharmacology

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

Research Summary

Skeletal system is the largest storage site for calcium in a living body and its metabolism is conducted by a complex cell society consisting of bone-forming osteoblasts and bone-resorbing osteoclasts as well as stromal cells and chondrocytes. In our department, we take molecular and cellular biological approaches to study the mechanisms of regulation of the development, differentiation, and function of each group of these cells.

Research Projects

1. TGF-β suppresses Ift88 expression in chondrocytic ATDC5 cells. (Kawasaki M, et al.).

Ift88 is an intraflagella transport protein, critical for the cilium. Here, we examined the effects of TGF-β, on the expression of Ift88 in ATDC5 cells as chondrocytes. TGF-β treatment suppresses the levels of Ift88 mRNA in a dose-dependent manner. TGF-β treatment reduces the number of cilia positive cells and suppresses average length of cilia. Knockdown of Ift88 by siRNA enhances TGF-β-induced increase in type II collagen mRNA expression in ATDC5 cells revealing the suppressive role of Ift88 on TGF-β-induced regulation of extracellular matrix protein expression. TGF-β also suppresses Ift88 mRNA expression in primary culture of rib chondrocytes. (J. Cell. Physiol., 2015).

2. Beta adrenergic receptor stimulation suppresses cell migration in association with cell cycle transition in osteoblasts-Live imaging analyses based on FUCCI system. (Katsumura S, et al.).

In disuse osteoporosis, sympathetic tone suppresses bone formation. However, how beta adrenergic stimulation affects osteoblastic migration and associated proliferation is not known. Here we introduced a live imaging system, fluorescent ubiquitination-based cell cycle indicator (FUCCI), in isoproterenol regulation of cell cycle transition and cell migration in osteoblasts. Isoproterenol treatment suppresses the cell cycle transition. The isoproterenol treatment also suppresses on the velocity of migration. This regulation is cell cycle phase specific in G1 phase but not in G1 /S nor in G2 /M phase. These observations on isoproterenol regulation of cell migration and cell cycle transition are opposite to the PTH actions in osteoblasts. (J. Cell Physiol. 2016)

3. BMP-2 enhances Lgr4 gene expression in osteoblastic cells. (Pawaputanon Na Mahasarakham C, et al.).

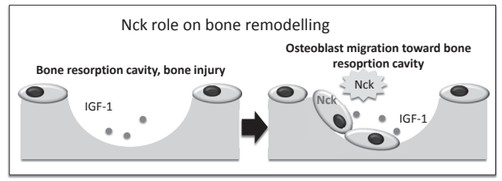
Bone morphogenetic protein (BMP) plays a key role in regulation of bone formation while its downstream targets are still incompletely understood. Lgr4 gene encodes an orphan receptor and has been identified as a genetic determinant for bone mass in osteoporotic patients. Here, we examine the effects of BMP on the expression of Lgr4 in osteoblastic cells, MC3T3E1. Lgr4 gene is expressed in MC3T3E1. BMP treatment enhances Lgr4 mRNA expression at least in part via transcriptional event. When Lgr4 mRNA is knocked down, the levels of BMP-induced increase in alkaline phosphatase (Alp) activity and Alp mRNA are suppressed. BMP enhancement of Lgr4 gene expression is suppressed by FGF and reversed by dexamethasone. BMP also enhances Lgr4 expression in primary cultures of calvarial osteoblasts. These data indicate that Lgr4 gene is regulated by BMP and is required for BMP effects on osteoblastic differentiation.

4. Profilin expression is regulated by bone morphogenetic protein (BMP) in Osteoblastic Cells. (Lin W, et al.).

Profilin 1 (Pfn1) regulates cytoskeletal reorganization and migration. Here, we examine the expression of Pfn1 in osteoblasts and its role in BMP-induced differentiation. In osteoblastic MC3T3-E1(MC) cells, Pfn1 expression declined during the culture in contrast to the increase in alkaline phosphatase activity. BMP treatment suppresses the levels of Pfn1 mRNA. This suppressive effect is time dependent and further down regulation was seen in a longer culture period. Pfn1mRNA knock down (KD) enhances BMP-induced increase in alkaline phosphatase (Alp) activity and Alp mRNA expression. Furthermore, Pfn1 KD enhances BMP-induced transcriptional expression of luciferase reporter activity via BMP response element in osteoblasts. These data indicate that Pfn1 is a novel target of BMP and suppresses BMP-induced differentiation of osteoblasts at least in part via transcriptional event. (J. Cell. Biochem. 2015)

Nck influences preosteoblastic/osteoblastic migration and bone mass

- Bone formation requires preosteoblast/osteoblast migration toward bone forming sites. However the molecular mechanisms connecting between cell migration and bone formation has not been elucidated.
- We discovered that Nck, which is a cytoskeletal regulatory molecule, is essential for osteoblast migration.
- Nck deficiency impairs osteoblast migration that results in suppression of bone formation.



Highlight

Nck influences preosteoblastic/osteoblastic migration and bone mass. (Aryal SAC, et al.).
Migration of the cells in osteoblastic lineage, including preosteoblasts and osteoblasts, has been postulated to influence bone formation. However, the molecular bases that link preosteoblastic/osteoblastic cell migration and bone formation are incompletely understood. Nck (noncatalytic region of tyrosine kinase; collectively referred to Nck1 and Nck2) is a member of the signaling adaptors that regulate cell migration and cytoskeletal structures, but its function in cells in the osteoblastic lineage is not known. Therefore, we examined the role of Nck in migration of these cells. Nck is expressed in preosteoblasts/osteoblasts, and its knockdown suppresses migration as well as cell spreading and attachment to substrates. In contrast, Nck1 overexpression enhances spreading and increases migration and attachment. As for signaling, Nck double knockdown suppresses migration toward IGF1 (insulin-like growth factor 1). In these cells, Nck1 binds to IRS-1 (insulin receptor substrate 1) based on immunoprecipitation experiments using anti-Nck and anti-IRS-1 antibodies. In vivo, Nck knockdown suppresses enlargement of the pellet of DiI-labeled preosteoblasts/osteoblasts placed in the calvarial defects. Genetic experiments indicate that conditional double deletion of both Nck1 and Nck2 specifically in osteoblasts causes osteopenia. In these mice, Nck double deficiency suppresses the levels of bone-formation parameters such as bone formation rate in vivo. Interestingly, bone-resorption parameters are not affected. Finally, Nck deficiency suppresses repair of bone injury after bone marrow ablation. These results reveal that Nck regulates preosteoblastic/osteoblastic migration and bone mass. (Proc. Natl. Acad. Sci. U.S.A. 2015).

Significance
This discovery provides a unique insight into the understanding of bone remodeling.

Publications

[Original articles]

1. Aryal A C S, Miyai K, Izu Y, Hayata T, Notomi T, Noda M, Ezura Y. Nck influences preosteoblastic/osteoblastic migration and bone mass. ***Proc. Natl. Acad. Sci. U S A.*** 2015, 112(50): 15432-7.
2. Kawasaki M, Ezura Y, Hayata T, Notomi T, Izu Y, Noda. TGF-β suppresses Ift88 expression in chondrocyteic ATDC5 cells. ***J. Cell. Biochem.*** 2015; 230 (11): 2788-2795.
3. Katsumura S, Ezura Y, Izu Y, Shirakawa J, Miyawaki A, Harada K, Noda M. Beta adrenergic receptor stiumulation suppresses cell migration in association with cell cycle transition in osteoblasts-live imaging analyses based on FUCCI system. ***J. Cell. Physiol.*** 2015; 231 (2), 496-504.
4. Lin W, Ezura Y, Izu Y, Smriti A, Kawasaki M, Pawaputanon C, Moriyama K, Noda M. Profilin expression is regulated by bone morphogenetic protein (BMP) in osteoblastic cells. ***J. Cell. Physiol.*** 2015; 117(3): 621-628.
5. Pawaputanon Na Mahasarakham C, Ezura Y, Kawasaki M, Smriti A, Moriya S, Yamada T, Izu Y, Nishimori K, Izumi Y, Noda M. BMP-2 enhances Lgr4 gene expression in osteoblastic cells. ***J. Cell. Physiol.*** 2015 *In press*
6. Nakamoto T, Izu Y, Kawasaki M, Notomi T, Hayata T, Noda M, Ezura Y. Mice Deficient in CIZ/NMP4 Develop an Attenuated Form of K/BxN-Serum Induced Arthritis. ***J. Cell. Physiol.*** 2015. *In press*
7. Moriya S, Izu Y, Arayal S, Kawasaki M, Hata K, Pawaputanon Na Mahasarakhahm C, Izumi Y, Saftig P, Kaneko K, Noda M, Ezura Y. Cathepsin K Deficiency Suppresses Disuse-Induced Bone Loss. ***J. Cell. Physiol.*** 2015. *In press*
8. Izu Y, Ezura Y, Koch M, Birk D, Noda M. Collagens VI and XII form complexes mediating osteoblast interactions during osteogenesis. ***Cell Tissue Res.*** 2015. *In press*

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.

Roles of WDR26 in the canonical Wnt signaling.

Wnt signaling plays important roles in multiple developmental events during embryogenesis. Canonical Wnt signaling is initiated by binding of the Wnt ligand to the cell-surface Frizzled and transmembrane LRP complex. This leads to the membrane recruitment and activation of Dishevelled (DVL), which inactivates the APC/Axin/GSK-3 complex in the cytoplasm, responsible for the degradation of β-catenin. As a result, β-catenin accumulates in the cytoplasm, translocates to the nucleus and associates with Tcf transcription factors, which activate the Wnt target genes. In *Xenopus*, Wnt signaling accompanied by β-catenin nuclear localization at the dorsal side is an important for axis formation during early embryogenesis. Ventral over-expression of *Xwnt-8* or *β-catenin* induces a secondary axis and promotes expression of Wnt target genes, such as *Siamois*, *Xnr3* and *Xtwn*. On the other hand, inhibition of canonical Wnt signaling induces head formation on neural stages. Axin1 contains four conserved regions known as a regulation of G-protein signaling (RGS) domain, which binds to APC, at the N-terminus; a dishevelled and axin (DIX) domain at the C-terminus; and binding domains of GSK-3β and β-catenin in the center region. In the canonical Wnt pathway, Axin1 is a component of the β-catenin destruction complex that negatively controls Wnt signaling. Axin1 down-regulates the amount of cytoplasmic β-catenin, to function as a tumor suppressor gene, and several mutations of Axin1 have been identified in tumor cell lines. In *Xenopus* development, Axin1 functions as a ventralizing gene. Axin1 is a multifunctional gene and its function is dependent on binding partners.

To identify novel proteins that may bind to Axin, we performed a high-throughput analysis of proteins that co-immunoprecipitated with human Axin1 in HEK 293 cells using direct nanoflow liquid chromatography-coupled tandem MS (LC-MS/MS). We identified WDR26 as a candidate protein that may physically interact with Axin1. WDR26 contains several protein-interacting domains: the LisH (lis homology domain); the CTLH (C-terminal to LisH motif) domain; and a WD40 repeat domain. A previous study suggested that WDR26 contributes to the MAPK signaling pathway. However, there are no reports that WDR26 is associated with the Wnt signaling pathway. In the yeast *Saccharomyces cerevisiae*, nine glucose-induced degradation-deficient (GID) genes (GID1–GID9) were isolated . The GID complex, which comprises GID1–GID9 except for GID6, acts as a polyubiquitination enzyme in yeast. Eight vertebrate homologs that share high similarities of domain architecture to yeast GID complex genes have been identified. The following are the yeast GID complex genes and their corresponding vertebrate homologs: GID1/RanBP9; GID2/Rmnd5; GID3/UBE2H; GID4/C17ors39; GID5/ARMc8; GID7/WDR26; GID8/TWA1; and GID9/MAEA. Recent studies showed that RMND5 and ARMc8 promote ubiquitination in vertebrates, but it is still unknown whether other vertebrate homologs including WDR26 are associated with the ubiquitination pathway.

We investigated roles of WDR26 and Axin1 in the canonical Wnt signaling pathway, and we performed several analyses to elucidate the mechanism of β-catenin degradation with WDR26, and obtained the following new results.

1. The interaction of ectopically expressed hWDR26 with hAxin1 was confirmed in HEK 293T cells (Figure 1A). We found that the N-terminal region including LisH domain was responsible for binding to Axin1. Conversely, WDR26 bound to Axin1 at the central region including GSK3β-binding domain.
2. In *Xenopus*, expression of *xWDR26* was gradually localized to the neural region at the early neurula stage. In the late neurula and tadpole stages, *xWDR26* was strongly expressed in the anterior neural region (Figure 1B).
3. The injection of *xWDR26*-MO (morpholino oligo) into dorso-animal blastomeres of eight-cell embryos, knochdoen of xWDR26, reduced both head formation at the tadpole stage (Figure 1C).
4. When *Xwnt-8* mRNA is injected into the ventral sides of four-cell embryos, the target genes of Wnt signaling are induced. Ventral injection of *xWDR26*-MO increased the expression of Wnt target genes that were induced by co-injection of *Xwnt-8* mRNA. Co-injection of *xWDR26* mRNA containing the MO-targeted site with 5-mismatched sequences was restituted the increasing of Wnt target genes by *xWDR26*-MO (Figure 1D).
5. The expression of WDR26 reduced the amount of β-catenin protein in cultured cells in a dose-dependent manner, and the knockdown of *WDR26* by siRNA increased the amount of endogenous β-catenin protein in cultured cells, especially in Wnt-stimulated cells.
6. We found that WDR26 did not bind to β-catenin, although Axin1 binds to WDR26. This suggests that WDR26 controls the stability of β-catenin through binding with Axin1. The expression of Wnt target genes induced by ventral injection with *Xwnt-8* mRNA was decreased by co-injection with *xWDR26* mRNA, but not by mRNA of the LisH domain-deleted construct, and the expression of the LisH domain-deleted construct hardly reduce the amount of β-catenin protein. These suggest that binding between WDR26 and Axin is necessary for β-catenin degradation.
7. Although the ubiquitination of x β-catenin was only slightly increased by co-transfection of *xAxin1* alone, the co-transfection of both *xWDR26* and *xAxin1* highly increased the ubiquitination of x β-catenin. These results suggest that WDR26 regulates the ubiquitination of β-catenin for its degradation, and that binding of WDR26 and Axin is important for this ubiquitination.

These results suggest that WDR26 plays a negative role in β-catenin degradation with Axin1/WDR26 binding in the Wnt signaling pathway (Figure 2).

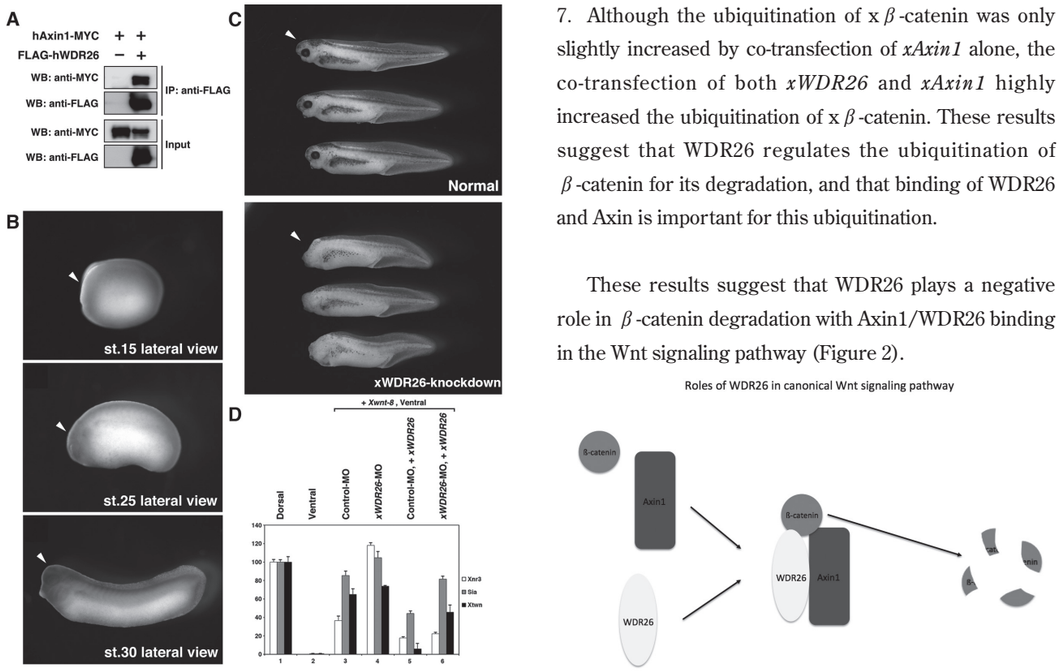


Fig.1

Fig.2

Publications

Fukuzono, T., Pastuhov, S. Iv., Fukushima, O., Li, C., Hattori, A., Iemura, S., Natsume, T., Shibuya, H., Hanafusa, H., Matsumoto, K. and Hisamoto, N. (2016). Chaperone complex BAG2–HSC70 regulates localization of Caenorhabditis elegans leucine-rich repeat kinase LRK-1 to the Golgi. **Genes Cells** in press.

Department of Molecular Neuroscience

Professor	Kohichi Tanaka
Associate Professor	Hidenori Aizawa (~2015/5/31)
	Tomomi Aida (2015/7/1~)
Assistant Professor	Tomomi Aida (~2015/6/30)
Assistant Professor	Masashi Ohmachi (~2015/6/15)

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.

In the cerebellum, both GLAST, a glial glutamate transporter, and CDC42EP4 the small GTPase-effector protein, is exclusively expressed in Bergmann glia and localizes beneath specific membrane domains enwrapping dendritic spines of Purkinje cells. We show that CDC42EP4 forms complexes with septin hetero-oligomers, which interact with GLAST. In *Cdc42ep4*$^{-/-}$ mice, GLAST is dissociated from septins and is delocalized away from the parallel fibre-Purkinje cell synapses. The excitatory postsynaptic current exhibits a protracted decay time constant, reduced sensitivity to a competitive inhibitor of the AMPA-type glutamate receptors (γDGG) and excessive baseline inward current in response to a subthreshold dose of a nonselective inhibitor of the glutamate transporters/EAAT1–5 (DL-TBOA). We propose that the CDC42EP4/septin-based glial scaffold facilitates perisynaptic localization of GLAST and optimizes the efficiency of glutamate-buffering and clearance.

Glutamate-mediated excitotoxicity that occurs due to a deficiency of the glial glutamate transport GLT is one of several potential pathogenic mechanisms of motor neuron death in amyotrophic lateral sclerosis (ALS). However, it remains unknown whether this deficiency is a primary cause or a secondary consequence of motor neuron degeneration. Here, we generated conditional knockout mice that lacked GLT1 specifically in the spinal cord (GLT1-cKO mice) using the Cre/LoxP system. GLT1-cKO mice showed motor deficits, motor neuron loss and nuclear TDP-43 loss. Thus, dysfunction of glial glutamate transporters is sufficient to phenocopy ALS in mice.

2. Development of genome editing technologies

Genetically modified mice such as knockout and knockin mice have drastically improved our understanding of the functions of genes in vivo. However, the generation of genetically modified mice relies on homologous recombination in ES cells, which is a time-consuming, laborious, and expensive process. Recent development of genome editing technologies has enabled direct manipulation of the genome in mouse zygotes without ES cells, thereby providing new avenues for simple, convenient, highly efficient, and ultra-rapid production of genetically modified mice. We developed highly efficient cloning-free CRISPR/Cas system for the production of genetically modified mice, especially for knockin mice carrying functional gene cassettes. Our novel method provides ultra convenient and highly efficient CRISPR/Cas-mediated genome editing and accelerates functional genomic research in vivo.

Publications

[Original papers]

1. Aida, T., Chiyo, K., Usami, T., Ishikubo, H., Imahashi, R., Wada, Y., Tanaka KF., Sakuma, T., Yamamoto, T., Tanaka, K. Cloning-free CRISPR/Cas system facilitates functional cassette knockin in mice. *Genome Biol* 16. 87, 2015.
2. Aida, T., Yoshida, J., Nomura, M., Tanimura, A., Iino, Y., Soma, M., Bai, N., Ito, Y., Cui, W., Aizawa, H., Yanagisawa, M., Nagai, T., Takata, N., Tanaka, KF., Takayanagi, R., Kano, M., Gotz, M., Hirase, H., Tanaka, K. Astroglial glutamate transporter deficiency increases synaptic excitability and leads to pathological repetitive behaviors in mice. *Neuropsychopharmacology* 40. 1569-1579, 2015.
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Department of Biodefense Research

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

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

1. Research on mononuclear phagocytes

1) Discovery of a novel source of mononuclear phagocytes

In 1968, Drs. Ralph van Fruth and Zanvil A. Cohn proposed a concept of mononuclear phagocytes that include monocytes and macrophages. In 1973, Dr. Ralph Steinman discovered dendritic cells (DCs), thereby redefining the mononuclear phagocytes as a population consisting of monocytes, macrophages and also DCs. It has been recently continuing epoch-making discoveries in the field of mononuclear phagocytes and their functions are now beyond classical Immunology and rather extend to broad life phenomenon, e.g. tissue development/regeneration, wound-healing, and establishment of various inflammatory diseases (**Fig. 1**).

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 identified human cMoP (unpublished). Human cMoP gives rise to only monocytes but not other hematopoietic cells including DCs (**Fig. 2**). Given that monocytes and monocyte-derived macrophages cause a variety of inflammatory disorders, including metabolic syndromes and tumor development (**Fig. 1**), our studies shed light on possible therapeutic applications for infectious diseases, cancers, and autoimmune diseases.

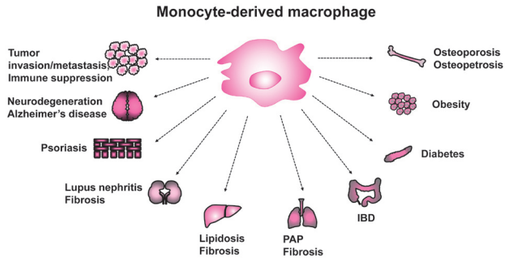


Fig.1 Role of monocyte-derived macrophage in disease

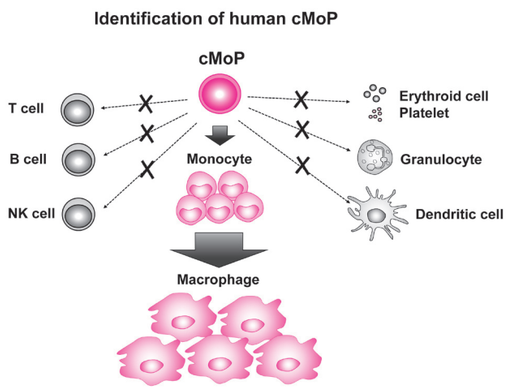


Fig.2 Human cMoP only gives rise to monocyte and macrophage

2) Roles of mononuclear phagocytes in inflammatory bowel disease

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

Using a mouse dextran sodium sulfate (DSS)–induced colitis model, we show here that commensal Gram-positive bacteria trigger the mobilization of inflammatory monocytes and macrophages into the colon (***Mucosal Immunol*** 2015). TNF-α is a representative cytokine that aggravates colitis, and predominantly produced by monocytes/macrophages. Interestingly, pretreating mice with vancomycin, which eliminated Gram-positive bacteria, particularly the Lachnospiraceae family, significantly reduced the severity of the colitis, evaluated by the body weight loss, colon length, pro-inflammatory cytokine level, massive leukocytic infiltration etc. As the sera from Crohn's disease patients and colitic mice react with *Lachnospiraceae bacterium A4* flagella, our findings provide a new environmental risk factor and new therapeutic approaches for IBD. We are currently trying to identify the molecular basis of how bone marrow-derived monocytes differentiate into inflammatory/effector monocytes and macrophages in the inflamed colon.

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, e.g. intestinal stem cells and hair follicle stem cells. Elucidation of detailed mechanisms is currently in progress.

3. Collaborative research with other institutes

In collaboration with National Center for Global Health and Medicine and Keio University School of Medicine, we reported that bacteria-derived bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP) promotes proliferation and mobilization of hematopoietic stem progeny cells (HPSCs) by acting on STING expressed on both HPSCs and their niches. These findings define machinery underlying the dynamic regulation of HSPCs and their niches during bacterial infection through c-di-GMP/STING signaling (***Cell Rep*** 2015).

Publications

[original papers]

1. Liu J, Guo YM, Onai N, Ohyagi H, Hirokawa M, Takahashi N, Tagawa H, Ubukawa K, Kobayashi I, Tezuka H, Minamiya Y, Ohteki T, and Sawada K. Cytosine-phosphorothionate- guanine oligodeoxynucleotides exacerbates hemophagocytosis by inducing tumor necrosis factor-α production in mice after bone marrow transplantation. ***Biol Blood Marrow Transplant*** 2015 [Epub ahead of print]
2. Kobayashi H, Kobayashi CI, Nakamura-Ishizu A, Karigane D, Haeno H, Yamamoto KN, Sato T, Ohteki T, Hayakawa Y, Barber GN, Kurokawa M, Suda T, and Takubo K. Bacterial c-di-GMP affects hematopoietic stem/progenitors and their niches through STING. ***Cell Rep*** 11, 71-84, 2015.
3. Nakanishi , Sato T, and Ohteki T. Commensal Gram-positive bacteria initiates colitis by inducing monocyte/macrophage mobilization. ***Mucosal Immunol*** 8, 152-60, 2015.

[Awards]

Nobuyuki Onai, TMDU Award for Excellence in Research in 2015
Shunsuke Kawamura (D4), Best Presentation Award of the 44th Annual Meeting of The Japanese Society for Immunology in 2015

[Personnel changes]

Hiroyuki Tezuka, Project Associate Professor of School of Pharmacy and Pharmaceutical Sciences, Hoshi University, Tokyo, Japan.

Department of Bio-informational Pharmacology

Professor Tetsushi Furukawa, M.D., Ph.D.
Associate Professor Junko Kurokawa, Ph.D.
Assistant Professor Yusuke Ebana, M.D., Ph.D. (until May 2015)
Kensuke Ihara, M.D., Ph.D. (since September 2015)

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

1. Pathogenesis of atrial fibrillation (AF)

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

We had carried out most extensive GWAS (genome-wide association study) in Japan with 610K SNPs to determine gene polymorphisms associated with AF. Since 2011, we have participated in the international Meta-analysis called as CHARGE study. Up to the last year, we identified totally 15 SNPs associated with AF (Nat. Genet. 2012;44;670-675, Circulation 2014;130:1225-1235). Using these SNPs, we could predict AF development with 65% sensitivity and 65% specificity, which is not sufficient to precede personalized medicine. This year, we carried out GWAS with 1,000K SNPs, and identified further AF-associated SNP. Furthermore, to identify rare SNPs associated with AF, we performed exon-wide association study (EWAS), and identified one SNP with a high odds ratio.

2. Cardiovascular diseases and microRNA

The growing body of evidence indicates that microRNAs play important roles in development of diseases, and that circulating microRNAs can be biomarkers for development and progression of diseases. This year, we used high-fat diet (HFD) mouse model and found that HFD increased expression of miR-27b. miR-27b down-regulated Cx40 in atrial myocytes, disturbed electrical conduction in atrium, and thus caused development of atrial arrhythmias. These findings were reported in J. Mol. Cell. Cardiol. (publication list #9).

3. Novel gene associated with exercise-related lethal ventricular arrhythmias (Fig. 1)

His-Purkinje system has been developed at birds and mammals, providing rapid and reversal propagation of electrical signals in ventricles. Growing body of clinical data indicates its role in pathogenesis of lethal ventricular arrhythmias; however, the mechanism linking His-Purkinje system and ventricular arrhythmias is largely unknown. In collaboration with Professor Miura in Hamamatsu Medical School, we examined electrical characteristics and arrhythmogenicity in mice deleted Irx3, a transcription factor specifically expressed in His-Purkinje system. We found that Irx3-null mice developed lethal ventricular arrhythmias associated with exercise and increased sympathetic nervous activity. We further looked for the association of IRX3 genetic defects with clinical arrhythmias in collaboration with Dr. Shimizu in National Cerebral and Cardiovascular Center. We found 2 novel IRX3 mutations in idiopathic ventricular fibrillation patients, but not in 250 healthy volunteers. In these 2 patients, ventricular fibrillation occurred in association with exercises. Thus, we propose that IRX3 genetic defect is a novel genetic risk for exercise-related lethal ventricular arrhythmias. These results were published in Eur. J. Pharmacol. (publication list #7), were press released in September 29, 2015, and appeared in several medias.

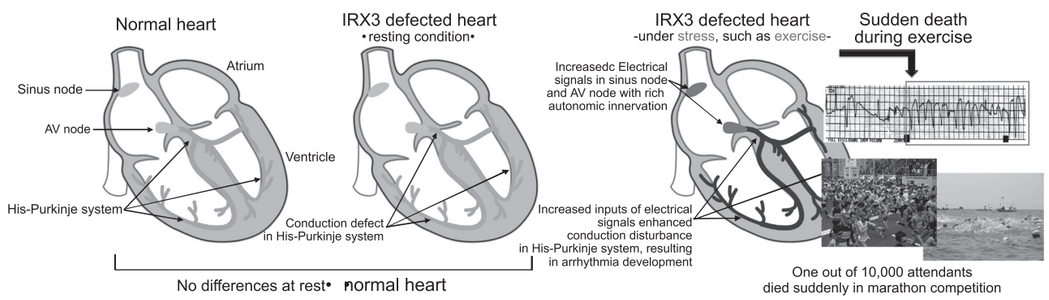


Fig.1 Mechanism of exercise-induced arrhythmias in IRX3 genetic defects

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

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

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

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

Prof. Hisada T. et al. in the University of Tokyo have developed a 3-D cardiac simulator (UT-heart). We have tried to broaden its application to screening of cardiac toxicity of medicines. This year, we examined 10 standard medicines (high risk, intermediate risk, and no risk), and found that the UT-heart predicted cardiac toxicity of medicines with a high accuracy. The UT-heart is a 3-D simulator and divides ventricular wall into 3 layers, endocardial, mid-myocardial, and epicardial layers. If we eliminated 3 layers structure from a simulation, then the accuracy to predict cardiac toxicity of medicines significantly dropped, suggesting the importance of a 3-D simulation for accurate prediction of cardiac toxicity of medicines. There results were reported in Sci. Advance 2015;1:e1400142 (publication list #2), were press released in May 1, 2015, and appeared in several medias.

Publications

[original articles]

1. Kawabata M, Yokoyama Y, Sasaki T, Tao S, Ihara K, Shirai Y, Sasano T, Goya M, Furukawa T, Isobe M, Hirao K. (2015). Severe iatrogenic bradycardia related to the combined use of beta-blocking agents and sodium channel blockers. ***Clin. Pharmacol.*** 7, 29-36.
2. Okada J, Yoshinaga T, Kurokawa J, Washio T, Furukawa T, Sawada K, Sugiura S, Hisada T. (2015). Screening system for drug-induced arrhythmogenic risk combining patch clamp and heart simulator. ***Sci. Advance*** 1, e1400142.
3. Kurokawa J, Sasano T, Kodama M, Li M, Ebana Y, Harada N, Honda S, Nakaya H, Furukawa T. (2015). Aromatase knockout mice reveal an impact of estrogen on drug-induced alteration of murine electrocardiography parameters. ***J. Toxil. Sci.*** 40, 339-348.
4. Yamakawa H, Muraoka N, Miyamoto K, Sadahiro T, Isomi M, Haginawa S, Kojima H, Umei T, Akiyama M, Kuishi Y, Kurokawa J, Furukawa T, Fukuda K, Ieda M. (2015). Fibriblast growth factors and vascular endothelial growth factor promote cardiac reprogramming under defined conditions. ***Stem Cell Reports*** 5, 1128-1142.
5. Saito Y, Nakamura K, Yoshida M, Sugiyama H, Ohe T, Kurokawa J, Furukawa T, Takano M, Nagase S, Morita H, Kusano K,F, Ito H. (2015). Enhancement of spontaneous activity by HCN4 overexpression in mouse embryonic stem cell-derived cardiomyocytes – A possible biological pacemaker. ***PLoS ONE*** 10, e0138193.
6. Ihara K, Sasaki T, Shirai Y, Tao S, Maeda S, Kawabata M, Sasano T, Yokoyama Y, Isobe M, Hirao K. (2015). High atrial debibrillation threshold with internal cardioversion indicates arrhythmogenicity of superior vena cava in non-long-standing persistent atrial fibrillation. ***Circ. J.*** 79, 1479-1485.
7. Koizumi A, Sasano T, Kimura W, Miyamoto Y, Aiba T, Ishikawa T, Nogami A, Fukamizu S, Sakurada H, Takahashi Y, Nakamura H, Ishikura T, Koseki H, Arimura T, Kimura A, Hirao K, Isobe M, Shimizu W, Miura N, Furukawa T. (2015). Genetic defects in a His-Purkinje system transcription factor, IRX3, cause lethal cardiac arrhythmias. ***Eur. Heart J.*** Epub 2015 Oct. 1.
8. Yoshikawa S, Usami T, Kikuta J, Ishii M, Sasano T, Sugiyama K, Furukawa T, Nakasho E, Takayanagi H, Tedder TF, Karasuyama H, Miyawaki A, Adachi T. (2015). Intravital imaging of Ca(2+) signals in lymphocytes of Ca(2+) biosensor transgenic mice:indication of autoimmune diseases before the pathological onset. ***Sci. Rep.*** Epub 2015 Dec. 9.
9. Takahashi K, Sasano T, Sugiyama K, Kurokawa J, Tamura N, Soejima Y, Sawabe M, Isobe M, Furukawa T. (2015). High-fat diet increases vulnerability to atrial arrhythmia by conduction disturbance via miR-27b. ***J. Mol. Cell. Cardiol.*** Epub 2015 Dec. 2.

Department of Stem Cell Regulation

Professor	Tetsuya TAGA
Associate Professor	Tetsushi KAGAWA (-March 2015), Ikuo NOBUHISA
Assistant Professor	Kouichi TABU (June 2015-)
Part-time Lecturer	Ryoichiro KAGEYAMA, Taichi KASHIWAGI
Administrative Assistant	Mako FUSHIMI (-February 2015)
Technical Assistant/Administrative Assistant	Kazuko INOUE
Graduate Student	Genki SUDO, Yasuhiro KOKUBU, Wenqian WANG, Yoshitaka MUROTA, Tomoyo IKENOUE, Ryosuke KIMURA, Kiyoka SAITO, Aoi MINOWA, Shunki NOMOTO, Yuki YOKOI, Haruka EISHI, Satomi TAKAHASHI
Research Student	Kazuo TERASHIMA

Research Outline

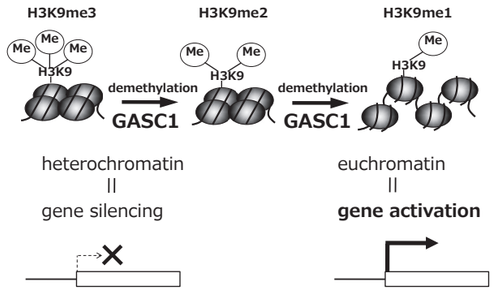
Our research has been conducted to elucidate the mechanisms by which stem cells are regulated. The major focus has been on neural stem cells, hematopoietic stem cells, and cancer stem cells. The study is aimed to understand development, maintenance, and regeneration of the central nervous system and the hematopoietic system, and to obtain a clue to tackle the problem of cancer recurrence. The projects performed in 2015 are categorized into three groups: 1. Understanding of molecular basis for self-renewal and fate determination of neural stem cells, 2. Characterization of fetal hematopoiesis, and 3. Characterization of cancer stem cells and their niche.

Research Projects

1. Epigenetic regulation of brain function

Physiological and pathological roles of histone modification, particularly histone methylation, in higher brain functions largely remained elusive. Gasc1 gene encodes a histone H3 lysine 9 (H3K9) demethylating enzyme, which is considered to lead to a wide range of epigenetic activation of gene expression. We are analyzing the mice with a hypomorphic mutation in the Gasc1 gene in collaboration with Professor Inazawa at Medical Research Institute, Tokyo Medical and Dental University (TMDU), and found that Gasc1 is strongly expressed in post-mitotic neurons in the embryonic and adult mouse forebrain. Although Gasc1 homozygous mutants showed no obvious histological abnormalities in the brain, they exhibited abnormal behaviors, including hyperactivity, stereotyped behaviors and impaired learning and memory. These symptoms appeared to be relevant to human neurodevelopmental disorders. In this year, we reported the increase in GFAP-positive astrocytes in the brain of Gasc1 hypomorphic mutant mice at 2-3 months of age, but not at postnatal day 14 and 30 by immunohistochemistry. Increases of GFAP-positive astrocytes were widely observed in the forebrain and prominent in such regions as cerebral cortex and caudate putamen. This observation of the increase in GFAP-positive astrocytes may help understand the phenotypes of Gasc1 homozygous mutants, because astrocytes are known to affect synaptic plasticity. This study may also help understand neurodevelopmental disorders.

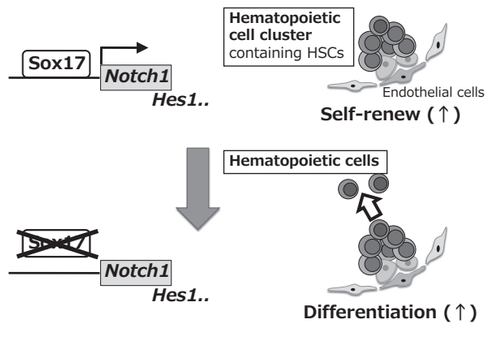
Gene Activation by Histone Demethylase GASC1



2. Analysis of the role of the Sox17-Notch1-Hes1 pathway in the maintenance of hematopoietic stem cells in the mouse embryo

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

【Dorsal aorta in midgestation embryos】



3. Characterization of cancer stem cells and their niche

"Cancer stem cells" (CSCs), a functional subset of tumor cells, are characterized by radio- and chemo-resistance and have been postulated as key drivers of tumor relapse and progression (Figure, upper panel). CSCs reside in a specialized microenvironment known as the niche composed of, for instance, various stromal cells. Elucidation of the CSC niche may help develop effective strategies of cancer therapy. However, to date, very little is known about the identity of niche components. As we have previously reported, C6 glioma cell line contains a sub-population of CSCs, which is enriched in the "side population (SP)" by Hoechst 33342 staining and FACS analysis. As we published in 2004, SP cells in C6 are tumorigenic, but cells in the major population (main population, MP) are not. In the recent couple of years, we searched for CSC niche mimicries from hundreds of synthetic polymers in collaboration with Professor Mark Bradley (University of Edinburgh). Out of nearly 400 polymers arrayed on slides, one urethane polymer PU10 was identified which enriches a higher tumorigenic cell fraction within SP when transplanted into the NOD/SCID mouse brain. TOF/MS analysis of the Pol10-binding proteins in collaboration with Professor Issay Kitabayashi (National Cancer Center Research Institute) further identified an iron-carrier transferrin as a niche candidate protein for CSCs. In mouse tumors formed by SP cells, iron was found to be stored in CD204(+) tumor-associated macrophages (TAMs), suggesting the pivotal contribution of TAMs to cancer progression. We further demonstrated that CSCs recruit bone marrow-derived monocytes and induce their differentiation into macrophages by secreting CCL2 and GM-CSF, respectively, indicating that CSCs have the prominent capacities to accelerate tumor expansion by self-constructing their own niche (Figure, lower panel). Such a polymer-based approach will provide clues to understand the molecular basis for CSC and CSC niche and to develop effective therapeutic strategies against cancers.

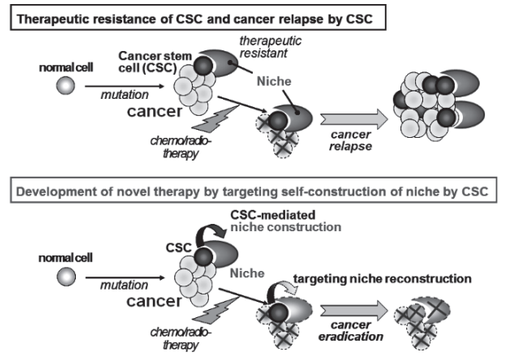


Figure. The concept of CSC-mediated niche construction and cancer eradication

Publications

[Original Article]

1. Sudo G, Kagawa T, Kokubu Y, Inazawa J, and Taga T. Increase of GFAP-positive astrocytes in histone demethylase GASC1/KDM4C/JMJD2C hypomorphic mutant mice. Genes to Cells, in press, 2015
2. Kokubu Y, Tabu K, Wang W, Muramatsu N, Murota Y, Nobuhisa I, Jinushi M, and Taga T. Induction of protumoral CD11c[high] macrophages by glioma cancer stem cells through GM-CSF. Genes to Cells, in press, 2015
3. Tabu K, Muramatsu N, Mangani C, Wu M, Zhang R, Kimura T, Terashima K, Bizen N, Kimura R, Wang W, Murota Y, Kokubu Y, Nobuhisa I, Kagawa T, Kitabayashi I, Bradley M, and Taga T. A synthetic polymer scaffold reveals the self-maintenance strategies of rat glioma stem cells by organization of the advantageous niche. Stem Cells, in press, 2015
4. Kimura T, Wang L, Tabu K, Tsuda M, Tanino M, Maekawa A, Nishihara H, Hiraga H, Taga T, Oda Y, and Tanaka S. Identification and analysis of CXCR4-positive synovial sarcoma-initiating cells. Oncogene, in press, 2015

Department of Structural Biology

Professor	Nobutoshi Ito
Associate Professor	Teikichi Ikura
Assistant Professor	Nobutaka Numoto
Technical Assistant	Michiko Hattori
Postdoctoral fellow	Kenrou Shinagawa

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

1. Molecular mechanisms of the sero-specificity of dengue viruses

Dengue fever is caused by the dengue virus infections transmitted by mosquitoes. Dengue is endemic throughout the tropics and subtropics regions, but recently autochthonous case of dengue in Japan has been reported. Dengue viruses are classified into four serotypes, as DEN1, DEN2, DEN3, and DEN4. Although an infection with one serotype for the first time usually cause mild fever, secondary infection with the other serotypes increase the risk of the severe disease such as dengue hemorrhagic fever or dengue shock syndrome by cross-reactivity. Since there is no vaccine to protect against all four dengue serotypes, the elucidation of detailed molecular mechanisms of sero-specificity will be useful for developing a dengue vaccine.

The ED3 domain of the envelope protein of dengue virus was identified as the antibody binding site, and two putative epitopes (E1 and E2) has been suggested. To elucidate the mechanisms of sero-specificity and cross-reactivity of the epitopes in ED3s, we have initiated the analyses of the immune response and crystallographic studies for the ED3 mutants. We expressed six ED3 mutants of DEN3 and DEN4, in which the E1 and/or E2 from DEN3 was switched to that of DEN4 and *vice versa*. Immune response analyses suggested that the E2 might be primarily responsible for the sero-specificity in DEN3, whereas the E1 might be primarily responsible in DEN4. We have determined the crystal structure of a DEN4 ED3 mutant in which the E2 residues were switched to those of DEN3 ED3 (Figure 1). The structure indicates that the E2 grafting induce no significant changes in the overall structure of ED3, while the electro static potentials of the molecular surface appear to be remarkably different from that of the wild type DEN4 ED3. We have modeled the structures of five remaining mutants by reference to its wild type structures. Comparisons among these structures reveal that the epitope grafted mutants vary in its electro static potentials. These findings and the immune response analyses strongly suggest that the electro static interaction between the antibody and ED3 is thought to be one of important contributing factor to the sero-specificity. We have also investigated the biophysical and structural analyses of the ED3 mutants in which the residue at the hydrophobic core is substituted. The results would provide insights into the effects of thermostability of each ED3s on the sero-specificity. This work is performed in collaboration with Associate Professor Kuroda at Tokyo University of Agriculture and Technology.

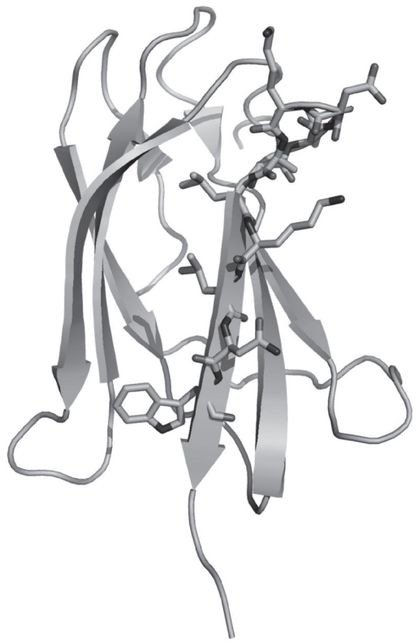


Fig.1 Crystal structure of a DEN4 ED3 mutant in which the E2 residues were switched to those of DEN3 ED3. Residues of the E2 are represented as stick model.

2. Alzheimer's disease-related study

In Alzheimer's, the disease-related protein, Tau, is hyperphosphorylated and aggregates into neurofibrillary tangles (NFT). Recent studies suggest that a peptidyl-prolyl isomerase, Pin1, prevents Tau from aggregating by catalyzing the *cis*-to-*trans* interconversion of phosphorylated Thr231-Pro232 (pThr231-Pro232) bond. Here, we synthesized a peptide comprising a phosphorylated region including Thr231-Pro232 and an aggregation-core region R1; moreover, by nuclear magnetic resonance and fluorescence measurements, we investigated how the catalytic activity of Pin1 prevented the peptide from aggregating. We observed that Pin1 did not catalyze isomerization of the peptide, of which 98% of the pThr231-Pro232 bond took *trans* isoform, but prevented its aggregation by shifting the equilibrium from oligomeric to monomeric form. This work is performed in collaboration with Professor Tate at Hiroshima University.

3. Improvement in Protein Data Bank

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

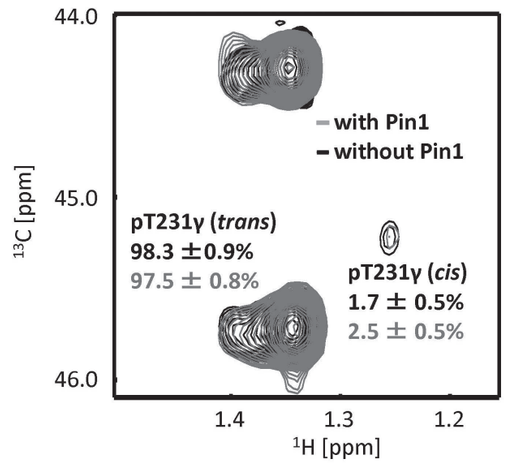


Fig.2 Superposition of the ^{1}H, ^{13}C HSQC spectra of the tau peptide without Pin1 (black) and with Pin1 (gray). The ••••and•• •••• isomers of the pThr231-Pro232 bond were distinguished with chemical shift correlation between γ-proton and γ-carbon ^{13}C of pTh231. The population of the • ••and •••• •isomers can be estimated by the peak intensities. The values (black and gray letters) indicate that the pThr231-Pro232 bond of the peptide is not catalyzed by Pin1.

Publications

1. Watarai Y, Ishizawa M, Ikura T, Zacconi FC, Uno S, Ito N, Mouriño A, Tokiwa H, Makishima M, Yamada S.: Synthesis, Biological Activities, and X-ray Crystal Structural Analysis of 25-Hydroxy-25(or 26)-adamantyl-17-[20(22),23-diynyl]-21-norvitamin D Compounds. J Med Chem, 58, 9510-9521, 2015.
2. Anami Y, Sakamaki Y, Itoh T, Inaba Y, Nakabayashi M, Ikura T, Ito N, Yamamoto K.: Fine tuning of agonistic/antagonistic activity for vitamin D receptor by 22-alkyl chain length of ligands: 22S-Hexyl compound unexpectedly restored agonistic activity. Bioorg Med Chem, 23, 7274-7281, 2015.
3. Kulkarni M.R., Islam M.M., Numoto N., Elahi M., Mahib M.R., Ito N., Kuroda Y.: Structural and biophysical analysis of sero-specific immune responses using epitope grafted Dengue ED3 mutants. Biochim. Biophys. Acta, 1854, 1438-1443, 2015.
4. Masaki S, Kii I, Sumida Y, Kato-Sumida T, Ogawa Y, Ito N, Nakamura M, Sonamoto R, Kataoka N, Hosoya T, Hagiwara M.: Design and synthesis of a potent inhibitor of class 1 DYRK kinases as a suppressor of adipogenesis. Bioorg Med Chem, 23, 4434-4441, 2015.
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Frontier Research Unit
Laboratory of Oxygen Biology

Associate Professor Koh Nakayama, Ph.D.

Aim of the Research

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

Research Projects

1. Signal transduction of hypoxic response

Hypoxia-Inducible Factor (HIF)-α is a transcription factor which plays a central role during hypoxic response. HIF-α is actively degraded during normoxic condition, whereas it is stabilized and activated under hypoxic conditions. PHD is a HIF-prolyl hydroxylase which hydroxylates and regulates the expression of HIF-α. There are three PHDs in mammals; namely PHD1, 2, and 3. These proteins hydroxylate HIF-α to negatively regulate its expression. Moreover, it is suggested that they have substrates besides HIF-α. We have been focusing on PHD3, and studying hypoxic cell signaling pathways.

2. Metabolic regulation under hypoxic condition

Cancer cells are known to exhibit glycolytic metabolism. We identified pyruvate dehydrogenase (PDH) as a component included in the hypoxia complex (Figure). 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. We now challenge to alter the cancer metabolism by changing the PHD3 expression and increasing the PDH activity, which would break up the glycolytic metabolism and suppress the tumor progression.

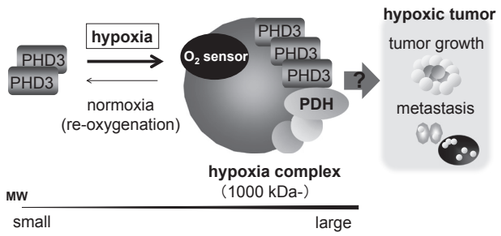


Figure Characterization of Hypoxia Complex

3. Crosstalk of CREB and ER stress response pathways during hypoxia.

We have previously demonstrated that CREB and NF-κB are activated during chronic phase of hypoxia. We focused on CREB to further elucidate the signaling machinery during chronic hypoxia. As a result, we identified that ER stress response pathway is activated during chronic hypoxia and cross talks with CREB. Knockdown of CREB in breast cancer cells reduced the expression of two ER stress responsive molecules, PERK and IRE1α, which led to decreased ER stress responses in these cells. Consequently, CREB-depleted cells exhibited less tumor metastasis in a tumor mouse model. This study highlights the possibility of CREB to be a therapeutic target for cancer, which we currently focus on.

Publications

1. Kikuchi D., Tanimoto K., and Nakayama K.* CREB is activated by ER stress and modulates the unfolded protein response by regulating the expression of IRE1α and PERK. ***Biochem. Biophys. Res. Commun.*** 469, 243-250, **(2016)**.

2. Katsuta E, Tanaka S, Mogushi K, Shimada S, Akiyama Y, Aihara A, Matsumura S, Mitsunori Y, Ban D, Ochiai T, Kudo A, Fukamachi H, Tanaka H, Nakayama K., Arii S, Tanabe M. CD73 as a therapeutic target for pancreatic neuroendocrine tumor stem cells. ***Int. J. Oncol.*** 48, 657-669, **(2016)**.

Tenure Track Research Unit
Department of Cellular and Molecular Medicine

Associate professor Yumiko Oishi MD, PhD
Assistant professor Daiki Taneichi PhD, Shinichiro Hayashi, PhD
Project Assistant professor Sumio Hayakawa, PhD
Research Technician Yukiko Hoshino, Yumi Suzuki

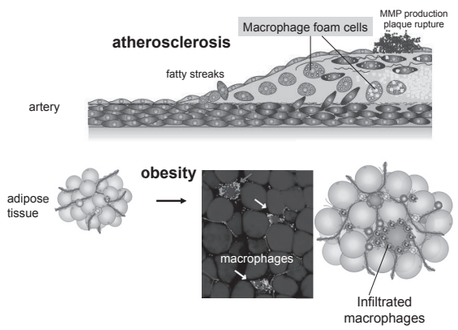
Research outline

Cardiovascular disease, as a consequent of the obesity related metabolic syndrome, remains a significant cause of morbidity and mortality in industrialized societies. A major effort of our laboratory has been to investigate the molecular mechanism of metabolic syndrome from the viewpoint of transcriptional regulation. We focus on macrophage and skeletal muscle in this context. The long term goals of our current study are to elucidate: 1) the mechanism of the link between cellular metabolism and immune response of macrophage 2) the mechanism of chronic inflammation that leads to metabolic syndrome, and 3) the mechanism responsible for pathogenesis of sarcopenia and skeletal muscle degeneration.

Research Project

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

Chronic low-grade inflammation has been recognized as a key contributing factor in the onset and progression of metabolic syndrome and atherosclerosis. As a multifunctional effector cell, macrophage play pivotal roles in both the enhancement and resolution of this inflammatory process (Figure). By utilizing molecular biology technique, lipidomics and bioinformatics, we found that the lipid homeostasis is coordinately regulated with inflammatory response in macrophage. TLR4 activation rapidly, and transiently inhibits Liver X receptor (LXR) signaling, and subsequently activates Sterol regulatory element-binding protein (SREBP). In the late phase of inflammation, LXR and SREBP work together to increase anti-inflammatory fatty acid synthesis, necessary for a resolution of inflammation. Thus, transcriptional/signaling network involving LXR and SREBP play a pivotal role in the regulation of lipid homeostasis and cellular function. By elucidating the crosstalk between cellar function and metabolism, we would be able to accumulate beneficial knowledge to develop novel therapeutic strategy targeting macrophages for the prevention and treatment of metabolic syndrome.



Macrophage is important for a chronic inflammation.

2. Mechanism of skeletal muscle degeneration

Skeletal muscle consume ~40% of total energy, playing a key role for the pathogenesis of metabolic syndrome. Sarcopenia is the degenerative loss of skeletal muscle mass, quality and strength associated with aging. Although the causes and mechanisms of sarcopenia still remains unclear, one of the hypotheses is reduction of the number of satellite cells, stem cells in adult muscle, and failure of satellite cell activation. We identified KLF5 as a novel factor that play a pivotal role in skeletal muscle degeneration. KlF5 is a Zinc-finger transcription factor involved in the self-renewal and proliferation of embryonic stem cell and cancer stem cell. KLF5 is transiently induced in the myoblast during differentiation and it plays critical role for muscle degeneration and repair. Now we are testing the hypothesis whether the dysregulation of Klf5 causes a mulfunction of satellite cells.

Publications

1. Oishi Y and Manabe I. Immunometabolic control of homeostasis and inflammation. ***Inflamation and Regeneration*** 35(4)185-192, 2015

2. Chal J, Hayashi S, et al. Differentiation of pluripotent stem cells to muscle fiber to model Duchenne muscular dystrophy. ***Nat Biotechnol.*** 33(9):962-9, 2015

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】

- Establishment of *in vivo* imaging technique to observe macroautophagy in living brains.
- Direct proof of insufficient macroautophagy in Alzheimer's disease model mice.

【Pathological Cell Biology】

- Identification of a novel compound that inhibits both mitochondria-mediated necrosis and apoptosis.
- Discovery of a novel role of Atg5-dependent autophagy: retardation of cell motility by controlling Rho GTPase.

【Developmental and Regenerative Biology】

- Discovery of flat medaka mutant solves a 100-year-old mystery of 3D body morphogenesis against gravity
- A Modified Murine Embryonic Stem Cell Test for Evaluating the Teratogenic Effects of Drugs on Early Embryogenesis

【Stem Cell Biology】

- Identification of stem cells in the skin
- Mechanisms of skin and hair aging

【Immunology】

- Development of novel lupus-prone mouse model in which autoimmune response can be addressed at the cellular level.
- Elucidation of the role of glycan cis-ligand in CD22/Siglec-2-mediated regulation of B lymphocyte signaling.

【Molecular Pathogenesis】

- Discovery of an α-myosin heavy chain gene mutation as a novel mechanism for sick sinus syndrome via affecting myofibrinogenesis.
- Identification of APOBEC3H haplotypes as a novel regulator of susceptibility to HIV-1 infection and development of AIDS in Japanese and Asian Indian populations.

Department of Neuropathology

Professor	Hitoshi Okazawa
Practical professor	Kazuhiko Tagawa
Project Lecturer/Part-time Lecturer	Nobuyuki Nukina, Haruhisa Inoue, Toshiki Uchihara, Masaki Sone
Assistant professor	Takuya Tamura
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Technical assistant	Tayoko Tajima
Office work assistant	Shigemi Sato, Hiroyo Fujii
Secretary	Rumi Innami, Ayako Seki
Graduate Student	Juliana Bosso Taniguchi, Mao Ying, Eriko Hoshino, Hikari Tanaka
Research Student	Zhang Xuemei

Research contents

Following studies have been intensively carried out in our laboratory.

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

Below is the brief report of this year's progress.

Starvation induced autophagy enhances accumulation of amyloid-beta (Aβ) in brain

We developed a new technique to observe macroautophagy in the brain in vivo, and examined whether fasting induced macroautophagy in neurons and how the induction was different between Alzheimer's disease (AD) model and control mice. Lentivirus for EGFP-LC3 injected into the brain successfully visualized autophagosome in living neurons by two-photon microscopy. The time-lapse imaging revealed that fasting increased the number, size and signal intensity of autophagosome in neurons. In AD model mice, these parameters of autophagosome were higher at the basal levels before starvation, and increased more rapidly by fasting than in control mice. However, metabolism of exogenous labeled Aβ evaluated by the new technique suggested that the activated macroautophagy was insufficient to degrade the intracellular Aβ increased by enhanced uptake from extracellular space after fasting. Ordinary immunohistochemistry also revealed that fasting increased intracellular accumulation of endogenous Aβ, triggered cell dysfunction but did not mostly decrease extracellular Aβ accumulation. Moreover, we unexpectedly discovered a circadian rhythm of basal level of macroautophagy. These results revealed new aspects of neuronal autophagy in normal/AD states and indicated usefulness of our method for evaluating autophagy functions *in vivo*.

Neurodegenerative diseases, including AD, have a unique pathological feature which is abnormal protein accumulation inside or outside of cells. Two distinct abnormal proteins accumulate in AD brain. The senile plaques consist of abnormal protein called beta-amyloid (Aβ) outside cells, and the neurofibrillary tangles that tau protein aggregates inside the cell. On the other hand, two distinct protein degradation systems are well-studied as cellular mechanism to remove abnormal proteins, ubiquitin-proteasome and autophagy systems. Autophagy system is further classified to "basal autophagy" which works at a constant level and "induced autophagy (macroautophagy)" which is activated in calorie restriction.

Macroautophagy has been known to play a significant role in tissues other than the brain. While a report suggested that macroautophagy in brain tissue is not observed (Mizushima et al, Mol Biol Cell 2004; while there are, etc.), except for the aggregation of the abnormal protein-induced autophagy by calorie restriction, etc. in neurodegenerative disease, a result of improving the symptoms has been reported by many (Ravikumar et al, Nat Genet 2004 ; etc.) that the presence or absence of induced autophagy in nerve cells was not settled. Furthermore, our proof of the induced autophagy in higher animals was important, since the signaling pathway from the insulin receptor via mTOR (mammalian target of rapamycin) is considered important, and diabetes and high calorie are said risk factors of Alzheimer's disease pathology. Therefore, in this study it was the first object of the present invention is to clarify the "presence or absence of induced autophagy in nerve cells".

Highlight

"Excessive dietary restriction can accelerate the Alzheimer's disease"

In the Alzheimer's disease, the activation of macroautophagy is insufficient to degrade amyloid-beta inside neurons and it was implied that it could lead to the cell death along with cell expansion due to the accumulation of amyloid-beta in neurons. Therefore, this research suggested that activating autophagy excessively by limiting calories after the increase of amyloid-beta outside neurons could worsen the Alzheimer's disease symptoms.

Publications

1. Chen, X., Kondo, k., Motoki, k., Homma, H., Okazawa, H. (2015) Fasting activates macroautophagy in neurons of Alzheimer's disease mouse model but is insufficient to degrade amyloid-beta. *Scientific Reports*. 5, Article number: 12115. doi:10.1038/srep12115

2. Kamagata, K., Kerever, A., Yokosawa, S., Otake, Y., Ochi, H., Hori, M., Kamiya, K., Tsuruta, K., Tagawa, K., Okazawa, H., Aoki, S., Arikawa-Hirasawa, E. (2016) Quantitative Histological Validation of Diffusion Tensor MRI by Two-Photon Microscopy of Cleared Mouse Brain. *Magnetic Resonance in Medical Sciences*.

3. Shiwaku, H., Okazawa, H. (2015) Impaired DNA Damage Repair as a Common Feature of Neurodegenerative Diseases and Psychiatric Disorders. *Current Molecular Medicine*. 2015; Vol.15 (2) pp119-128. doi: 10.2174/1566524015666150303002556

Department of Pathological Cell Biology

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

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

〈**Research Projects**〉

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

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

In this year, we discovered that alternative macroautophagy is phylogenetically conserved from yeast to mammal. We also identified four novel molecules that is crucial for the induction of alternative macroautophagy.

Furthermore, we found that Atg5-dependent autophagy controls cell motility.

2, Molecular mechanisms of programmed cell death

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

In this year, we discovered the small compound that inhibits apoptosis and necrosis. In various pathological events, particularly in oxygen radical-mediated cell injury, both apoptosis and necrosis play essential roles. Apoptosis and some types of necrosis are induced via increases in mitochondrial membrane permeability, called mitochondrial outer membrane permeabilization (MOMP) and permeability transition pore (PTP) opening, respectively. To search for small compounds that inhibit both MOMP-mediated apoptosis and PTP-mediated necrosis, we performed a mitochondria-based high-throughput screening of a chemical library. We identified TMD#7538, a small compound that inhibits both MOMP and PTP opening. Consistent with the fact that this compound inhibited both apoptosis and necrosis, it efficiently suppressed H_2O_2-induced cell death in mouse embryonic fibroblasts and rat neonatal cardiomyocytes.

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. In this year, we discovered the small compound that regulates mitochondrial function, including membrane potential and oxygen consumption rate.

Figure 1

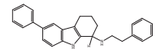

Figure 2

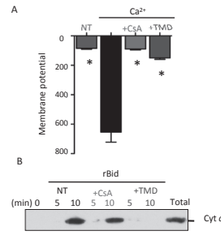

Figure 3

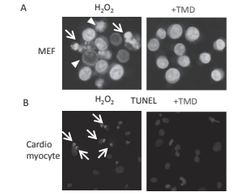

Figure. Identification of a novel compound that inhibits both mitochondria-mediated necrosis and apoptosis.
Figure 1: Structure of TMD#7538. **Figure 2**: (A) Suppression of Ca^{2+}-induced PTP opening by TMD#7538 in mitochondria. Isolated mitochondria were incubated with Ca^{2+} in the presence or absence of CsA or TMD#7538, and Rh123 intensity (membrane potential) at 15 min was measured. (B) Suppression of rBid-induced cytochrome *c* release by TMD#7538. Isolated WT mitochondria were incubated with rBid in the presence or absence of CsA or TMD#7538 for the indicated times, and cytochrome *c* release was examined. **Figure 3**: (A) MEFs were treated with H_2O_2 in the presence or absence of TMD#7538. After 12 hr, cells were stained with PI and Hoechst333542. Necrotic and apoptotic cells had round pink nuclei (arrowheads) and fragmented nuclei (arrows), respectively. (B) Isolated cardiomyocytes were treated with H_2O_2 in the presence or absence of TMD#7538. After 24 hr, cells were examined by the TUNEL assay. Cells with pink puncta (arrows) indicate TUNEL-positive (apoptotic) cells.

List of Publications

[Original paper]

1. A mild and facile synthesis of aryl and alkenyl sulfides via copper-catalyzed deborylthiolation of organoborons with thiosulfonates. S. Yoshida, Y. Sugimura, Y. Hazama, Y. Nishiyama, T. Yano, S. Shimizu, T. Hosoya. Chemical Commun. 51:16613-16616, 2015
2. Identification of a novel compound that inhibits both mitochondria-mediated necrosis and apoptosis. S. Arakawa, I. Nakanomyo, Y. Kudo-Sakamoto, H. Akazawa, I. Komuro, S. Shimizu. Biochem. Biophys. Res. Commun. 467, 1006-1011, 2015
3. Direct Thioamination of Arynes via Reaction with Sulfilimines and Migratory N-Arylation. S. Yoshida, T. Yano, Y. Misawa, Y. Sugimura, K. Igawa, S. Shimizu, K. Tomooka, T. Hosoya. J. Am. Chem. Soc. 137:14071–14074, 2015

[Review paper]

1. S. Shimizu. "Innovative Medicine : Basic Research and Development" Autophagic Cell Death and Cancer Chemotherapeutics. Springer Press, 219-226, 2015

Department of Developmental and Regenerative Biology

Professor Hiroshi Nishina, Ph.D.
Associate Professor Jun Hirayama, Ph. D.
Assistant Professor Norio Miyamura, Ph.D.

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

1. SAPK/JNK Signaling Pathway

Stress-activated protein kinase (SAPK)/c-Jun NH_2-terminal kinase (JNK) is activated by many types of cellular stresses and extracellular signals. We showed that two JNK activators, MKK4/SEK1 and MKK7, are required for full JNK activation and fetal liver formation. MKK4-deficient embryos die between E10.5 and E12.5 with impaired liver formation and massive apoptosis. MKK7-deficient embryos die between E11.5 and E12.5 with similar defects in liver formation. These results indicate that MKK4 and MKK7 cannot substitute for one another *in vivo* and that both are important for hepatoblast proliferation and survival during mouse embryogenesis. Furthermore, we found that the Tyr and Thr residues of JNK are sequentially phosphorylated by MKK4 and MKK7, respectively. We propose the hypothesis that the strong or weak activation of SAPK/JNK is induced by MKK4 and MKK7 to regulate the cell fate.

2. Hippo Signaling Pathway

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

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

3. Circadian Clock

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

Highlight

Vertebrates have a unique 3D body shape in which correct tissue/organ shape and alignment are essential for function. For example, vision requires the lens to be centred in the eye cup which must in turn be correctly aligned in the head. Tissue tension is essential for tissue morphogenesis via three processes: force generation, force transmission through the tissue, and response of tissues and extracellular matrix (ECM) to force. Little is known about how these mechano-morphogenetic processes are orchestrated to form a 3D body. We reported a mechanism of animal morphogenesis essential for adopting a 3D body shape. Via exhaustive mutant screening in medaka, we identified a unique medaka mutant, *hirame* (*hir*), with a markedly flattened body caused by mutation of YAP, a nuclear executor of Hippo-signaling that regulates cell proliferation. We showed that the flattened body phenotype arises from global reduction in actomyosin-mediated tissue tension, rendering *hir* mutant embryos sensitive to deformation by gravity. Reduced tissue tension in *hir* mutants led to tissue flattening and misalignment, both of which contribute to body flattening. By analyzing YAP function in 3D-spheroids of human cells, we identified the RhoGAP ARHGAP18 as a mediator of YAP function essential for tissue tension. Together, these findings revealed a previously unrecognized role of YAP for proper tissue shape and alignment to ensure correct 3D organ/body shape. Understanding of this function of YAP could facilitate use of embryonic or induced pluripotent stem cells to generate complex organs requiring correct alignment of multiple tissues.

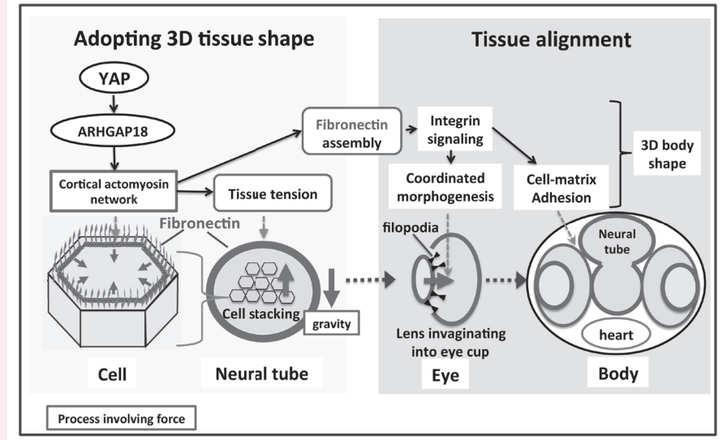

Fig.1. A proposed model for the function of Yap in vertebrate 3D body shape and alignment.

Publications

1. Ruoxing Yu, Norio Miyamura, Yoshimi Okamoto-Uchida, Norie Arima, Mari Ishigami-Yuasa, Hiroyuki Kagechika and Hiroshi Nishina (2015) A Modified Murine Embryonic Stem Cell Test for Evaluating the Teratogenic Effects of Drugs on Early Embryogenesis. ***PLoS ONE*** 10 e0145286

2. Sean Porazinski[1], Huijia Wang[1], Yoichi Asaoka[1], Martin Behrndt[1], Tatsuo Miyamoto[1], Hitoshi Morita, Shoji Hata, Takashi Sasaki, S.F. Gabby Krens, Yumi Osada, Satoshi Asaka, Akihiro Momoi, Sarah Linton, Joel B. Miesfeld, Brian A. Link, Takeshi Senga, Atahualpa Castillo-Morales, Araxi O. Urrutia, Nobuyoshi Shimizu, Hideaki Nagase, Shinya Matsuura, Stefan Bagby, Hisato Kondoh, Hiroshi Nishina*, Carl-Philipp Heisenberg* and Makoto Furutani-Seiki* (2015) YAP is essential for tissue tension to ensure vertebrate 3D body shape. ***Nature*** 521, 217-221 ([1]Contributed equally; *Corresponding authors)

3. Koichi Fujisawa, Shuji Terai, Toshihiko Matsumoto, Taro Takami, Naoki Yamamoto, Hiroshi Nishina, Makoto Furutani-Seiki and Isao Sakaida (2015) Evidence for a Role of the Transcriptional Regulator Maid in Tumorigenesis and Aging. ***PLoS ONE*** 10 e0129950

4. Shoudai Kawano, Junichi Maruyama, Shunta Nagashima, Kazutoshi Inami, Wenzhe Qiu, Hiroaki Iwasa, Kentaro Nakagawa, Mari Ishigami-Yuasa, Hiroyuki Kagechika, Hiroshi Nishina, Yutaka Hata (2015) A cell-based screening for TAZ activators identifies ethacridine, a widely used antiseptic and abortifacient, as a compound that promotes dephosphorylation of TAZ and inhibits adipogenesis in C3H10T1/2 cells. ***J. Biochem.*** 158, 413-423.

5. Yuta Motimaru, Morio Azuma, Natsuki Oshima, Yuta Ichijo, Kazuhiro Satou, Kouhei Matsuda, Yoichi Asaoka, Hiroshi Nishina, Takashi Nakakura, Chihiro Mogi, Koichi Sato, Fumikazu Okajima and Hideaki Tomura (2015) Extracellular acidification activates ovarian cancer G-protein-coupled receptor 1 and GPR4 homologs of zebrafish. ***Biochem. Biophys. Res. Commun.*** 457, 493-499.

Department of Stem Cell Biology

Professor	Emi K. Nishimura, M.D., Ph. D.
Associate Professor	Daisuke Nanba, Ph D.
Assistant Professor	Hiroyuki Matsumura, Ph. D.
	Yasuaki Mohri, Ph. D.
JSPS Postdoctoral Fellow (SPD)	Hironobu Morinaga, Ph. D.
Project Assistant Professor	Makoto Fukuda.,Ph. D.

Stem cell systems play fundamental roles in tissue turnover and homeostasis. Our goal is to reveal the mechanisms of tissue homeostasis driven by stem cell systems and to apply the knowledge to better understand the mechanisms underlying tissue decline, cancer development and other diseases associated with aging. We further aim to apply this knowledge to develop new therapeaufic strategies for age-associated diseases.

1) Identification of stem cells in the skin

The skin is the largest organ in the body. Hair follicles in the skin constantly renew themselves by alternate phases of growth, regression and rest. During this process, mature melanocytes (pigment cells) in hair follicles are replaced by a new cell population every hair cycle. We previously identified the source of those melanocytes, "melanocyte stem cells" (McSC), which are located in the hair follicle bulge and supply mature melanocytes required for hair and skin pigmentation (Nishimura EK et al. Nature 2002). We currently identified McSCs in eccrine sweat glands in non-hair-bearing skin areas as well. Also we are currently searching for the prospective method for identification of epidermal keratinocyte stem cells in mouse and human skin.

2) Mechanisms of stem cell maintenance

The underlying mechanisms of stem cell maintenance is a fundamental issue in stem cell biology and medicine. We previously demonstrated that the niche microenvironment plays dominant role in melanocyte stem cell fate determination (Nishimura EK et al. 2002). We then revealed that hair follicle stem cells (HFSC), which surround McSCs in the hair follicle bulge-subbulge area, serve as a functional niche for McSC maintenance through transforming growth factor β (TGF-β) (Nishimura EK et al. Cell Stem Cell, 2010) (Tanimura S et al. Cell Stem Cell 2011). As intrinsic defects in stem cells such as caused by *Mitf* or *Bcl2* deficiency also induces McSC depletion which leads to the progressive expression of hair graying phenotype, incomplete maintenance of McSCs either by defective signaling from the stem cell niche or by intrinsic defects in stem cells induces the progressive hair graying phenotype.

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

Physiological hair graying is the most obvious outward sign of aging in mammals, yet it has been unclear what causes the incomplete maintenance of MsSCs during the course of aging (Nishimura EK et al. Science 2005). We have found that genotoxic stress abrogates renewal of McSCs by triggering their differentiation without inducing stem cell apoptosis nor cellular senescence. Our findings indicated that a "stem cell renewal checkpoint" exists to maintain the quality of the MCSC pool (Inomata K, Aoto T et al. Cell 2009). Interestingly, a similar mechanism actually underlies epithelial tissue aging as well (See Highlight).

4) Development of skin regeneration technology with human skin stem cells

Human epidermal keratinocyte stem cells can be cultivated under suitable conditions, and generate a progeny large enough to entirely reconstitute the epidermis of an adult human. This has enabled the autologous transplantation of cultured epidermal sheets onto patients with extensive burns. However, the cultured keratinocytes can regenerate only the epidermis and cannot suppress dermal scarring. To develop novel skin regeneration technology, we have investigated human epidermal keratinocytes and dermal fibroblasts, and obtained the following results. 1) Human epidermal keratinocyte stem cells can be identified *in situ* by analyzing cell motion during their cultivation (Nanba et al., J. Cell Biol., 2015, Tate et al., J. Dermatol. Sci. 2015). The identification of keratinocyte stem cells by image analysis is a valid parameter for quality control of cultured keratinocytes for transplantation, and improves the clinical outcome of cell therapy and the efficiency of cell manufacturing for regenerative medicine. 2) Human dermal fibroblasts can be categorized at least two functional clonal types by comprehensive phenotypic and gene expression profiling (Hiraoka et al., J. Dermatol. Sci. *in press*). One is highly proliferative, while the other is less proliferative but has the ability to remodel the tissue architecture. The proliferative clones are predominant in infants, but decrease with physiological aging. These data have implications regarding the functional heterogeneity of dermal fibroblasts and skin repair and aging.

Highlight

Hair follicle aging is driven by transepidermal elimination of stem cells via COL17A1 proteolysis

Hair thinning is a prominent aging phenotype but has an unknown mechanism. We show that hair follicle stem cell (HFSC) aging causes the stepwise miniaturization of hair follicles and eventual hair loss in wild-type mice and in humans. *In vivo* fate analysis of HFSCs revealed that the DNA damage response in HFSCs causes proteolysis of Type XVII Collagen (COL17A1/BP180), a critical molecule for HFSC maintenance, to trigger HFSC aging, characterized by the loss of stemness signatures and by epidermal commitment. Aged HFSCs are cyclically eliminated from the skin through terminal epidermal differentiation, 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).

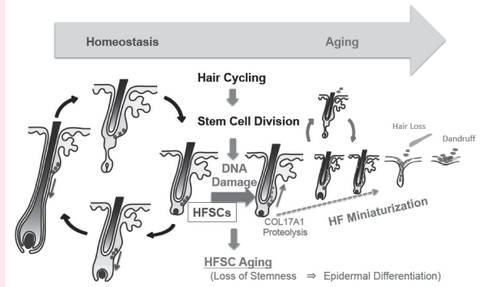

Figure 1 Mechanisms of hair follicle aging

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Invited lecture/presentation at international meetings

1. Emi K. Nishimura:Stem cells in skin appendages and their fate change by aging:23rd Wolrd Congress of Dermatology:(Vancouver, Canada)June 8-13, 2015.
2. Emi K. Nishimura:Hair follicle aging program in hair follicle stem cells orchestrates dynamic tissue aging and associated hair loss:Gordon Research Conferences-Epithelial Differentiation & keratinization:(Boston, USA)July 12-17, 2015.

Department of Immunology

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

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

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

1. Study on the cis-ligand-mediated regulation of B lymphocyte activation by synthetic sialoside.

Immune cells express various lectin molecules such as members of Siglec family and C-type lectins on the surface. Some of these lectins bind to the glycan-ligand expressed on the same cell (cis-ligand), and cis-ligands regulate cell signaling when the lectins carry signaling function. However, little is known about cis-ligand mediated signal regulation.

CD22 (also known as Siglec-2) is a member of Siglec family mostly expressed in B cells. CD22 specifically recognizes α2,6 sialic acid, and constitutively bind to the cis-ligand. We have been developing synthetic sialoside that binds to CD22 with high affinity in collaboration with Prof. Ishida at Gifu University, and generated the sialoside that binds to CD22 with 1000 fold higher affinity than the natural ligand. By inhibiting the interaction between CD22 and cis-ligand by this compound, we demonstrated that the cis-ligand negatively regulates CD22 (Fig. 1). We are currently analyzed how cis-ligand regualts CD22. Moreover, we are developing drugs based on this compound as this compound is able to regulate B cell activation

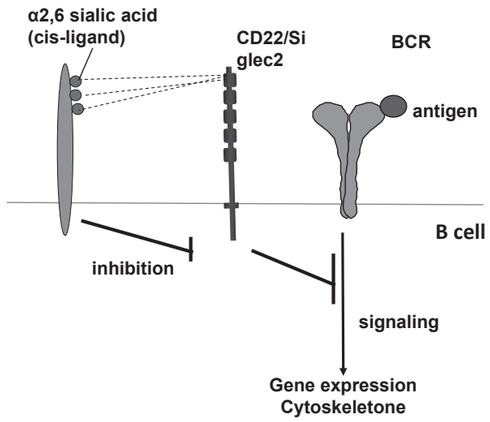

Figure 1. regulation of CD22/Siglec-2 by cis-ligand

2. Study on regulatory mechanisms for pathogenic autoantibody production and their defects in lupus.

Systemic lupus erythematosus (SLE) is a prototype of systemic autoimmune diseases, and characterized by production of autoantibodies to various nuclear components such as DNA. Among these autoantibodies, antibodies reactive to RNA-related antigens such as the Sm antigen play a pathogenic role. However, little is known how anti-Sm B cells are tolerized in normal individuals, and how these cells circumvent self-tolerance and produce the autoantibody. We have been studying the regulation mechanisms for anti-Sm B cells and its defect (see Highlight)

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

ROS play a various roles including in induction of cell activation and cell death. We have demonstrated that ligation of B cell antigen receptor (BCR) induces prolonged ROS generation, which plays a crucial role in B cell activation. NOX2, a member of NADPH oxidase (NOX) family is involved in BCR ligation-induced ROS production, but there is also involvement of other molecules/mechanisms in this process. We are currently elucidating molecular mechanisms for NOX2-independent ROS production in B cells.

Highlight

Analysis of the mechanisms for autoantibody production by visualization of self-reactive B cells.

Identification of antigen-specific lymphocytes after immunization advanced the understanding of immune responses. This approach has been taken for the analysis of self-reactive B cells such as anti-DNA antibody-producing B cells, and revealed that self-reactive B cells are deleted, edited or anergized at the immature B cell stage in the bone marrow. However, little is known about how self-reactive B cells produce autoantibodies by escaping from tolerance mechanisms. To answer this question, detection of pathogenic self-reactive B cells in autoimmune models is required. Systemic lupus erythenmatosus (SLE) Systemic lupus erythematosus (SLE) is a prototype of systemic autoimmune diseases, and characterized by production of autoantibodies to various nuclear components such as DNA. Among these autoantibodies, antibodies reactive to RNA-related antigens such as the Sm antigen play a pathogenic role. We have developed a system to identify the anti-Sm B cells in mice using mice transgenic for anti-DNA H chain 56R in collaboration with Dr. Weigert at the University of Chicago, and addressed the response of these pathogenic B cells.

In patients with SLE and its mouse model, CD40L (CD154), a costimulatory molecule for B cells, is excessively produced, and CD72 polymorphism is associated with the disease. We demonstrated that anti-Sm B cells mature and migrate to the peripheral lymphoid organs such as spleen, and then removed. Interestingly, among the two major mature B cell compartments follicular (FO) and marginal zone (MZ) B cells, excess CD40L inhibits deletion of anti-Sm marginal zone but not follicular B cells, whereas CD72-deficiency inhibits deletion of anti-SM follicular but not marginal zone B cells (Fig. 2). These findings clearly demonstrate that the regulation of anti-Sm B cells is distinct from previously demonstrated regulatory mechanisms for other self-reactive B cells, and suggest that pathogenic self-reactive B cells are generated by the defect in peripheral self-tolerance of B cells, which has been poorly understood.

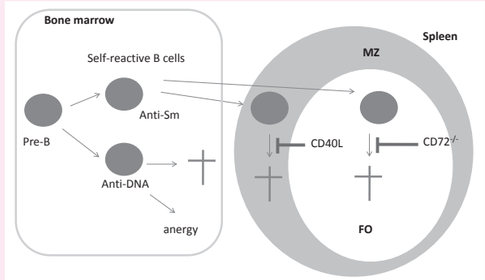

Figure 2. Self-tolerance of anti-Sm B cells

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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	Daisuke Sakurai, Ph.D.
Assistant Professor	Jianbo An, PhD
Research Associate	Taeko Naruse, Ph.D.

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

1. Molecular mechanisms for hereditary cardiomyopathy

We have developed a screening system of mutations in the known 67 disease genes for hereditary cardiomyopathy. Among the sporadic children cases of hypertrophic cardiomyopathy, a considerable portion could be explained by *de novo* mutations in the sarcomere genes. In addition, we analyzed subjects with sportive heart for sarcomere mutations and found about 5% carried mutations, of which 1% was considered to have a pathogenic mutation.

2. Molecular mechanisms for atherosclerosis

We generated transgenic mouse lines expressing coronary atherosclerosis-associated *MKL1* under the CD68 promoter and found that these mice exhibited abnormality in function of macrophages.

3. Molecular mechanisms for arrhythmia

We identified an alpha-myosin heave chain (*MYH6*) mutation (delE933) in a case with sick sinus mutation. The *MYH6* mutation increased binding of alpha-myosin heavy chain and myosin binding protein C. Over-expression of the *MYH6* mutation impaired the sarcomere integrity in rat cardiomyocytes and decreased electrical velocity in HL-1 cells. In addition, involvement of *MYH6* mutation in bradycardia was proven in a zebrafish model. On the other hand, we identified *IRX3* mutations in patients with cardiac arrhythmia and revealed functional alterations caused by the mutations.

4. Analysis of *MHC* genes in human, rhesus monkey and penguins

We have analyzed *MHC* class I diversities in macaque model for SIV vaccination in detail. In addition, we analyzed divergence and diversity of *MHC* class I genes in penguins, which revealed molecular evolution of *MHC* in penguins.

5. Genome diversity in association with HIV/AIDS

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

Highlight

We have searched for pathogenic mutations causing familial sick sinus syndrome (SSS) and identified a *MYH6* mutation, delE933, in a large family with SSS. The mutation was suggested to disrupt 3D structure of *MYH6* leading to decreased binding to myosin binding protein C (MYBPC). We revealed *in vitro* the decreased interaction of MYH6 protein with MYBPC3 protein by the mutation. In addition, introduction of the mutation caused disorganization of sarcomere in rat cardiomyocytes and the same change was also caused by SSS-associated polymorphism R731W. Furthermore, we demonstrated that the delE933 mutation delayed conductance at the cellular level and formation of heart in zebrafish model. These data decipher the molecular mechanisms for pathological role of *MYH6* mutation in SSS.

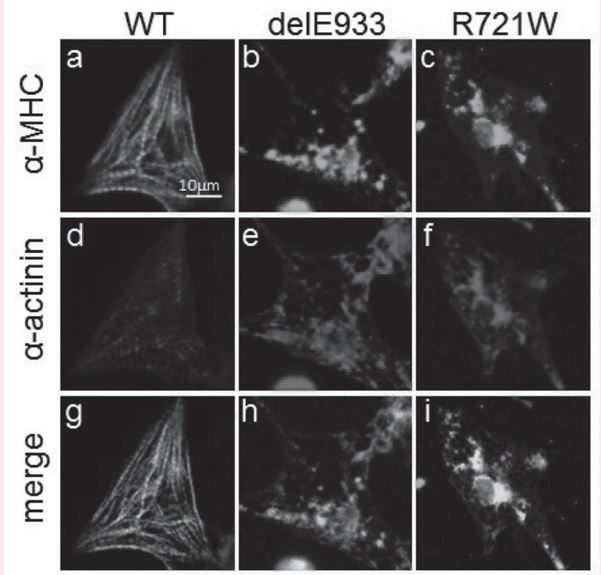

Figure Disorganization of sarcomere caused by *MYH6* mutations
We introduced a *MYH6* mutation (delE933) found in familial SSS and a *MYH6* rare variant (R721W) reported to be associated with sporadic SSS into rat primary cardiomyocytes. It was revealed both mutations induced sarcomere disorganization.

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Division of Medical Genomics

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

【Molecular Cytogenetics】

The principal aim of our reserach is to understand the molecular basis underlying cancer and genetic diseases including chromosome aberration syndromes. We have contributed as follows;

1. Identification of novel genes including microRNAs responsible for cancer and unknown genetic diseases.
2. Understanding the pathogenesis of intractable cancers and genetic disorders based on the integrative omics approach including systems biology.
3. Establishment of practical tools for diagnosis in personalized medicine of cancer and genomic disorders in the clinical setting.

【Biochemical Genetics】

Our lab is focusing on basic transcriptional mechanism and its biological function and pathogenesis of human disease.

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

【Molecular Genetics】

BRCAs, products of hereditary breast cancer genes, are associated with genome stability. We analyze functions of BRCAs and other related proteins to reveal the mechanism of breast carcinogenesis.

1. We aimed to establish novel synthetic lethal interactions between BRCA2 and chemical compounds provided by Chemical Biology Screening Center at TMDU.
2. We found that the enhancement of the ATPase activity of non-muscle myosin (NM)-IIC by BRCA2 was required for completion of the cytokinesis.
3. We analyzed the intramolecular BRCA2 region concerning the numerical integrity of centrosomes by an automated centrosome counting system

【Molecular Epidemiology】

1. We identified that a non-synonymous polymorphism in the DNA repair gene CHD4, pD140E associates with cancer risk.
2. We are studying the effect of predictive genetic test on the conception of health and disease of the applicants, when the medical doctor is involved in returning of the results.

【Genomic Pathology】

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

【Epigenetics】

1. We reported the existence of sushi-ichi-related retrotransposon homologue family of genes (*SIRH* family genes) and demonstrated that *Peg10*, *Peg11/Rtl1, Sirh7* and *Sirh11*, play essential eutherian-specific functions, such as placental and brain cognitive functions.
2. We have recently reported that most of *SIRH* and *PNMA* family genes are eutherian-specific, thus, suggesting that these newly acquired genes deeply contributed to diversification and establishment of eutherian mammalians, including humans.
3. Assisted reproductive technologies, such as *in vitro* fertilization and intracytoplasmic sperm injection, become very popular, however, their effects on human development remain unclear. We have extensively examined pre-and postnatal epigenetic effects caused by such technologies.

【Medical Science Mathematics】

1. As one of striking progresses of big medical-omic data analysis, we exhaustively analyzed whole genomes of 300 hepatocellular carcinoma, and identified six sub-classifications, which revealed significantly different outcomes.
2. The genetic cause of approximately 20% of LQTS patients remains elusive. With LQTS cases, we performed whole-exome sequencing (WES) and protein-protein interaction network analyses. They revealed new pathogen candidates, half of which directly interact with calmodulin.
3. We compared and characterized four latest commercial WES kits, improved coverage of WES combining the haloplex method, and identified novel pathogenic mutations for congenital neurological diseases and hearing loss.

Department of Molecular Cytogenetics

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

The principal aim of the Department of Molecular Cytogenetics is to understand the molecular mechanism underlying cancer and genetic diseases including multiple congenital anomalies and/or intellectual disability (MCA/ID). Our research interests are as follows (1) Identification of genes responsible for cancer and unknown genetic diseases, (2) Development of innovative techniques for 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 patients.

I. Precision cancer medicine based on omics and functional research

1. Molecular basis for the development of novel strategies to inhibit tumor metastasis

Cancer metastasis is a multistep process, including genetic and epigenetic events that result in the activation of metastasis-associated genes together with the activation of oncogenes and/or the inactivation of tumor suppressor genes, but the underlying mechanisms of metastasis are still poorly understood. We established a highly metastatic cell subline from oral squamous cell carcinoma cell line using *in vivo* selection and analyzed its genomic and transcriptional status by system biological approach. As a result, the hypusine cascade which is involved in protein synthesis was identified as a novel cancer therapeutic target (Muramatsu T et al. Oncogene. 2016 in press). The epithelial-mesenchymal transition (EMT) also contributes to cancer progression including metastasis. We established a cell-based reporter system for identifying EMT-inducing microRNAs (miRNAs) with a gastric cancer cell line transfected with a reporter construct containing a promoter sequence of *VIM*. Function-based screening using this reporter system was performed with a 328 miRNA library, and resulted in the identification of *miR-544a* as an EMT-inducing miRNA (Yanaka Y et al. Carcinogenesis. 2015).

2. Molecular basis for autophagy-based personalized cancer medicine

It has been suggested that autophagy might contribute to tumor progression through cell survival of cancer cells in microenvironment and resistance to cancer therapy. However, we have found that autophagy was impaired by genetic or epigenetic aberrations in a subset of cancers, suggesting that autophagy activity may be individually different in patients. Hence, it is important to develop molecular basis for an autophagy-based therapeutic concept. It has been considered that p62/SQSTM1, a substrate of autophagic degradation, may become a molecular marker for the impaired autophagy. In 2015, we found that high expression of p62 is associated with poor prognosis in endometrial cancers (Iwadate R et al. Am J Pathol 2015). This suggests that examining p62 expression may be a useful strategy to monitor autophagy activity in human cancers.

3. MicroRNA therapy against NRF2-activated tumors

Some tumor-suppressing miRNAs target multiple oncogenes concurrently and therefore may be useful as cancer therapeutic agents. Further, such miRNAs may be useful to address chemotherapeutic resistance in cancer, which remains a primary clinical challenge in need of solutions. Thus, cytoprotective processes upregulated in cancer cells that are resistant to chemotherapy are a logical target for investigation. Here, we report that overexpression of miR-634 activates the mitochondrial apoptotic pathway by direct concurrent targeting of genes associated with mitochondrial homeostasis, antiapoptosis, antioxidant ability, and autophagy. In particular, we show how enforced expression of miR-634 enhanced chemotherapy-induced cytotoxicity in a model of esophageal squamous cell carcinoma, where resistance to chemotherapy remains clinically problematic. Our findings illustrate how reversing miR-634-mediated cytoprotective processes may offer a broadly useful approach to improving cancer therapy.

II. Cancer omics research

Though the "tailor-made Medical treatment Program", we have explored tumor susceptible genes and cancer biomarkers of malignancy for esophageal squamous cell cancer (with Tokyo Med. Dent. Univ. and Aichi Cancer Center), breast cancer (with Cancer Institute Hospital and the Univ. of Tokushima), pulmonary cancer (Nagoya Univ. and Shiga Univ. of Medical Science), colorectal cancer (with CIH and Osaka Univ.), prostate cancer (with Kyoto Univ. and Iwate Medical Univ.) and gastric cancer (with National Cancer Center and UT) in order to establish a personalized cancer medicine.

Furthermore, under the "Project for development of innovative research on cancer therapeutics (P-DIRECT)", we have performed an integrative analysis of genomics, epigenomics, and gene expression in esophageal squamous cell cancers.

III. Molecular investigation of congenital disorders

Intellectual disability (ID) is a heterogeneous condition affecting 2-3% of the population, often associated with multiple congenital anomalies (MCA). The genetic cause remains largely unexplained for most cases. Since 2005, we have been investigating the causes of ID/MCA of unknown etiology in 645 subjects through the use of chromosomal microarrays. First, we performed a two-stage screening by two in-house bacterial artificial chromosome (BAC) arrays, which identified pathogenic copy number variants (CNVs) in 133 patients. Next, we performed a third screening by SNP arrays in 450 negative cases from the previous screenings, and smaller causative CNVs were detected in 22 subjects. Overall, our three-stage screening allowed the identification of pathogenic CNVs in 155 subjects, which means that 24% of the cases can be explained by alterations in the copy-number state (Uehara et al. J Hum Genet. 2016). Through this project, we also carried out a parallel research 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 *CASK* aberrations in 35, then clarifying the etiology in almost 70% of the cases.

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Department of Molecular Genetics

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Assistant Professor — Miho Takaoka, Ph.D.
Tokunin Assistant Professor — Ken Miyaguchi, Ph.D.

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

1. Microtubule stabilization by the loss of BRCA2 is attributed to MAP4 assembly

Synthetic lethality is defined as a genetic combination of mutations in two genes that leads to cell death. We have reported the potential synthetic lethality relationships between BRCA2 deficiency and paclitaxel (PTX). To date, compared with control siRNA-treated cells, PTX treatment of BRCA2-siRNA knockdown cells has been found to result in a significant increase in microtubule polymer mass. However, the mechanism underlying the synthetic lethality is unclear. To investigate whether the BRCA2 protein forms the microtubule cytoskeleton complex, we performed immunoprecipitation experiments using anti-BRCA2 antibody in T47-D cell lysate. Analysis of the immunoprecipitate by mass spectrometry identified the microtubule-associated proteins (MAP2, MAP4, and Tau) that directly bind microtubules to promote microtubule stabilization. Furthermore, a cell-based tubulin polymerization assay following the treatment of the siRNA knockdown of BRCA2 revealed microtubule stabilization by the effect of MAP4. In this study, we identified MAP4 as a new binding partner of BRCA2, suggesting that a synergistic effect of PTX and MAP4 on tubulin assembly contributes to microtubule stabilization.

2. Potential role of FKBP51 in cancer cell migration and invasion

FKBP51 has been identified as a target molecule binding to the immunosuppressive drug FK506. In recent studies, the upregulation of FKBP51 has been reported to be associated with drug resistance in various cancers (including breast cancer, prostate cancer, myeloma, and melanoma) following treatment with antineoplastic agents. However, the expression of FKBP51 in various human cancers is not uniform, and the role of FKBP51 in cancer biology has not been clearly defined. Here we show that FKBP51 contributes to cell migration and invasion. The knockdown of FKBP51 by siRNA inhibited the migration and invasion of U2OS cells, whereas the overexpression of FKBP51 promoted the cell migration and invasion. The depletion of FKBP51 caused the inhibition of lamellipodia structure assembly and the breakdown of actin stress fiber, which was revealed by visualizing actin with phalloidin–Alexa 488 (Figure 1). Furthermore, we identified STARD13 from the anti-FKBP51 immunoprecipitate by LC–MS/MS analysis and validated the interaction between FKBP51 and STARD13 of the Rho-GAP protein by immunoblotting of similarly prepared fractions. These results suggest that FKBP51 regulates cell migration and invasion through the Rho-GAP activity of STARD13.

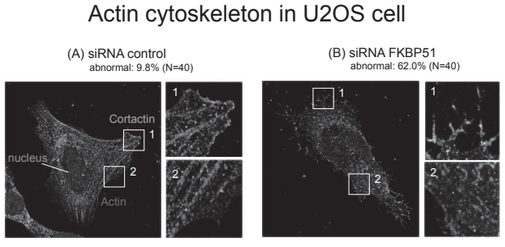

Figure 1. FKBP51 knockdown inhibits actin cytoskeleton assembly and lamellipodia morphology
Control (A) and FKBP51 (B) siRNA-transfected U2OS cells were stained with cortactin antibody for lamellipodia (green) and phalloidin for F-actin (red). Boxes indicate the high-magnification images in which the abnormal formation of lamellipodia and F-actin are increased in FKBP51 siRNA-transfected cells compared to control.

3. Estrogen-mediated BRCA2 localizes in the cytoplasm

Germline mutations in *BRCA2* correlate with an increased risk of breast and ovarian cancer. Although BRCA2 has essential functions in all cell types, the reason for which BRCA2 loss or mutation has a higher risk for cancer development mainly in estrogen-regulated tissues remains unclear. Recent studies have reported that estrogen activates *BRCA2* transcription in an estrogen receptor (ER)-dependent manner and promotes the expression of the BRCA2 protein for 24 h. Here we report the effect of estrogen–ER on the subcellular distribution of BRCA2 in MCF-7 cells, as revealed by confocal immunofluorescence microscopy. The BRCA2 content was quantified by a Z-stack gallery acquired for each cell using MetaMorph image analysis software based on binary image. BRCA2 localization patterns obtained by the treatment of estrogen were preferentially exhibited in the cytoplasm, and there was a significant difference between estrogen-treated and untreated cells. On the other hand, the nuclear translocation of BRCA2 did not differ significantly by the treatment of estrogen (Figure 2). Subcellular fractionation experiments using immunoblot analysis also confirmed the immunostaining results in MCF-7 cells. Our results suggest that an increasing effect of the BRCA2 protein in estrogen-treated MCF-7 cells is not associated with the response to DNA repair.

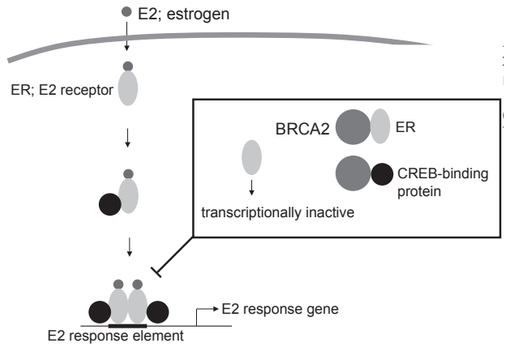

Figure 2. Model for the role of BRCA2 in E2-ERa signaling pathway
In this study, we suggest that BRCA2 inhibits ERa activity through interactions with ER or CREB-binding protein, implying the existence of a negative feedback mechanism.

4. The possibility of new subcellular function of BRCA2 at recycling endosome

We already reported that BRCA2 is involved in the abscission between two daughter cells during cytokinesis. Many common proteins are known as having some role in cytokinesis and endocytosis. Besides some of these proteins are reported as a partner of BRCA2, so we made hypothesis that BRCA2 might have some role at endosome. We isolated endosome lysates from HeLa S3 cells and analyzed the lysates by mass spectrometry or immunoblotting using anti-BRCA2 antibody. The results indicated that BRCA2 exists in endosome. Then, we tried the mass spectrometry analysis of the anti-BRCA2 immunoprecipitates from endosome lysates. This results suggested that the possibility of new BRCA2 function at recycling endosome. We picked up Rab11-FIP3 from the list of mass spectrometry analysis data. Rab11-FIP3 is one of the effector protein of Rab11, recycling endosome marker, and interacts with dynein for transport of recycling endosome. We already confirmed the interaction between BRCA2 and dynein. Besides, Rab11-FIP3 is involved in the spindle formation and cytokinesis during M-phase. We analyzed the reciprocal influence of these proteins in the cells. In the result, BRCA2 depletion inhibited the localization of Rab11-FIP3 at centrosome. The mechanism of this phenomenon is not unclear and this is the issue should be resolved in the future.

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Department of Molecular Epidemiology

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Associate Professor Noriko Sato M.D.& Ph.D.
Assistant Professor Shinobu Ikeda, DMD. Ph.D.

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

1. Association of the Chromodomain Helicase DNA-Binding Protein 4 (CHD4) Missense Variation p.D140E with Cancer: Potential Interaction with Smoking

Chromodomain helicase DNA-binding protein 4 (CHD4) plays a pivotal role in chromatin remodeling and has been implicated in the development of cancer. The aim of this study is to determine the association of *CHD4* gene variants with cancer. Nine missense single nucleotide variations (SNVs) in *CHD4* were retrieved from genotyping, by an exome-chip, 2,343 consecutive autopsy cases, in which the presence or absence of cancer was pathologically reviewed. The association of *CHD4* variants with the presence of cancer and with different types of cancer was determined. Interaction with smoking was also determined. There were 1,446 patients with cancer and 897 patients without cancer. Of the 9 SNVs, 7 SNVs were monomorphic, while two non-synonymous SNVs; rs7479004 (p.D140E) and rs1639122 (p.E139D) were further verified by direct sequencing. The p.D140E was associated with the presence of cancer (adjusted odds ratio [OR], 2.17; 95% confidence interval [CI], 1.37-3.44, P = 0.001), but not p.E139D. The effect size was larger in the smokers (adjusted OR, 4.66; 95% CI, 1.82-11.9; P = 0.001), suggesting that there may be a gene environment interaction. For individual cancer types, p.D140E was associated with lung cancer (adjusted OR, 3.99; 95% CI, 2.07-7.67; P < 0.001), malignant lymphoma (adjusted OR, 3.24; 95% CI, 1.43-7.33; P = 0.005), and rectum cancer (adjusted OR, 6.23; 95% CI, 2.31-16.8; P < 0.001). A non-synonymous SNV of *CHD4*, p.D140E, confers a risk of cancer and may interact with smoking habit to increase the risk.

2. Genetic risk score based on the lifetime prevalence of femoral fracture in 924 consecutive autopsies of Japanese males.

A genetic risk score (GRS) was developed for predicting fracture risk based on lifetime prevalence of femoral fractures in 924 consecutive autopsies of Japanese males. A total of 922 non-synonymous single nucleotide polymorphisms (SNPs) located in 62 osteoporosis susceptibility genes were genotyped and evaluated for their association with the prevalence of femoral fracture in autopsy cases. GRS values were calculated as the sum of risk allele counts (unweighted GRS) or the sum of weighted scores estimated from logistic regression coefficients (weighted GRS). Five SNPs (α-L-iduronidase rs3755955, C7orf58 rs190543052, homeobox C4 rs75256744, G patch domain-containing gene 1 rs2287679, and Werner syndrome rs2230009) showed a significant association (P < 0.05) with the prevalence of femoral fracture in 924 male subjects. Both the unweighted and weighted GRS adequately predicted fracture prevalence; areas under receiver-operating characteristic curves were 0.750 [95 % confidence interval (CI) 0.660-0.840] and 0.770 (95 % CI 0.681-0.859), respectively. Multiple logistic regression analysis revealed that the odds ratio (OR) for the association between fracture prevalence and unweighted GRS ≥3 (n = 124) was 8.39 (95 % CI 4.22-16.69, P < 0.001) relative to a score <3 (n = 797). Likewise, the OR for a weighted GRS of 6-15 (n = 135) was 7.73 (95 % CI 3.89-15.36, P < 0.001) relative to scores of 0-5 (n = 786). The GRS based on risk allele profiles of the five SNPs could help identify at-risk individuals and enable implementation of preventive measures for femoral fracture.

3. A missense single nucleotide polymorphism, V114I of the Werner syndrome gene, is associated with risk of osteoporosis and femoral fracture in the Japanese population.

Werner syndrome is a rare autosomal recessive disorder caused by mutations in the human WRN gene and characterized by the early onset of normal aging symptoms. Given that patients with this disease exhibit osteoporosis, the present study aimed to determine whether the WRN gene contributes to the etiology of osteoporosis. A genetic association study of eight non-synonymous polymorphisms in the WRN gene and the incidence of femoral fracture was undertaken in 1,632 consecutive Japanese autopsies in which 140 patients had experienced the fracture during their lifetime. The results were validated in 251 unrelated postmenopausal Japanese women with osteoporosis and 269 non-institutionalized, community-dwelling Japanese adults. A statistically significant association was observed between rs2230009 (c.340G > A)–which results in a Val to Ile substitution–and fracture risk; the incidence of femoral fracture increased dose-dependently with the number of A alleles (p = 0.0120). Femoral neck bone and whole bone densities were lower among postmenopausal women with osteoporosis and community-dwelling adults, respectively, if they were of the AG instead of the GG genotype. The results suggest that Japanese subjects bearing at least one A allele of rs2230009 of the WRN gene are at a significantly higher risk of femoral fracture, possibly due to decreased bone density.

4. Development of a simple genotyping method for the HLA-A*31:01-tagging SNP in Japanese.

HLA-A*31:01 is confirmed to be significantly associated with definite/probable cases of CBZ-related Stevens-Johnson syndrome/toxic epidermal necrolysis (SJS/TEN) (p = 0.0040). In this study, we aimed to construct a simple, low-cost typing method for the surrogate marker of HLA-A*31:01, a risk factor for carbamazepine (CBZ) related (SJS/TEN).

DNAs from Japanese SJS/TEN patients were used for genotyping and developing the assay. Three single nucleotide polymorphisms, rs1150738, rs3869066 and rs259945, were in absolute linkage disequilibrium with HLA-A*31:01 in 210 Japanese SJS/TEN patients. Robust genotyping of rs3869066 in ZNRD1-AS1 was developed using polymerase chain reaction-restriction fragment length polymorphism assays. Single nucleotide polymorphism genotyping is less time consuming and cheaper than conventional HLA typing, and would be useful for identifying Japanese patients at risk of CBZ-related SJS/TEN.

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Department of Biochemical Genetics

Professor Shigetaka Kitajima MD, PhD.
Associate Professor Yujiro Tanaka MD, PhD.

Scope of research :

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

Research 1: Analysis of Wnt-ATF3 pathway and tumor metastasis in human colon cancer

The mutation of components of the Wnt pathway is found in colorectal cancers. We have shown that ATF3 is the direct target of Wnt pathway in normal cells and is over-expressed in human colon cancer cells harboring mutated β-catenin. Moreover, ATF3 did not affect proliferation but inhibited migration and invasion. Additional, global analysis have shown that ATF3 activates a number of Wnt target genes, whereas plays role negative regulator as suppress tumor metastasis and Epithelial-Mesenchymal Transition (EMT)-related genes (Fig. 1). We newly have shown that ATF3 suppresses a number of inflammation-related factors included Monocyte Chemotactic Protein-1 (MCP-1) and further study association their regulation with metastasis in vivo.

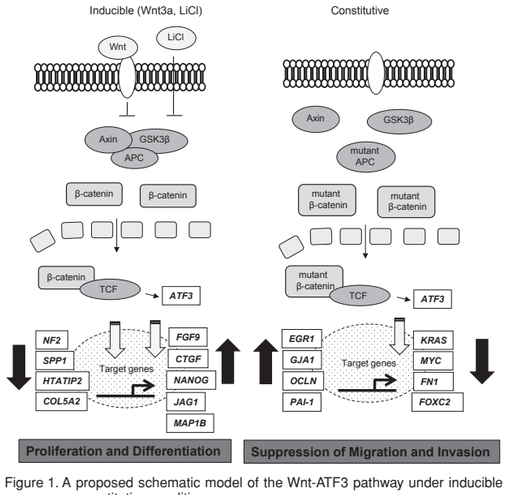

Figure 1. A proposed schematic model of the Wnt-ATF3 pathway under inducible or constitutive conditions.

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

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

Research 3: ASH1 and epigenetic mechanisms of facioscapulohumeral muscular dystrophy

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

Highlight

1. Wnt target gene ATF3 is tumor suppressor

First, we revealed whether ATF3 is directly Wnt target gene in colon cancer. The expression of ATF3 was strongly induced in colon cancer cells harboring aberrant Wnt signaling pathway including HCT116 (Fig.2). Actually, ATF3 Luciferase assay, DNA affinity pre-precipitation and β-catenin ChIP assay showed that TCF4/β-catenin binding site existed within ATF3 gene promoter from -30 to -40 base region and β-catenin was recruited to it.

Next, we showed biological function of Wnt target gene ATF3. ATF3-knockdown cells promoted cell migration and invasion ability in vitro. Moreover, RT2 profiler PCR array analysis of Wnt target genes, tumor metastasis or EMT-related genes identified 21 candidate genes included KRAS and FN. Taken together, we indicated that Wnt target gene ATF3 plays as tumor suppressor in colon.

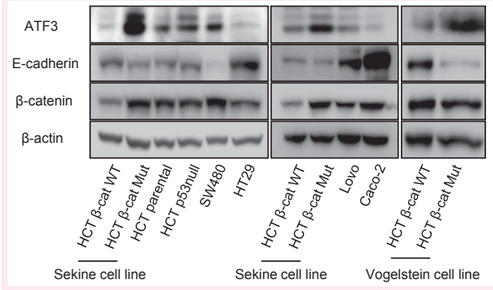

Figure 2. ATF3 is induced by aberrant Wnt signaling pathway.

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

We are trying to reveal the biological meaning of p53-ATF3 regulatory pathway. As a first step, we have performed microarray analysis using the doxorubicin treated embryonic fibroblasts from wild type, Atf3 KO, p53 KO and Atf3-p53 double KO mice. We illustrated heatmaps of stress response pathways included DNA damage response, unfolded protein response etc. (Fig. 3). The results show that most of these pathway genes were down-regulated by ATF3 KO, therefore implying that ATF3 generally up-regulates genes that are implicated in stress response.

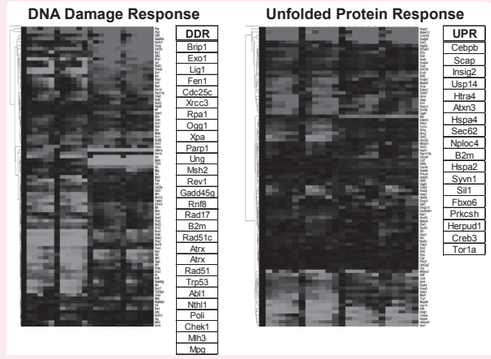

Figure 3. Heatmaps of stress response pathway

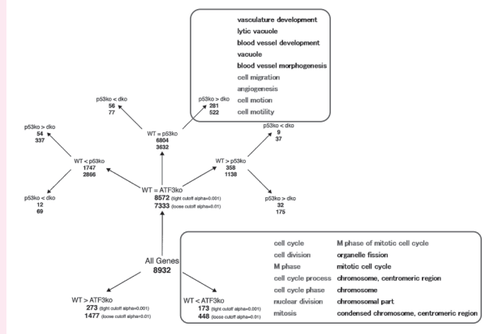

Figure 4. The role of ATF3 in the presence or absence of p53

Publications

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2. Uchida Y et al. Systems analysis of DNA damage response of p53-ATF3 pathway in a mouse model. manuscript in preparation 2015;
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5. Sood V et al. ATF3 hyper-induced during Japanese encephalitis virus infection negatively regulates the antiviral signalling in the absence of type 1 interferons. submitted

Department of Genomic Pathology

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Research content

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

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

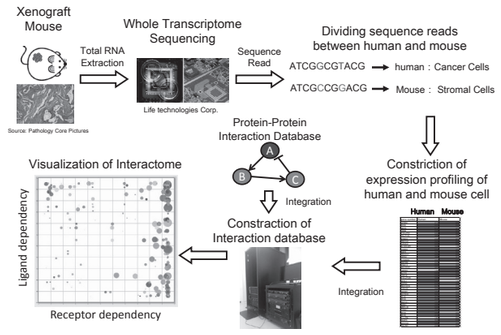

Fig 1. Genomic approach for cancer - stromal interaction

Research introduction

1. Genomic approach for the cancer-stromal interaction

In the department of genomic pathology, we have developed a new method to analyze a wide range of cancer-stromal interactions in tumor tissues which are composed of various types of cells (tumor-stroma interactome). 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.

Furthermore, by using a direct xenograft model (PDX: Patient Derived Xenograft, collaborated with Central Institute for Experimental Animals (CIEA), we are investigating the interactome analysis of multiple clinical tumors in order to make it possible to clarify the cell-cell interactome in primary human tumors.

2. Genomics Analysis for Clinical Disease Tissues

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

3. Analysis of Antigen Receptor Repertoire in Tumor Infiltrating Lymphocytes

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.

In the department of genomic pathology, we try to uncover the functions of TILs in cancer environment by analyzing their antigen receptor sequences using massively-parallel sequencing technology (Fig. 2). Our main focus is on diffuse gastric cancer, which is characterized by very poor prognosis and few actionable driver mutations for molecular targeting therapy. Due to its low mutation load, diffuse gastric cancer is expected to respond poorly to immune checkpoint blockade such as anti-PD-1 therapy. Our analysis provides a promising approach to define TIL characteristics and may lead to the development of improved cancer immunotherapy.

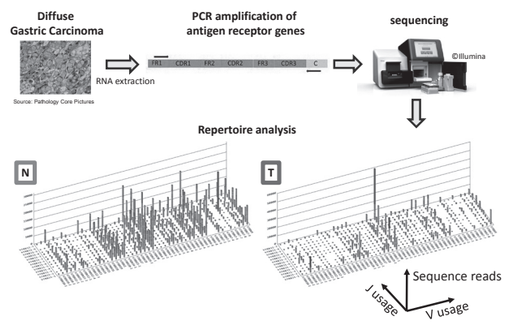

Fig 2. Antigen Receptor Analysis of Tumor infiltrating lymphocytes

4. Functional Genomics Screening

In the Department of Genomic Pathology, 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 (Fig. 3). 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 human gastric cancers, pancreatic cancers, colon cancers, liver cancers and so forth, having identified some candidate therapeutic target genes. We will continue genomics screenings and identify novel therapeutic targets against otherwise devastating human cancers.

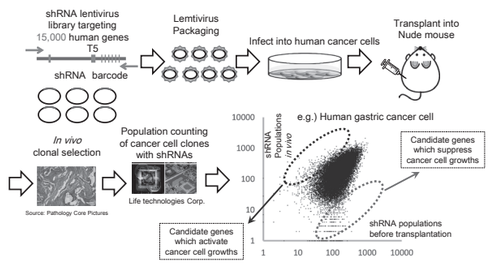

Fig 3. Functional Genomics Screening to Identify Novel Therapeutic Targets against Cancers

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Original Paper

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RHOA mutation in diffuse-type gastric cancer: a comparative clinicopathology analysis of 87 cases.
Gastric Cancer. 2015 Apr 1. [Epub ahead of print] PubMed PMID: 25823974.

International Conference

1. Komura D, Isagawa T, Sato R, Kishi K, Suzuki R, Ishikawa S.
Comprehensive analysis of tumor - stromal interactome
CSH-ASIA/AACR JOINT MEETING: BIG DATA, COMPUTATION, AND SYSTEMS BIOLOGY IN CANCER, Suzhou, China, Dec.2-Dec.5, 2015

2. Kato H, Komura D, Konishi H, Yamamoto A, Fukayama M, Ishikawa S.
Immunogenomic Characterization of Tumor Infiltrating TCR Repertoire in the Gastric Carcinoma Enviroments using Archived Histopathological Specimens
3rd Annual immungenomics 2015 Shaping the Future of Human Health, HUNTSVILLE, ALABAMA, USA, Sep.28-Sep.30, 2015

3. Kato H, Komura D, Konishi H, Yamamoto A, Fukayama M, Ishikawa S.
Immunogenetic profiling of tumor infiltrating T cells among human gastric cancer
IMMUNE PROFILING IN HEALTH AND DISEASE, Seattle, USA, Sep.9 - Sep.11, 2015

Department of Epigenetics

Professor	Fumitoshi ISHINO
Associate Professor	Takashi KOHDA
Assistant Professor	Hirosuke SHIURA
	Yuki KAWASAKI
Tokunin Lecturer	Jiyoung LEE
Tokunin Assistant Professor	Masahito IRIE
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Introduction: Department of Epigenetics

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

Research subjects

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

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

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

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

3. Biology of haploid ES cells in mammals

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

4. New method of analyzing DNA methylation status in genomes

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

Highlight

Cognitive function related to the *Sirh11/ Zcchc16* gene acquired from an LTR retrotransposon in eutherians.

Gene targeting of mouse Sushi-ichi-related retrotransposon homologue 11/Zinc finger CCHC domain-containing 16 (*Sirh11/Zcchc16*) causes abnormal behaviors related to cognition, including attention, impulsivity and working memory (Figure 1). *Sirh11/Zcchc16* encodes a CCHC type of zinc-finger protein that exhibits high homology to an LTR retrotransposon Gag protein. Upon microdialysis analysis of the prefrontal cortex region, the recovery rate of noradrenaline (NA) was reduced compared with dopamine (DA) after perfusion of high potassium-containing artificial cerebrospinal fluid in knockout (KO) mice (Figure 2). These data indicate that Sirh11/Zcchc16 is involved in cognitive function in the brain, possibly via the noradrenergic system, in the contemporary mouse developmental systems. Interestingly, it is highly conserved in three out of the four major groups of the eutherians, euarchontoglires, laurasiatheria and afrotheria, but is heavily mutated in xenarthran species such as the sloth and armadillo, suggesting that it has contributed to brain evolution in the three major eutherian lineages, including humans and mice (Figure 3).

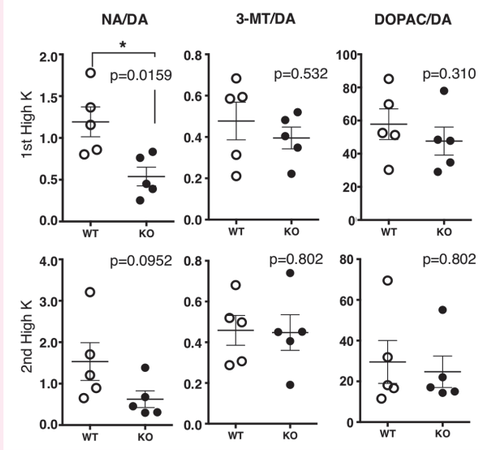


Figure 2. **Abnormality of monoamine levels in brain of *Sirh11/Zcchc16* KO mice.** Microdialysis analysis in the prefrontal cortex in the cerebrum. The levels of various monoamines, including DA, NA, 3-MT and DOPAC were measured after perfusion of high potassium-containing artificial cerebrospinal fluid in the prefrontal cortex. Each plot shows the ratio of the DA metabolites, NA, 3-MT and DOPAC, to DA.

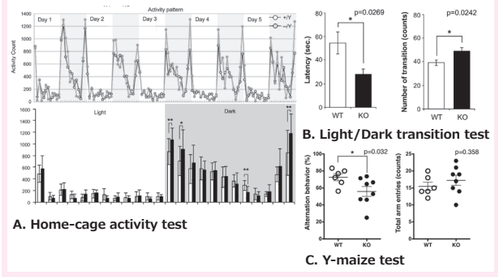


Figure 1. **Abnormal behavior of *Sirh11/Zcchc16* KO mice.** A. Home-cage activity test. The plots show the activity counts every hour over 5 days. The white and grey areas indicate the light and dark phases, respectively. B. Light/Dark transition test. The left panel shows the latency time before entering into the light chamber. The right panel shows the number of transitions. C. Y-maze test. Left: each plot shows the percentage of alternation behavior. Right: each plot shows the number of total arm entries. T

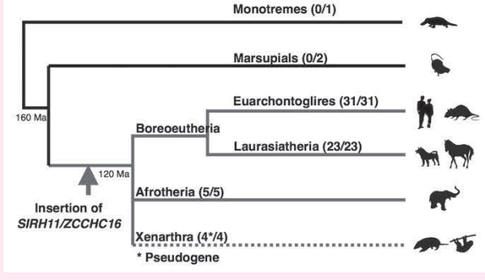


Figure 3. **Conservation of *Sirh11/Zcchc16* in eutherian mammals.** The *SIRH11/ ZCCHC16* sequence was confirmed in three out of four major eutherian groups, euarchontoglires, laurasiatheria, xenarthra and afrotheria, but became a pseudogene in xenarthra (the dashed line), indicating that the insertion of *SIRH11/ZCCHC16* occurred in a common eutherian ancestor. The number of species possessing *SIRH11/ZCCHC16* (front) and those which in total were analyzed (back) are noted in parentheses. An asterisk means *pseudoSIRH11/ZCCHC16*.

Publications (Original papers)

1. Kagami M, Kurosawa K, Miyazaki O, Ishino F, Matsuoka K, Ogata T. Comprehensive clinical studies in 34 patients with molecularly defined UPD(14) pat and related conditions (Kagami-Ogata syndrome). Eur J Hum Genet **23**(11), 1488-1498 (2015).
2. Ito M, Sferruzzi-Perri AN, Edwards CA, Adalsteinsson BT, Allen SE, Loo T-H, Kitazawa M, Kaneko-Ishino T, Ishino F, Stewart CL and Ferguson-Smith AC. A trans-homologue interaction between reciprocally imprinted *miR-127* and *Rtl1* regulates placenta development. Development **142**(14), 2425-2430 (2015).
3. Ono R, Ishii M, Fujihara Y, Kitazawa M, Usami T, Kaneko-Ishino T, Kanno J, Ikawa M and Ishino F. Double strand break repair by capture of retrotransposon sequences and reverse-transcribed spliced mRNA sequences in mouse zygotes. Sci Rep. **5**:12281 (2015).
4. Irie M, Yoshikawa M, Ono R, Iwafune H, Furuse T, Yamada I, Wakana S, Yamashita Y, Abe T, Ishino F* and Kaneko-Ishino T*. Cognitive function related to the *Sirh11/Zcchc16* gene acquired from an LTR retrotransposon in eutherians. PLoS Genet **11**(9):e1005521 (2015).

Publications (Review)

1. Kaneko-Ishino T and Ishino F. Mammalian-specific genomic functions: Newly acquired traits generated by genomic imprinting and LTR retrotransposon-derived genes in mammals. Proc Jpn Acad Ser B Phys Biol Sci. 2015;**91**(10):511-538.

Department of Medical Science Mathematics

Professor Tatsuhiko Tsunoda
Junior Associate Professor Daichi Shigemizu
Assistant Professor Fuyuki Miya

Research Summary

Recently, medical application of rapidly progressing omic profiling technologies and, in particular, the promotion of personalized/precision/preventive medicine have been keenly desired. Our department overcomes such medical science issues by using a combination of mathematics and computational sciences. Nowadays, biomedical big data of clinical and omic profiles are collected from hospitals and medical institutions. First, applying data-mining methodologies, we explore etiologies of intractable diseases, e.g. cancer, common diseases, and neurodegenerative diseases. Next, we classify each disease into finer categories through molecular profiles, and understand disease causing mechanisms through a systems approach. Last, we apply mathematical methods to optimize therapy prediction for each patient when she/he visits a hospital/medical institute, and we can also apply these methods to disease prevention based on an individual's health check records.

Research Projects

1. Exploring etiologies, sub-classification, and risk prediction of diseases based on big-data analysis of clinical and whole omics data in medicine (CREST, JST)

Toward the goal of personalized/precision medicine, we fully integrate omics, clinical, and molecular datasets into big data. We develop methodologies and utilize advanced statistics and computer science for medical big data analysis. Our plan: (a) standardization, integration, and management of big data, (b) exploration of the compound factors of disease, (c) sub-classification of disease, and (d) prediction of disease and the optimum therapy for individuals. Data resources: (a) BioBank data, which includes genomic and clinical data, (b) multi-omics and clinical data of cancer patients, (c) whole genome/exome sequences and clinical data of liver cancer patients, (d) prospective genome cohort, with abundant drug application data, of liver disease patients, (e) prospective genome cohort, with precise follow-ups after drug application, of rheumatoid arthritis patients, and (f) molecular DBs. Our department proposes new methodologies towards these goals (e.g. Lyons J. et al. *J. Theor. Biol.* **2016**; Sharma R. et al. *IEEE* Trans Nanobioscience **2015**). In addition to successful results from the analysis of biliary phenotype liver cancer (Fujimoto A. et al. *Nature communications* **2015**), we recently observed a significant correlation between the molecular clustering of omic data and clinical information, e.g. survival time, from the liver cancer omic analysis.

2. Exome analyses of long QT syndrome reveal candidate pathogenic mutations in calmodulin-interacting genes

Long QT syndrome (LQTS) is an arrhythmogenic disorder that can lead to sudden death and has an estimated prevalence as high as one in 2,000 people. To date, mutations in 15 LQTS-susceptibility genes have been implicated. However, the genetic cause of approximately 20% of LQTS patients remains elusive. Here, we performed whole-exome sequencing analyses on 59 LQTS and 61 unaffected individuals in 35 families (120 individuals) and 138 unrelated LQTS cases, after genetic screening for known LQTS genes. Protein-protein interaction (PPI) network analyses revealed ten new pathogenic candidates that directly or indirectly interact with proteins encoded by known LQTS genes. Moreover, half of the newly identified candidates directly interact with calmodulin. These results suggest an important role of calmodulin and its interacting proteins in the pathogenesis of LQTS.

3. Performance comparison of four commercial human whole-exome capture platforms

Whole exome sequencing (WES) is widely used to identify causative genetic mutations of diseases. Currently, there are four major exome enrichment platforms: Roche/NimbleGen's SeqCap EZ Human Exome Library, Illumina's Nextera Rapid Capture Exome, Agilent's SureSelect XT Human All Exon and Agilent's SureSelect QXT. Several performance comparison studies among these exome capture platforms have been reported. However, substantial updates have been released for each of these platforms over the past few years. Therefore, we comprehensively evaluated the latest version of the four major exome platforms from three manufactures with respect to five parameters. This performance comparison analysis of current exome enrichment platforms will be helpful to investigators when selecting the best platform for their research.

4. Investigation of pathogenic mutations for congenital neurological diseases and hearing loss.

We recently established a consortium with the aim of identifying disease-causing mutations and applying that knowledge to clinical diagnosis of congenital neurological diseases and hearing loss between a group of research institutes and hospitals in Japan. To that end, we performed targeted resequencing and whole-exome sequencing (WES) analysis on ~160 families (~500 individuals). We identified some novel pathogenic mutations for the diseases, and published 7 reports (e.g. Okamoto N., Miya F., *et al. Clin. Genet.* **88**, 288-292 (2015)) in 2015.

5. Combination of multiple methodologies for exome sequencing reveals novel pathogenic mutations.

As above, WES is a useful method to identify disease-causing mutations, however, often no candidate mutations are identified using commonly available targeted probe sets. One possible cause for this lack of candidates is that standard WES cannot sequence all protein-coding sequences (CDS) due to capture probe design and regions of low coverage. We combined a selective circularization-based target enrichment method with a hybrid capture method, and achieved a more complete coverage of CDS regions (~97% of all CDS). We applied this "CCCS" (Complementary Custom CDS Sequencing) approach, to 7 pedigrees with no candidate mutations identified through standard WES analysis and identified novel pathogenic mutations in one pedigree (see Fig., Miya F. *et al. Sci. Rep.* **5**, 9331 (2015)). The application of this effective combination of targeted enrichment methodologies can be expected to aid in the identification of novel pathogenic mutations previously missed by standard WES analysis.

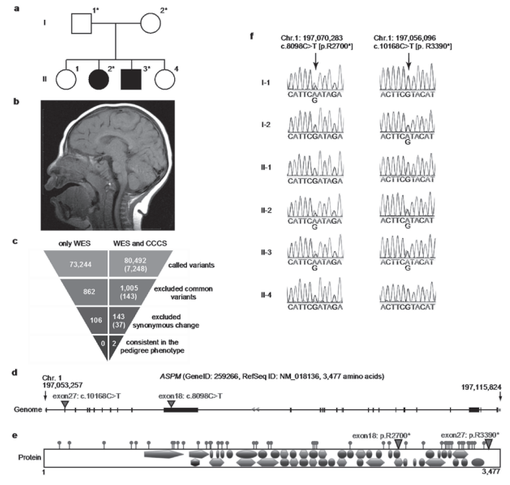


Figure. **Identified mutation by using combination of WES and CCCS in a family with microcephaly.**
(a) Family tree of the pedigree with microcephaly. Shaded symbols denote affected individuals. Asterisks denote NGS was performed. (b) Sagittal T1-weighted brain magnetic resonance image (MRI) of the II-2 individual at 4 years of age shows frontal sloping and reduced volume of the brain, particularly the frontal lobe of the cerebrum. (c) Filtering the candidate mutations for the II-2 individual. The numbers in parenthesis represent the number of called variants with CCCS. Overlapping variants between WES and CCCS are not excluded. (d) ASPM gene in human genome. Red triangles indicate loci of identified mutation. (e) Domains and mutations in the ASPM protein. Red pins indicate loci of known nonsense mutations. Red triangles indicate loci of identified mutation. Colored diagrams in box indicates repository domains. (f) Sanger sequencing data of the identified mutation.

Publications

[Original articles]

1. James Lyons, Kuldip K Paliwal, Abdollah Dehzangi, Rhys Heffernan, Tatsuhiko Tsunoda, Alok Sharma. Protein fold recognition using HMM-HMM alignment and dynamic programming. J. Theor. Biol.. 2016.03; 393 67-74.
2. Maki Morishita, Tomoki Muramatsu, Yumiko Suto, Momoki Hirai, Teruaki Konishi, Shin Hayashi, Daichi Shigemizu, Tatsuhiko Tsunoda, Keiji Moriyama, Johji Inazawa. Chromothripsis-like chromosomal rearrangements induced by ionizing radiation using proton microbeam irradiation system. Oncotarget. 2016.02;
3. Atsuko Konta, Kouichi Ozaki, Yasuhiko Sakata, Atsushi Takahashi, Takashi Morizono, Shinichiro Suna, Yoshihiro Onouchi, Tatsuhiko Tsunoda, Michiaki Kubo, Issei Komuro, Yoshinobu Eishi, Toshihiro Tanaka. A functional SNP in FLT1 increases risk of coronary artery disease in a Japanese population. J. Hum. Genet.. 2016.01;
4. Ronesh Sharma, Abdollah Dehzangi, James Lyons, Kuldip Paliwal, Tatsuhiko Tsunoda, Alok Sharma. Predict Gram-Positive and Gram-Negative Subcellular Localization via Incorporating Evolutionary Information and Physicochemical Features Into Chou's General PseAAC. IEEE Trans Nanobioscience. 2015.12; 14 (8): 915-926.
5. Akihiro Ito, Tadahiro Shimazu, Satoko Maeda, Asad Ali Shah, Tatsuhiko Tsunoda, Shun-Ichiro Iemura, Toru Natsume, Takafumi Suzuki, Hozumi Motohashi, Masayuki Yamamoto, Minoru Yoshida. The subcellular localization and activity of cortactin is regulated by acetylation and interaction with Keap1. Sci Signal. 2015; 8 (404): ra120.

[Review]

1. Tatsuhiko Tsunoda. Big data analysis and mathematical research in medical science Clinical Immunology & Allergology. 2015.12; 64 (6); 565-571.

Frontier Research Unit
Laboratory of Gene Expression

Associate Professor **Hidehito KUROYANAGI**
Project Assistant Professor **Yumiko YAMASAKI-KATO (-August, 2015), Sharmin HASAN (November, 2015-)**

Recent whole genome sequence analyses revealed that a high degree of proteomic complexity is achieved with a limited number of genes. This surprising finding underscores the importance of alternative pre-mRNA splicing, through which a single gene can generate structurally and functionally distinct protein isoforms. Based on recent transcriptome analysis, >90% of human multi-exon genes produce multiple mRNA isoforms. Regulation of the splice site choice through so called "splicing codes" provide a versatile mechanism for controlling gene expression and for generation of the proteome diversity. We are trying to decipher the splicing codes in living organisms.

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

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

Global Search for Target Events of Tissue-Specific Splicing Factors.

We are searching for alternative splicing events that are affected in the splicing factor mutants through transcriptome analyses by utilizing a next generation sequencer. We found new target events for a neuron-specific splicing factor UNC-75 and identified its *cis*-elements through bioinformatic and reporter analyses (Nucleic Acids Res, 2013) (see figure). We have also identified many endogenous mRNA isoforms that are rapidly degraded by nonsense-mediated mRNA decay (NMD) *in vivo*.

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

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

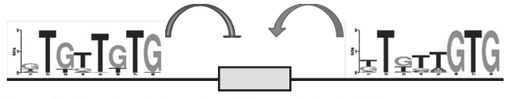


Figure. Position effect of alternative splicing regulation by UNC-75.

Publications

Original Articles

1. Takei, S, Togo-Ohno, M, Suzuki, Y, KUROYANAGI, H. Evolutionarily conserved autoregulation of alternative pre-mRNA splicing by ribosomal protein L10a. Nucleic Acids Research (2016). DOI: 10.1093/nar/gkw152.

Meeting Presentations

1. Satomi Takei, Yutaka Suzuki and Hidehito Kuroyanagi. Evolutionarily conserved autoregulation of alternative pre-mRNA splicing by ribosomal protein L10a. Cold Spring Harbor Meeting on Eukaryotic mRNA Processing, Cold Spring Harbor, NY, USA, August, 2015.

Project Research Unit

Associate Professor Michinori Kubota

Effects of salicylate on neural activities to frequency-modulated (FM) sounds with different FM sweep rates were investigated in the primary auditory cortex (AI) using optical imaging with a voltage-sensitive dye (RH795). Activity patterns to the FM sounds with different sweep rates (0.5-16.5 kHz in 16-160 ms duration: sweep rate 0.1-1 kHz/ms) were recorded from the AI on both sides before (control) and 8 hours after the intraperitoneal injection of salicylate (200 mg/kg). Amplitude differences between the 16-kHz frequency-band (FB) and other FBs at the 16-ms duration were larger for the upward FM sounds than the downward FM sounds under both salicylate and control conditions. The peak of the response to the FM sound showed the maximum amplitude at the 16-ms duration in the 16-kHz FB. The peak amplitude decreased when the FM duration increased up to 40 ms duration under the control condition.

However, it continued to decrease when the FM duration increased up to 64 ms under the salicylate condition.

Publications

Hosokawa Y, Kubota M, Sugimoto S, Horikawa J. Salicylate-induced neural changes of the FM function in the primary auditory cortex of guinea pigs observed by optical recording. J Physiol Sci, Vol. 65, Suppl. 1, S262 (2015).

Laboratory for Integrated Research Projects on Intractable Diseases
Advanced Technology Laboratories

Laboratory for Integrated Research Projects on Intractable Diseases

Research Projects on Intractable Diseases in Medical Research Institute

Research Project Head: Professor　Akinori Kimura

Five different inter-division and inter-department research projects were done in 2015.

1. Research project on inflammatory bowel diseases
2. Research project on striated muscle diseases
3. Research project on hypoxia-related pathological changes in breast cancer
4. Research project on epigenetic regulation in cancers
5. Research project on rapid diagnosis method of intractable disease genes

Followings are summaries of each research projects except that main summaries for projects 2 and 3 are described in the Frontier Research Unit (Laboratory of Gene Expression) and Frontier Research Unit (Laboratory of Gene Expression) and Frontier Research Unit (Laboratory of Oxygen Biology), respectively.

IBD project, Laboratory for Integrated Research Projects on Intractable Diseases

Professor　Shigeomi SHIMIZU
Akinori KIMURA
Toshiaki OHTEKI
Assistant Professor　Yusuke NAKANISHI

Summary

Inflammatory bowel disease (IBD) primarily includes ulcerative colitis and Crohn's disease. Both usually involve chronic inflammation, severe diarrhea, pain and bloody stool. Our goal is to understand mechanism of IBD development and find the new therapies and treatments of the disease.

Research Project

Breakdown of the intestinal epithelial layer's barrier function results in the inflow of commensal flora and improper immune responses against the commensal flora, leading to IBD development. Indeed, in most spontaneous colitis models, antibiotics or a germ-free state can block the development of colitis.

Using a mouse dextran sodium sulfate (DSS)-induced colitis model, we found that commensal Gram-positive bacteria trigger the mobilization of inflammatory monocyte and macrophage (*Mucosal Immunol* 2015). TNF-α is a representative inflammatory cytokine that aggravates colitis and a target for therapy, predominantly produced by monocyte and macrophage. Interestingly, pretreating mice with vancomycin, which eliminated Gram-positive bacteria, significantly reduced the severity of colitis, evaluated by the colon length, cytokine level, histological analysis etc.

In addition, 16S rRNA analysis showed that vancomycin treatment dramatically reduced Lachnospiraceae, the most abundant order of Clostridiales in untreated control mice. We also suggested that this strain stimulated the local production of chemokine by colonic epithelial cells, which induced the mobilization of monocyte/macrophage into the inflamed colon.

In addition to the genetic factors, our finding implies that the presence of certain Gram-positive bacteria is an environmental risk factor for IBD development. We are now trying to develop new strategies that block any step of monocyte/macrophage mobilization and/or inflammatory response.

Publications

Nakanishi Y, Sato T, and Ohteki T. Commensal Gram-positive bacteria initiates colitis by inducing monocyte/macrophage mobilization. Mucosal Immunology 2015.152-60.

R&D project of rapid diagnosis method of intractable disease genes

Project members　Norio Shimizu (principal researcher)
Tomohiro Morio
Ayako Arai
Kohsuke Tanimoto

Research project

The goals of our research project is development of an exhaustive pathogenic microbial screening system using multiples PCR technology. We have modified our exhaustive virus screening system (capable of screening dozens of viruses simultaneously) which is currently under development so that, in addition to viruses, it can also detect various other kinds of pathogens and pathogenic genes such as bacteria, protozoa, fusion genes of leukemia and genes of intractable diseases within one hour. Other goals are to improve the sensitivity of the screening system and put it to practical use by conducting clinical investigations.

Research findings

1. Clinical investigation methods of eye infection and opportunistic viral infection are approved by Ministry of Health, Labor and welfare as advanced medical care.
2. Patent application (2): Mycoplasma detection system, New Quantitative PCR reagent.
3. Technology transfer to universities and companies (20)
4. Reagent kit (2): Mycoplasma detection kit, Virus detection kit
5. Multi-institutional joint research of new eye infection detection system
6. Development of new exhaustive pathogenic microbial screening system: Viral hepatitis (9 viruses), Respiratory tract infection (20 viruses), Mycosis (16 fungi)

Research Project on Intractable Cancer Epigenomics

Associate Professor　Takashi Kohda
Lecturer　Jun Inoue
Assistant Professor　Yuki Kawasaki

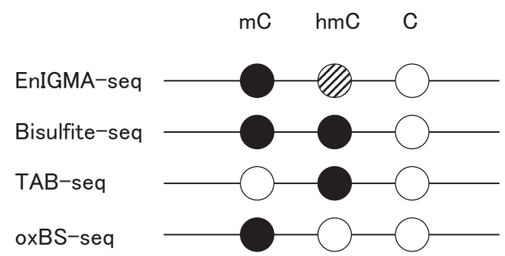
Research project

Molecular biological method to acquire a precise epigenetic information of the genome is indispensable tool in the medical and biological researches. Among them, analysis of the cytosine modification of DNA molecule is one of the most important techniques in the field. Recently, the discovery of the oxidation process by which mC is changed to hmC by the Tet enzymes leads to the understand the mechanism of DNA demethylation pathway. hmC also receive attention as a new modified nucleotide with distinct role in transcriptional regulation.

Therefore, single base resolution level analysis for hmC is indispensable tools needed in epigenetic studies. In this research project, we have been developed a novel method for the simultaneous identification of mC, hmC and C using DNMT1 enzyme specificity. We named this method the Enzyme-assisted Identification of Genome Modification Assay (EnIGMA), and it was demonstrated that this method could be identified with greater than 95% accuracy.

We have also developed a protocol to apply this method to the genome-wide analysis of hmC using massive parallel sequencers, improved the identification accuracy and adopted for small amount of DNA samples.

We successfully applied EnIGMA method for the analysis of MIR106A-363 gene cluster CpG island in oral squamous cell carcinoma cell lines. There was no hmC modification level difference between normal and cancer cells in this region. However, we identified a single CpG with extremely high hmC modification (37 -46 %) in this island. This suggests that the oxidation of CpG methylation by TET enzyme is not necessarily occurred entire CpG island but each single CpG modified independently.

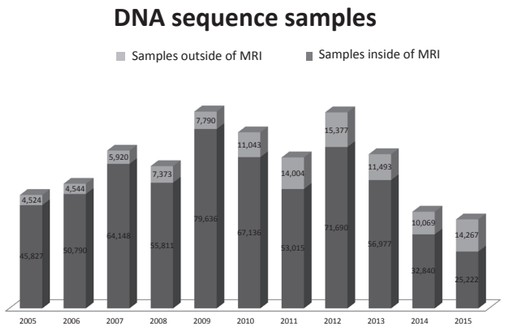


Conventional bisulfite sequencing can't distinguish mC and hmC. Recently developed methods such as TAB-seq and oxBS-seq enable to identify hmC or mC independently. We have developed a novel method to identify mC, hmC and C simultaneously at single base resolution and named "EnIGMA-seq".

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 2015.



1. Sequencing analyses

A total of 39,489 samples from 2,938 researchers were sequenced in the year of 2015. Among them 14,267 (36.1%) samples were requested by researchers outside the medical Research Institute (see below). Analysis by using next generation sequencing equipment (Ion Torrent PGM) has been started in 2013 and 27 runs was done in the year of 2015.

2. Equipment under the management of the Genome Laboratory.

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

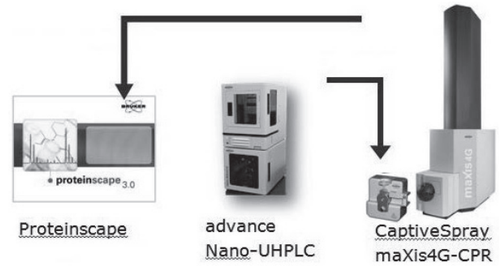
3. Introductory seminars

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

Laboratory of Cytometry and Proteome Research

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

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



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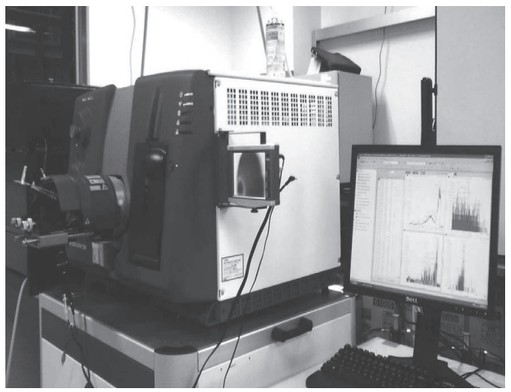

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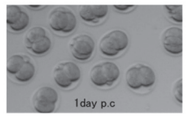
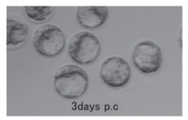
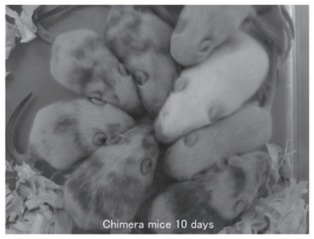
Laboratory of Recombinant Animals

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

<<Common equipment>>

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

Laboratory of Bioresource

Bioresource Lab. of Medical Research Institute was established in 2004, to support researchers and help postgraduates in cell culture. The center safely supply domestically or internationally well-recognized cultured cell lines as research materials. All of the cell lines handled are collected after exchanging MTA with original developers.

EB-virus transformed cell lines are also established with B-lymphocytes from patients with intractable diseases including genomic disorders after written informed consent from each of the patients or their parents and also with approval of the Internal Review Board on ethical issues.

Laboratory for Structure Analysis

The Laboratory for Structure Analysis is a small member of the Facilities and equipped with a high-brilliancy X-ray generator and an image plate X-ray detector. The Laboratory is also equipped with a dynamic light scattering (DLS) instrument, enabling the measurements of the particle size (thus 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 this and is now open for users from the outside of the university.

Stem Cell Laboratory

In order to assist researchers working on stem cell-related subjects across Medical Research Institute, Stem Cell Laboratory was established as of December, 2009. Now the Laboratory is equipped with basic and state-of-the-art research facilities. For instance, we have high-speed cell sorters (MoFlo Legacy and MoFlo XDP), time-lapse confocal laser scanning microscopes (FV10i-W and FV10i-DOC), sonicator, and hybridization oven.

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.

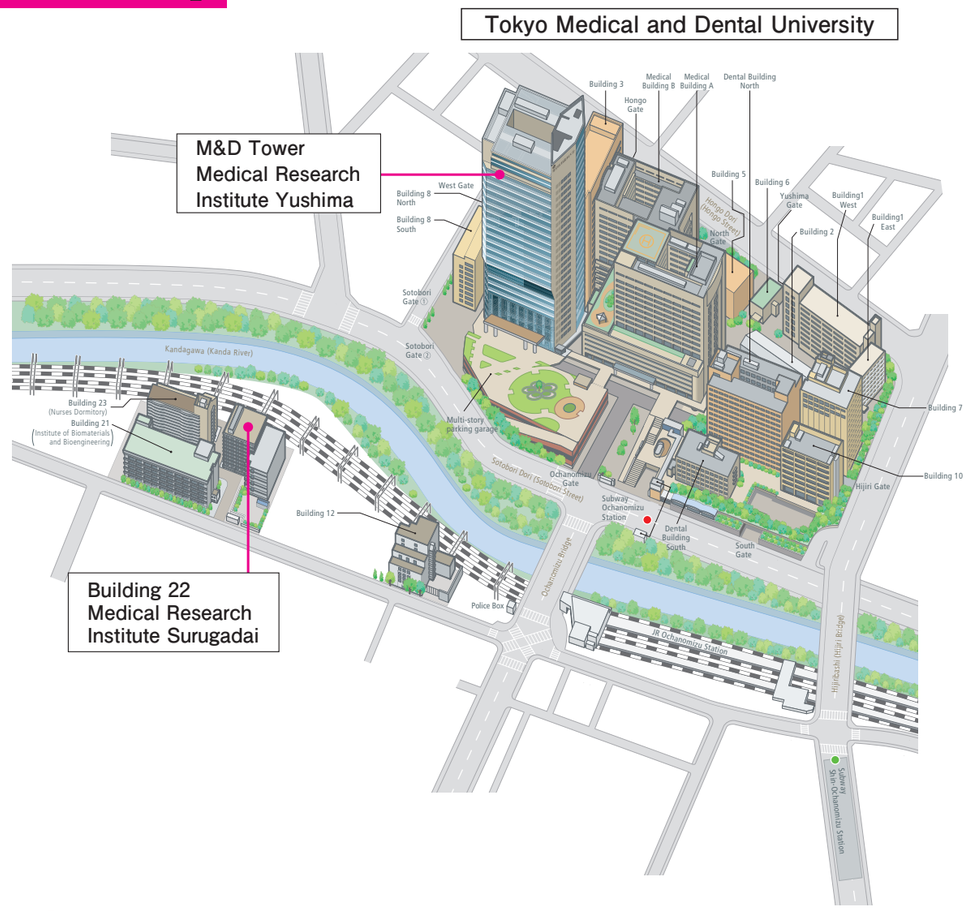
From August 1, 2013, the use of the equipment and services is opened newly to researchers in other departments within the University and those outside. Moreover, sorting service has started. The number of users is increasing gradually.

Throughout 2015, the number of overall use cases was 471. We held 9 short courses for beginners to help them use the equipment.

Advisory Committee Members

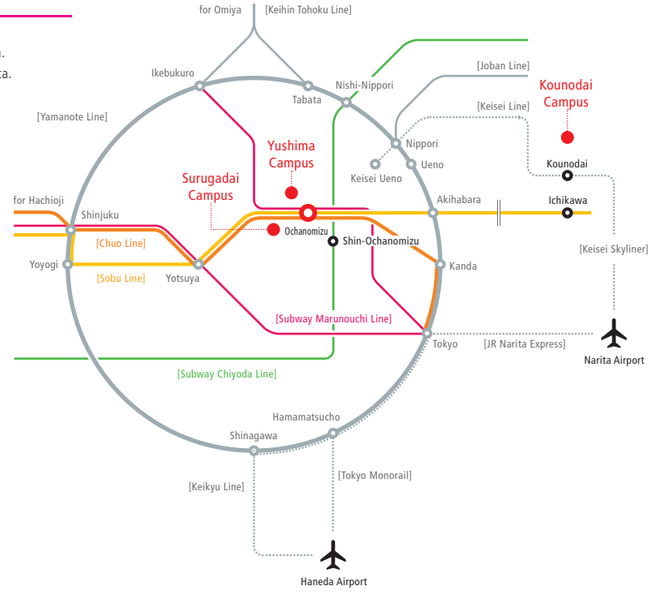
GO Mitiko	Trustee Nagoya University
SASAZUKI Takehiko	University Professor Kyushu University
TANAKA Takaharu	President Hoshi University
TANIGUCHI Masaru	Director RIKEN Center for Integrative Medical Sciences
NAGAI Ryozo	President Jichi Medical University
NAKAGAMA Hitoshi	Director National Cancer Center Research Institute
NAGANO Tetsuo	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.



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