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ANNUAL REPORT 2010

Tokyo Medical and Dental University

Medical Research Institute / School of Biomedical Science / Biomedical Science PhD Program / Tokyo Medical and Dental University

Annual Report 2010

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Medical Research Institute
School of Biomedical Science
Biomedical Science PhD Program
Tokyo Medical and Dental University

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School of Biomedical Science

Laboratory of Chemical Bioscience, Laboratory of Organic and Medicinal Chemistry

Medical Research Institute

Director
- Shigetaka Kitajima
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Dean of School of Biomedical Science — Takeshi Tsubata
- Faculty Meeting

Dean of Biomedical Science PhD Program
Hiroshi Tanaka

Research Divisions

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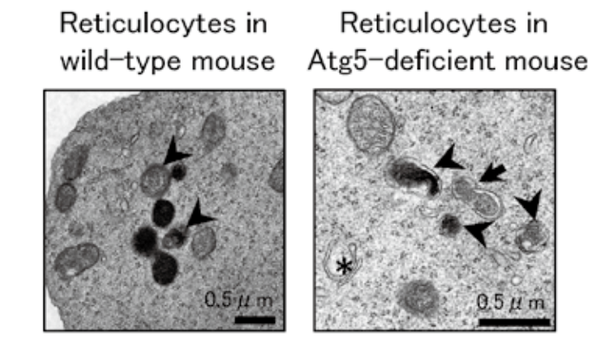
	Laboratory of Immunology	Professor	Takeshi Tsubata
	Laboratory of Biosystem Modeling	Professor	Tadashi Masuda

Hilight

Nishida et al. (2009) Discovery of Atg5/Atg7-independent alternative macroautophagy. Nature 461, 654-658

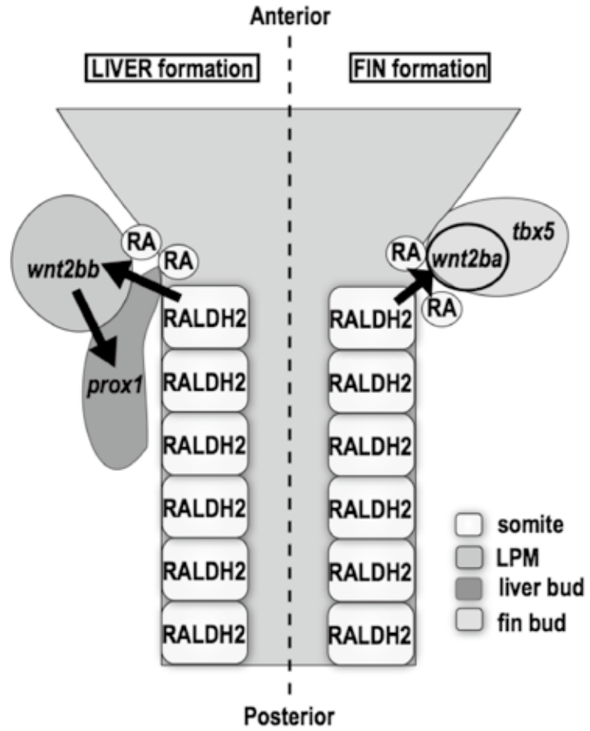
Macroautophagy is a process that leads to the bulk degradation of subcellular constituents through the creation of autophagosomes/autolysosomes. This mechanism is involved in the provision of nutrients when cells face starvation, as well as contributing to the turnover of cytoplasmic components. Studies of yeasts have identified a number of genes, designated ATG, that are required for the formation of autophagosomes. Many mammalian homologues of the yeast ATG genes have also been identified, and studies of mice lacking certain ATG genes, including ATG5 and ATG7, have confirmed that these genes are essential for induction of macroautophagy. Recently, we show that cells lacking ATG5 or ATG7 can still form autophagosomes/autolysosomes and perform autophagy-mediated protein degradation 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 process of macroautophagy. We also found that this alternate process of macroautophagy was regulated by several autophagic proteins, including ULK1 and Beclin-1. *In vivo*, ATG5-independent alternate macroautophagy was detected in several embryonic tissues. It was also found to play a role even in the clearance of mitochondria during erythroid maturation. 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.



Autophagic vacuoles in Atg5-deficient mouse engulfed and digested mitochondria.

Negishi et al. (2010) Retinoic acid signaling positively regulates liver specification by inducing wnt2bb gene expression in medaka. Hepatology 51, 1037-1045

During vertebrate embryogenesis, the liver develops at a precise location along the endodermal primitive gut tube due to signaling delivered by adjacent mesodermal tissues. Although several signaling molecules have been associated with liver formation, the molecular mechanism that regulates liver specification is still unclear. We previously performed a screen in medaka to isolate mutants with impaired liver development. The medaka *hio* mutants exhibit a profound (but transient) defect in liver specification that resembles the liver formation defect found in the zebrafish *prometheus* (*prt*) mutants, whose mutation occurs in the *wnt2bb* gene. In addition to their liver abnormality, *hio* mutants lack pectoral fins and die after hatching. Positional cloning revealed that the *hio* mutation affects the *raldh2* gene encoding retinaldehyde dehydrogenase type2 (RALDH2), the enzyme principally responsible for retinoic acid (RA) biosynthesis. Mutations of *raldh2* in zebrafish preclude the development of pectoral fins. Interestingly, in *hio* mutants, expression of *wnt2bb* in the lateral plate mesoderm (LPM) directly adjacent to the liver-forming endoderm was completely lost. *Conclusion*: Our data reveal the unexpected finding that RA signaling positively regulates the *wnt2bb* gene expression required for liver specification in medaka. These results suggest that a common molecular mechanism may underlie liver and pectoral fin specification during piscine embryogenesis.



Schematic model of RA signaling during liver and pectoral fin formation in a medaka embryo

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 approach. Topics of research projects in each Department are as follows:

【Molecular Medicine and Metabolism】

- Elucidation of a novel function of ATF3 as a negative regulator of the saturated fatty acid/TLR4/NF-κB pathway in obese adipose tissue
- Identification of cathepsin L as a FOXO1 target gene during skeletal muscle atrophy

【Molecular Pharmacology】

- Identification of ANA, an antiproliferative molecule that belongs to Tob/BTG family, as a negative regulator of BMP-induced ectopic bone formation
- Identification of Dicer, an RNase that processes microRNA precurosor into mature microRNA, as a negative regulator of bone remodeling by promoting osteoclast differentiation in osteoclasts and indirectly promoting bone formation
- Identification of methylation status of CpG islands in the promoter regions of signature genes during chondrogenesis of human synovium-derived mesenchymal stem cells

【Molecular Cell Biology】

- Identification of the mode of action of NLK, an essential effector for anterior formation: NLK functions downstream of p38 MAP kinase
- Elucidation of conservation of the WNK-OSR1 pathway among many species

【Molecular Neuroscience】

- Interleukin-1-mediated attenuation of normal tension glaucoma-like retinal degeneration in EAAC1 deficient mice
- Severely impairment of LTD at mossy fibers-CA3 synapses by upregulation of GLT-1

【Biodefense Research】

- Finding of type I IFNs-mediated regulation of the function and fate of hematopoietic stem cells (HSCs) such as self-renewal capacity and differentiation into progenitor cells
- Elucidation of Nod-like receptor signaling-induced enhancement of dendritic cell-mediated cross-priming in vivo
- Comprehensive introduction of recent findings about the maintenance of mucosal immune system homeostasis.

【Bio-informational Pharmacology】

- Elucidation of cross-talk between sex hormone non-genomic pathway and sympathetic nervous stimulation as one of the mechanisms for gender-difference in cardiac arrhythmias
- Establishment of mouse model for intractable lethal familiar arrhythmia “Brugada syndrome”

【Stem Cell Regulation】

- Identification of the cross-talk between FGF2 signaling and Wnt signaling, which promotes neural stem cell proliferation and, at the same time, inhibits neuronal differentiation
- Elucidation of a novel role of the FGF2-Wnt signaling crosstalk, which induces neural stem cell proliferation while inhibiting astroglial differentiation via cyclin D1 expression
- Identification of the Hoechst dye-effluxing “side population” among CD45 positive c-Kit positive placental cells, which shows high hematopoietic potential

Division of Advanced Molecular Medicine
Department of Molecular Medicine and Metabolism

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MTT Associate Professor	Yukio Yamamoto Ph.D.
GCOE Associate Professor	Naoki Sawada M.D., Ph.D.
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A. The metabolic syndrome and chronic inflammation

(1) Role of activating transcription factor 3 in adipose tissue inflammation (Fig. 1)

Through a combination of cDNA microarray analyses of saturated fatty acid-stimulated macrophages *in vitro* and obese adipose tissue in vivo, here we identified ATF3, a member of the ATF/cAMP response element-binding protein family of basic leucine zipper-type transcription factors, as a target gene of saturated fatty acids/TLR4 signaling in macrophages in obese adipose tissue. Importantly, ATF3, when induced by saturated fatty acids, can transcriptionally repress TNF α production in macrophages *in vitro*. Furthermore, transgenic overexpression of ATF3 specifically in macrophages results in the marked attenuation of pro-inflammatory M1 macrophage activation in the adipose tissue from genetically obese KKA^y mice fed high-fat diet. This study provides evidence that ATF3, which is induced in obese adipose tissue, acts as a transcriptional repressor of saturated fatty acids/TLR4 signaling, thereby revealing the negative feedback mechanism that attenuates obesity-induced macrophage activation. Our data also suggest that activation of ATF3 in macrophages offers a novel therapeutic strategy to prevent or treat obesity-induced adipose tissue inflammation (**Circ. Res.** 105: 25-32, 2009).

(2) Role of central leptin signaling in renal macrophage infiltration

Leptin acts directly on the hypothalamus, thereby regulating food intake and energy expenditure. The leptin receptor, a single transmembrane protein that belongs to the gp130 family of cytokine receptor superfamily, is expressed not only in the hypothalamus but in a variety of peripheral tissues, suggesting the role of leptin as a pro-inflammatory adipocytokine in peripheral tissues. Here, we show that deficiency of leptin signaling reduces renal macrophage infiltration after UUO. Bone marrow transplantation studies using leptin signaling-deficient *db/db* mice revealed that leptin signaling in bone marrow cells may not play a major role in the UUO-induced renal macrophage infiltration. Interestingly, central leptin administration reverses the otherwise reduced UUO-induced renal macrophage infiltration in leptin-deficient *ob/ob* mice. This is effectively abolished by central co-administration of SHU9119, a melanocortin-3 receptor/melanocortin-4 receptor antagonist. This study demonstrates that central leptin administration in ob/ob mice accelerates renal macrophage infiltration through the melanocortin system, thereby suggesting that the central nervous system, which is inherent to integrate information from throughout the organism, is able to control peripheral inflammation (**Endocr. J.** 57: 61-72, 2010).

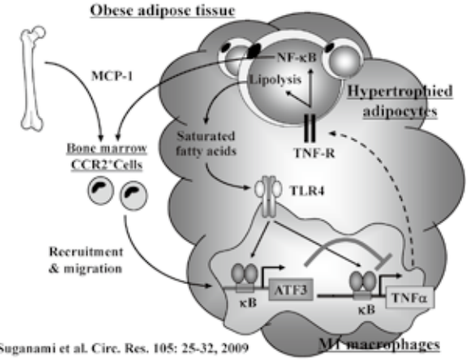

Fig. 1.

B. Epigenetic regulation of the metabolic syndrome

Obesity arises from a complex interaction between genetic and environmental factors, but the molecular basis of the interaction is poorly understood. Epigenetic mechanisms are likely to be involved in the development of obesity. DNA methylation is a key epigenetic contributor to the maintenance of gene silencing. Studies with Dnmts have focused mainly on development and cancer. However, their role in normal adult tissues, including the adipose tissue, remains largely unclear. Here, we show that *Dnmt3a* mRNA expression is markedly increased in obese adipose tissue. The data of this study also demonstrates that transgenic overexpression of *Dnmt3a* in the adipose tissue increases expression of some proinflammatory genes during a high-fat diet. This study highlights the potential role of Dnmt3a in the adult tissue as well as in the developing embryo and cancer (**Obesity** 18: 314–321, 2010).

C. Identification of target genes of FOXO1 in skeletal muscle atrophy (Fig. 2)

Previously, we found that a transcription factor FOXO1 is markedly up-regulated in skeletal muscle during muscle atrophy (streptozotocin-induced diabetes and starvation). We previously demonstrated that FOXO1 is a major regulator of skeletal muscle atrophy *in vivo*. Here, we show that FOXO1 enhances gene expression of cathepsin L, a lysosomal proteinase, *in vivo* and *in vitro*. Our data indicate that cathepsin L is a direct target of FOXO1 in the skeletal muscle and suggest that the FOXO1/cathepsin L pathway plays a role in diabetes and starvation-induced skeletal muscle metabolic change and atrophy. This study would provide further insight into new therapeutic strategies against skeletal muscle atrophy (**Biochem. J.** 427: 171-178, 2010).

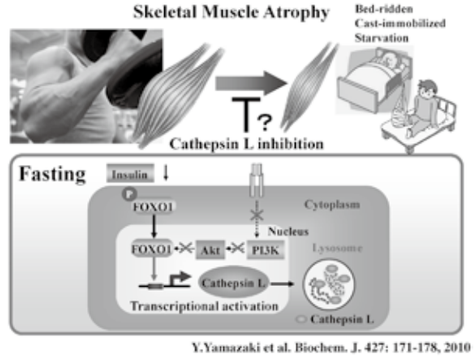

Fig. 2. Skeletal Muscle Atrophy

Publications

1. T. Suganami, X. Yuan, Y. Shimoda, K. Uchio-Yamada, N. Nakagawa, I. Shirakawa, T. Usami, T. Tsukahara, K. Nakayama, Y. Miyamoto, K. Yasuda, J. Matsuda, Y. Kamei, S. Kitajima, and Y. Ogawa. Activating transcription factor 3 constitutes a negative feedback mechanism that attenuates saturated fatty acid/Toll-like receptor 4 signaling and macrophage activation in obese adipose tissue. **Circ. Res.** 105: 25-32, 2009.
2. M. Kawamura, H. Itoh, S. Yura, H. Mogami, T. Fujii, H. Makino, Y. Miyamoto, Y. Yoshimasa, S. Aoe, Y. Ogawa, N. Sagawa, N. Kanayama, and I. Konishi. Isocaloric high-protein diet ameliorates systolic blood pressure increase and cardiac remodeling caused by maternal caloric restriction in adult mouse offspring. **Endocr. J.** 56: 679-689, 2009.
3. T. Chiba, Y. Kamei, T. Shimizu, T. Shirasawa, A. Katsumata, L. Shiraishi, S. Sugita, Y. Ogawa, S. Miura, and O. Ezaki. Overexpression of FOXO1 in skeletal muscle does not alter longevity in mice. **Mech. Ageing Dev.** 130: 420-428, 2009.
4. N. Satoh, A. Shimatsu, K. Kotani, A. Himeno, H. Yamakage, T. Majima, K. Yamada, T. Suganami, and Y. Ogawa. Highly purified eicosapentaenoic acid reduces cardio-ankle vascular index in association with decrease in serum amyloid A-LDL in metabolic syndrome. **Hypertens. Res.** 32: 1004-1008, 2009.
5. H. Mogami, S. Yura, H. Itoh, M. Kawamura, T. Fujii, A. Suzuki, S. Aoe, Y. Ogawa, N. Sagawa, I. Konishi, and S. Fujii. Isocaloric high-protein diet as well as branched-chain amino acids supplemented diet partially alleviates adverse consequences of maternal undernutrition on fetal growth. **Growth Hormone & IGF Res.** 19:478-485, 2009.

Department of Molecular Pharmacology

Professor	Masaki Noda, M.D., Ph.D.
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Assistant Professor	Tadayoshi Hayata, Ph.D.
GCOE Research Instructor	Takuya Notomi, Ph.D.
GCOE International Coordinator	Tetsuya Nakamoto, M.D., Ph.D.

Research Summary

In order to contribute to the establishment of therapy and prevention for osteoporosis and the other calcium-related disorders, we are elucidating molecular mechanisms underlying regulation of calcium metabolism with emphases on bone formation and resorption. Skeletal system is a 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. Angiotensin II type 2 receptor blockade increases bone mass (Izu Y, Hayata T, Ezura Y, Noda M).

Renin angiotensin system (RAS) regulates circulating blood volume and blood pressure systemically, whereas RAS also plays a role in the local milieu. In this study, we examined the presence of RAS components in adult bone and the effects of angiotensin II type 2 (AT2) receptor blocker on bone mass. Immunohistochemistry revealed that AT2 receptor protein was expressed in both osteoblasts and osteoclasts. In addition, renin and angiotensin II-converting enzyme were expressed in bone cells in vivo. Treatment with AT2 receptor blocker significantly enhanced the levels of bone mass, and this effect was based on the enhancement of osteoblastic activity as well as the suppression of osteoclastic activity in vivo. These results indicate that RAS components are present in adult bone and that blockade of AT2 receptor results in alteration in bone mass (J Biol Chem, 2009).

2. Osteoclast-specific Dicer gene deficiency suppresses osteoclastic bone resorption (Mizoguchi F, Hayata T, Ezura Y, Noda M).

MicroRNAs are produced by Dicer cleavage an emerging regulatory system for cell and tissue function. Here, we examine the effects of Dicer deficiency in osteoclasts on bone mass in vivo. We specifically knocked out Dicer in osteoclasts by crossing Dicer flox mice with cathepsin K-Cre knock-in mice. Dicer deficiency in osteoclasts decreased the number of osteoclasts and osteoclast surface, and suppressed the levels of TRAP positive multinucleated cell development in culture and also reduced NFATc1 and TRAP gene expression. Dicer deficiency in osteoclasts suppressed osteoblastic activity and also suppressed expression of genes encoding type I collagen, osteocalcin, Runx2, and Efnb2 in vivo. Dicer deficiency in osteoclasts increased the levels of bone mass indicating that the Dicer deficiency-induced osteoclastic suppression was dominant over Dicer deficiency-induced osteoblastic suppression. These results indicate that Dicer in osteoclasts controls activity of bone resorption in vivo (J Cell Biochem, in press).

3. Methylation status of CpG islands in the promoter regions of signature genes during chondrogenesis of human synovium-derived mesenchymal stem cells. (Ezura Y & Noda M).

The purpose of this study was to investigate the CpG methylation status in human synovium-derived MSCs during experimental chondrogenesis. The methylation status of 12 regions in the promoters of 10 candidate genes was analyzed. 10 of the 11 CpG-rich regions analyzed were hypomethylated in human progenitor cells before and after 3 weeks of pellet culture, regardless of the expression levels of the genes. The methylation status was consistently low in SOX9, RUNX2, CHM1, CHAD, and FGFR3 following an increase in expression upon differentiation and was low in GREM1 and GPR39 following a decrease in expression upon chondrogenesis. The hypermethylation status of a 1-kb upstream sequence of SDF1 was reduced after 3 weeks of pellet culture. This study is the first to perform DNA methylation analyses in MSCs culture by in vitro chondrogenic assay (Arthritis Rheum, 2009).

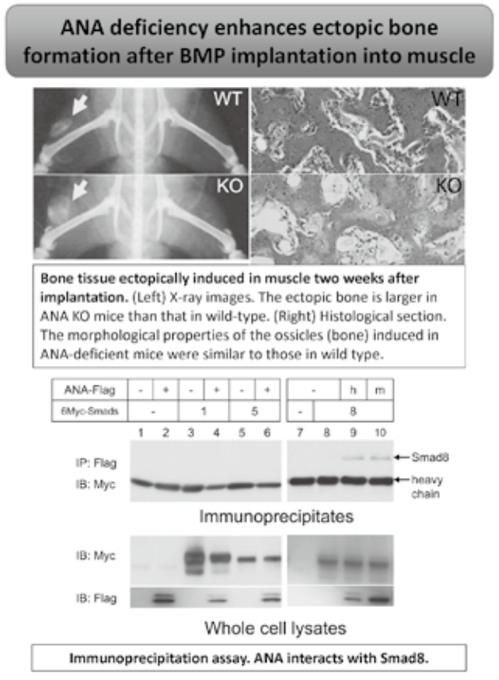
4. Osteoblastic bone formation is induced by using nanogel-crosslinking hydrogel as novel scaffold for bone growth factor (Hayashi C, Hayata T, Ezura Y, Noda M).

We examined the efficiency of nanogel-crosslinking hydrogel as a novel synthetic scaffold for BMP to stimulate osteoblasts to induce bone formation. Cholesterol-bearing pullulan nanogel-crosslinking hydrogel (CHPA/Hydrogel) was used to deliver BMP. The CHPA hydrogel pellets were implanted in vivo. Single implantation of CHPA/hydrogel containing low amounts of BMP induced osteoblastic activation and new bone formation in vivo. Furthermore, nanogel in a disc shape established recruitment of osteoblastic cells that vigorously formed bone to heal the calvarial defects, which did not heal spontaneously without it. In conclusion, CHPA/hydrogel serves as an efficient and versatile scaffold for the stimulation of osteoblasts to form bone and to repair defects via delivery of BMP (J Cell Physiol, 2009).

Highlight

ANA deficiency enhances bone morphogenetic protein-induced ectopic bone formation via transcriptional events. (Miyai K, Hayata T, Ezura Y, Noda M).

ANA is an antiproliferative molecule that belongs to Tob/BTG family, but its activity in bone metabolism has not been known. In ANA-deficient and wild-type mice, BMP2 was implanted to induce ectopic bone formation in muscle. ANA deficiency increased mass of newly formed bone in vivo compared with wild-type based on 3D-muCT analyses. Overexpression of ANA suppressed BMP-induced expression of luciferase reporter gene linked to BMP response elements in these cells. Conversely, ANA mRNA knockdown by small interference RNA enhanced the BMP-dependent BMP response element reporter expression. It also enhanced BMP-induced osteoblastic differentiation in muscle-derived C2C12 cells. Immunoprecipitation assay indicated that ANA interacts with Smad8. Thus, ANA is a suppressor of ectopic bone formation induced by BMP, and this inhibitory ANA activity is a part of the negative feedback regulation of BMP function (J Biol Chem, 2009).



Bone tissue ectopically induced in muscle two weeks after implantation. (Left) X-ray images. The ectopic bone is larger in ANA KO mice than that in wild-type. (Right) Histological section. The morphological properties of the ossicles (bone) induced in ANA-deficient mice were similar to those in wild type.

Immunoprecipitation assay. ANA interacts with Smad8.

Publications

[Original articles]

1. Miyai K, Yoneda M, Hasegawa U, Toita S, Izu Y, Hemmi H, Hayata T, Ezura Y, Mizutani S, Miyazono K, Akiyoshi K, Yamamoto T, Noda M. ANA deficiency enhances bone morphogenetic protein-induced ectopic bone formation via transcriptional events. J Biol Chem. 284:10593-600, 2009.
2. Izu Y, Mizoguchi F, Kawamata A, Hayata T, Nakamoto T, Nakashima K, Inagami T, Ezura Y, Noda M. Angiotensin II type 2 receptor blockade increases bone mass. J Biol Chem. 284:4857-64, 2009.
3. Ezura Y, Sekiya I, Koga H, Muneta T, Noda M. Methylation status of CpG islands in the promoter regions of signature genes during chondrogenesis of human synovium-derived mesenchymal stem cells. Arthritis Rheum. 60:1416-26, 2009.
4. Mizoguchi F, Izu Y, Hayata T, Hemmi H, Nakashima K, Nakamura T, Kato S, Miyasaka N, Ezura Y, Noda M. Osteoclast-specific Dicer gene deficiency suppresses osteoclastic bone resorption. J Cell Biochem (in press).
5. Hemmi H, Idoyaga J, Suda K, Suda N, Kennedy K, Noda M, Aderem A, Steinman RM. A new triggering receptor expressed on myeloid cells (Trem) family member, Trem-like 4, binds to dead cells and is a DNAX activation protein 12-linked marker for subsets of mouse macrophages and dendritic cells. J Immunol. 182:1278-86, 2009.
6. Hayashi C, Hasegawa U, Saita Y, Hemmi H, Hayata T, Nakashima K, Ezura Y, Amagasa T, Akiyoshi K, Noda M. Osteoblastic bone formation is induced by using nanogel-crosslinking hydrogel as novel scaffold for bone growth factor. J Cell Physiol. 220:1-7, 2009.
7. Saita Y, Nakamura T, Mizoguchi F, Nakashima K, Hemmi H, Hayata T, Ezura Y, Kurosawa H, Kato S, Noda M. Combinatory effects of androgen receptor deficiency and hind limb unloading on bone. Horm Metab Res. 41:822-8, 2009.

Division of Advanced Molecular Medicine
Department of Molecular Cell Biology

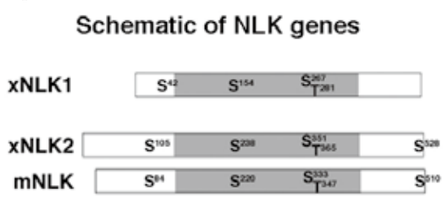
Professor Hiroshi Shibuya
Associate Professor Toshiyasu Goto
Assistant Professor Mi-sun Kim

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.

p38-NLK signaling regulates anterior formation in *Xenopus* development

Our previous studies have shown that NLK is involved in forebrain development and neural differentiation in *Xenopus*. However, there is no information about direct upstream regulators of NLK that may function in these processes. To explore potential regulators of NLK function, we initially performed a high-throughput analysis of proteins that co-immunoprecipitated with FLAG-tagged NLK in 293 cells using direct nanoflow liquid chromatography-coupled tandem MS. We identified a MAPK, p38 as a candidate protein that may physically interact with NLK. There are two NLK species in *Xenopus*: NLK1 and NLK2. Three of four putative p38-phosphorylation motifs, Ser-Pro or Thr-Pro, are conserved between these two NLKs. The putative phosphorylation site of NLK2 in the C-terminus is conserved among many different species including mouse and human. *in vitro* kinase assay using bacterially expressed GST-NLK indicated that p38 directly phosphorylates the specific Ser residue of NLK (Ser510 in mouse NLK, Ser42 in *Xenopus* NLK1 and Ser528 in *Xenopus* NLK2) in a kinase activity-dependent manner. p38 regulates the function of NLK via phosphorylation, and this modification can be abrogated by depletion of endogenous p38.

Schematic of NLK genes



In *Xenopus* embryos, p38 expression in the head region was detected during neural stages, and parallels expression of NLKs. Depletion of either p38 or NLK by antisense morpholino oligonucleotides results in a severe defect in anterior development and impaired expression of endogenous anterior markers. It is notable that morphants of *Xenopus* p38α another isoform of the p38 MAPK family, exhibited no obvious defects in anterior development. Defects in head formation or expression of anterior marker genes caused by suppression of endogenous p38β expression could be rescued by expression of wild-type NLK, but not mutant NLK lacking the p38β phosphorylation site. In contrast, defects in head formation or expression of anterior marker genes caused by suppression of endogenous NLK expression could not be rescued by expression of p38. These results provide the first evidence that p38 is an upstream kinase and activator of NLK, and specifically regulates NLK function required for the anterior formation in *Xenopus* development.

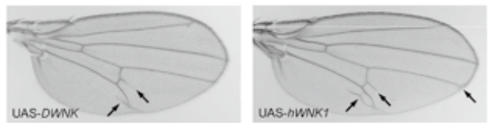
WNK protein kinases, the causative genes of pseudohypoaldosteronism type II (PHAII) disease

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

1. Evolutional conservation of WNK signaling pathway

We generated the over-expression system of human WNK, mouse OSR1 and *Drosophila* OSR1 homologue Fray, as well as Drosophila WNK (DWNK). When these were over-expressed at the posterior compartment of wing by *hh-Gal4,* each over-expression caused similar phenotypes such as ectopic wing veins and delta phenotype of the tip of vein 4. These results suggest that WNK pathway is conserved among many species. In addition, since OSR1/Fray are kinases and both over-expression phenotypes are similar, the substrate of the OSR1/Fray, the downstream target, may be also conserved.

Evolutional conservation of WNK pathway



2. The downstream transcription factor

The mosaic analysis using *DWNK* mutant showed the defect of abdominal development. The overexpression of the kinase dead form of DWNK, which might work as a dominant negative, also showed the similar phenotype. These phenotypes are similar to those of a mutant of gene X, which encodes the transcription factor. These data suggest that the transcription factor X and WNK are genetically involved. When we simultaneously overexpressed the kinase dead form of DWNK and the transcription factor X, the defect of abdominal development was rescued. This result indicates that the transcription factor X works at the downstream of WNK signaling pathway.

The transctiption factor X may work at the downstream of WNK signaling.

The homologues of gene X are conserved in mammals. In mouse, gene X is known to be involved in the development of the palate and the nervous system. Since the pathological conditions of PHAII showed a dental abnormalities and a mental retardation, these may suggest that this gene X is involved in the pathogenesis of PHAII. We analyzed the relationship between WNK and the transcription factor X using the cell culture system. WNK is known to be activated by the hypertonic condition. In NIH3T3 cells, the transcription of the transcription factor X was activated by the hypertonic condition. And when we knocked down both *WNK1* and *WNK4* transcripts by siRNA, the transcription of the transcription factor X was not activated under same hypertonic condition. These results suggest that the transcription factor X is the new downstream target of WNK signaling pathway.

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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.

Glaucoma, one of the leading causes of irreversible blindness, is characterized by progressive degeneration of retinal ganglion cells (RGCs) and optic nerves. Although glaucoma is often associated with elevated intraocular pressure, recent studies have shown a relatively high prevalence of normal tension glaucoma (NTG) in glaucoma patient populations. In the mammalian retina, glutamate/aspartate transporter (GLAST) is localized to Müller glial cells, whereas excitatory amino acid carrier 1 (EAAC1) is expressed in neural cells, including RGCs. Since the loss of GLAST or EAAC1 leads to retinal degeneration similar to that seen in NTG, we examined the effects of interleukin-1 (IL-1) on RGC death in GLAST- and EAAC1-deficient mice. IL-1 promoted increased glutamate uptake in Müller cells by suppressing intracellular Na^+ accumulation, which is necessary to counteract Na^+-glutamate cotransport. The observed trends for the glutamate uptake increase in the wild-type (WT), GLAST- and EAAC1-deficient mice were similar; however, the baseline glutamate uptake and intracellular Na^+ concentration in the GLAST-deficient mice were significantly lower than those in the wild-type mice. Consistently, pretreatment with IL-1 exhibited no beneficial effects on glutamate-induced RGC degeneration in the GLAST-deficient mice. In contrast, IL-1 significantly increased glutamate uptake by Müller cells and the number of surviving RGCs in the wild-type and EAAC1-deficient mice. Our findings suggest that the use of IL-1 for enhancing the function of glutamate transporters may be useful for neuroprotection in retinal degenerative disorders including NTG.

2. Role of Notch-dependent and –independent RBP-J signaling pathway in the cerebellar development

It is well known how to promote differentiation of neural stem cells to neural progenitors, but how to promote differentiation of neural progenitors is largely unknown. We found that basket and stellate cells were nearly absent in the RBP-J-deficient cerebellum. The basket and stellate cells arise from small Pax2-positive neural progenitors. Recently, it is suggested that there may exist proliferating Pax2-negative progenitors contributing to the generation of small Pax2-positive neural progenitors. In this study, we identified that Sox2 expressing Pax2-negative neural progenitor is the Pax2-negative neural progenitor and we revealed that RBP-J plays a crucial role in promoting cell differentiation from Sox2-positive/Pax2-negative neural progenitors to small Pax2-positive neural progenitors without activation of Notch. Furthermore, we disclosed that Ptf1a might not be involved in generation of these cells. Taken together, we found a novel RBP-J function to promote differentiation of neural progenitor to more mature progenitors.

3. Function of glutamate transporters in skeletal muscle development

The amino acid glutamate plays significant roles in a number of fundamental metabolic pathways including protein synthesis, energy production, glutathione synthesis (anti-oxidant molecule) and neurotransmitter. Glutamate is delivered to cells primarily by glutamate transporters, which actively take it up from the circulation. However, except for the neural transmission, few studies as yet have focused on the specific role played by glutamate metabolisms.

Recently we found glutamate transporters GLAST and GLT1 were transiently expressed in embryonic somite which is known as the origin of many peripheral tissues. By using mice which can label GLAST-expressing cells, we found these cells especially contribute to skeletal muscle, and dermis, bone and cartilage.

Further, we inactivated these glutamate transporters in order to elucidate the function of glutamate metabolism in somite-derived peripheral tissues. Because normal development of skeletal muscle is observed in mutant mice lacking GLAST or GLT1, we generated double-mutant mice lacking both GLAST and GLT1 (GLAST(-/-)&GLT1(-/-) mice). GLAST(-/-)&GLT1(-/-) embryos are lethal around embryonic day 16.5 and show a severe reduction of skeletal muscle. In mutant skeletal muscle, the second phase of myoblast fusion, which is the step of myoblast fusion that forms mature myotubes, is perturbed, leading to the elimination of unfused myoblasts and immature myotubes by apoptosis. These data suggest that glutamate transporters promote second phase of myoblast fusion by regulating the cellular glutamate metabolisms via glutamate provision. This is the first report providing direct in vivo evidence of the function of glutamate transport in peripheral tissue. These findings shed light on important functions of glutamate transporters, not only as regulators of the extracellular glutamate concentration but also as providers of intracellular glutamate.

Disturbances in muscular glutamate metabolism are consistently reported in catabolic conditions known as cachexia related to chronic or acute diseases accompanied by skeletal muscle wasting. In fact, reduced skeletal muscle glutamate uptake and disrupted glutamate metabolisms are reported in cachexia patients. Further, besides being crucial to embryonic muscle development, myoblast fusion and maturation are obligatory events for postnatal muscle hypertrophy as well as the regenerative responses of muscle tissue to injury. Thus, glutamate transporters may be an attractive therapeutic target in intractable diseases accompanied by skeletal muscle wasting.

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Our research projects focus on biodefense and maintenance of immunological homeostasis. Our goal is to define the molecular mechanism of immune cell differentiation and activation under healthy conditions as well as conditions of disease. To accomplish this goal, we are trying to clarify the molecular basis of induction and failure of immunological tolerance by focusing on dendritic cells and mucosa-associated lymphoid tissues. 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. Mechanism of tolerance induction and its failure in the gut-associated lymphoid tissues

Dendritic cells (DCs), composed of plasmacytoid DCs (pDCs) and conventional DCs (cDCs), are representative antigen presenting cells (APCs) and play integral roles in balancing tolerance to self-Ags and immunity to pathogens in peripheral lymphoid tissues. In the intestine, DCs are requested to keep the balance even more sharply such that DCs should be tolerogenic in the presence of numerous commensal bacteria while retain the capacity to respond to episodic pathogens (**Immunol Rev** 234, 247-258(2010)). We have recently found that pDCs play a critical role in mucosal T cell-independent (TI) IgA production. When cultured with naïve B cells to induce TI IgA CSR, mucosal pDCs are capable of inducing IgA production at substantially higher levels compared with mucosal cDCs and non-mucosal pDCs, which was caused by the predominant expression of APRIL and BAFF, critical regulators for TI IgA CSR. Detailed analysis are currently in progress to further identify the molecular basis of mucosal pDC-mediated IgA production.

2. Differentiation and homeostasis of dendritic cells

DCs are divided into two major subsets. It is currently accepted that pDCs, characterized by a capacity of high type I IFN production, and cDCs in lymphoid tissues are continuously regenerated from hematopoietic stem cells through the macrophage and DC precursor (MDP) and common DC precursor (CDP), the latter is a DC-restricted developmental intermediate. Interestingly, we have most recently succeeded to identify another DC-restricted developmental intermediate with a strong capacity for pDC differentiation. Consistent with the potential, the newly identified DC pecursors express elevated level of E2-2 and IRF8, critical transcription factors for pDC differentiation and function. We are currently analyzing the details of this DC precursors.

3. Regulation of hematopoiesis by immune system

Hematopoietic stem cells (HSCs) are pluripotent cells with the capacity for the life-long production of the entire lineage of mature hematopoietic cells. Under steady-state conditions, HSCs are present at 1~3 cells in 10^5 BM cells, and most HSCs are quiescent residents of the BM niche, a state that preserves their capacity to self-renew (Fig. 1). Serial BM transfers supports at most 4 to 5 rounds of reconstituted hematopoiesis in irradiated recipients, suggesting that excessive replicative stress results in HSC exhaustion.

Type I interferons (IFNs), a family of cytokines, are produced by mammalian cells and orchestrate numerous biological and cellular processes. Although it is well known that type I IFNs are essential for establishing the host antiviral state, their role in hematopoietic homeostasis remains unstudied. Interestingly, type I IFN receptor and interferon regulatory factor-2 (IRF2), a transcriptional suppressor of type I IFN signaling, are both expressed in dormant HSCs. We found that type I IFNs induce proliferation and exhaustion in hematopoietic stem cells (HSCs), and that IRF2 preserves the self-renewal and multi-lineage differentiation capacity of HSCs (**Nat Med** 15, 696-700 (2009)). The number of HSCs is substantially decreased in the bone marrow (BM) of *Irf2*$^{-/-}$ mice. *Irf2*$^{-/-}$ HSCs that show enhanced cell-cycling status fail to produce hematopoietic cells in competitive repopulation assays, and the reconstituting capacity of *Irf2*$^{-/-}$ HSCs are restored by the additional loss of type-I IFN signaling. Consistent with these findings, in wild-type (WT) mice, injection of poly I:C or IFN-α induces proliferation in HSCs, and chronic type I IFN-signaling further induces a reduction in quiescent HSCs (Fig. 2).

Our findings may offer a molecular basis for the maintenance of HSC quiescence and lead to improvements for BM-transplantation and type-I IFN-based therapies for viral infections and cancer. In particular, chronic myeloid leukemia (CML), caused by a chromosomal translocation called Philadelphia chromosome at a HSC level, is an attractive target of type-I IFN-based therapies (Fig. 3). Recent findings suggested the presence of leukemia-initiating cells (LICs), which are similar to HSCs in terms of a self-renewal capacity, pluripotency, and quiescence. Due to the similarities, LICs are resistant to anti-cancer drugs that target proliferating cells and cause a recurrence of the disease. Type-I IFNs might be able to make LICs sensitive to anti-cancer drugs by inducing their proliferation and lead to the extermination of CML.

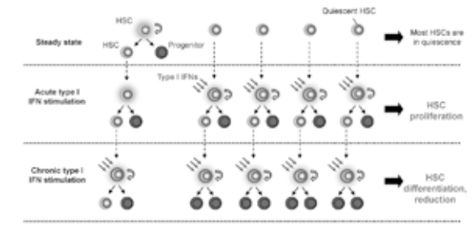

Fig.2 Mechanism of type I IFN action on HSC
Transient type I IFN signaling induces HSC proliferation, whereas chronic type I IFN signaling leads to HSC exhaustion.

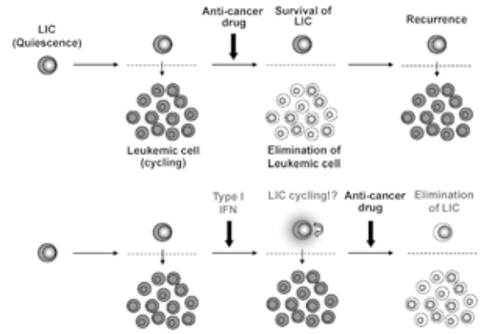

Fig.3 Type I IFN-based therapies targeting the eradication of LIC
Type-I IFNs might be able to induce LIC proliferation and lead to the extermination of CML.

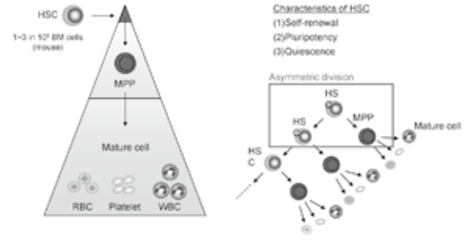

Fig.1 HSC maintains hematopoiesis during a lifetime
While most HSCs are in a quiescent state, a part of HSCs are mildly cycling, which generate both HSC and progenitor cell called "Asymmetric division".

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Department of Bio-informational Pharmacology

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This laboratory focuses on understanding fundamental pathophysiological roles of ion channels and transporters in cardiovascular system. We employ multidisciplinary approach (patch-clamp, cell biology, optical imaging, genetic analysis and computational analysis) in order to seek novel regulatory mechanisms and modulatory molecules/compounds of ion channels and transporters in cardiac myocytes, vascular smooth muscle and endothelial cells, and circulating cells in vessels (monocytes and macrophages). Our ultimate goal is to discover novel diagnostic and therapeutic strategy for intractable and common cardiovascular diseases.

1. Gender-specific medicine (GSM) for cardiac arrhythmias

a. Sex hormone receptors responsible for the non-genomic pathway (Furukawa T)

Despite intensive researches, sex hormone receptors responsible for the non-genomic pathway are still controversial. In cardiac myocytes, we show that N-terminal truncated form of sex hormone receptors co-localizes with signaling molecules for non-genomic pathway in lipid raft/caveola fraction. Thus, in reconstituted system we characterized full-length androgen receptor (fAR) and N-terminal truncated AR (AR45). Testosterone induced translocation of fAR, but not of AR45, into the nucleus and transcription of genes containing androgen responsive element (ARE). On the other hand, testosterone induced phosphorylation of ERK, a down-stream signal of the non-genomic pathway in AR45-transfected cells, but not in fAR-transfected cells. In sucrose gradient fractionation experiments, both fAR and AR45 locate in the plasma membrane; fAR migrates into the high density fraction, while AR45 migrates into the lipid raft/caveola fraction. Anti-AR antibody detected 2 positive bands for AR45. The high molecular band was abolished by treatment with a palmitoylation inhibitor, 2 bromo-palmitate, and the location of AR45 shifted from the lipid raft/caveola fraction to the high density fraction. Thus, palmitoylation makes AR45 being localized to the lipid raft/caveola fraction, and acting for the non-genomic pathway.

b. Spatial integration of signaling molecules for the non-genomic pathway (Kurokawa J, Kurobane E, Asayama M, Furukawa T)

Many signal molecules are coordinated in a spatio-temporal-dependent manner to exhibit the non-genomic action. In the non-genomic pathway, nitric oxide (NO) inhibited L-type Ca^{2+} channels, only when they are activated by cAMP. Pharmacological experiments suggest that cGMP-dependent phosphodiesterase (PDE) induced hydrolysis of cAMP to antagonize cAMP-induced enhancement of L-type Ca^{2+} channels. Among cGMP-dependent PDE (PDE2 and PDE3), PDE2 is responsible for this action. Then, we examined the special coordination of various types of PDE and the α-subunit of the L-type Ca^{2+} channel, $Ca_V1.2$ using sucrose density gradient fractionation and PLA (proximity ligation assay). In sucrose density gradient fractionation experiments, PDE3 migrated into the higher fraction, while PDE2 migrated into the fraction 5, which is the lipid raft/caveola fraction in our experiments. $Ca_V1.2$ migrated both in the lipid raft/caveola fraction and the higher density fraction. PLA assay showed that both PDE2 and PDE3 interacted with $Ca_V1.2$ within 50 nm proximity, but PDE2 and PDE3 did not. These data indicate that the $Ca_V1.2$ has at least 2 types of fraction; one co-localized with PDE2 and the other with PDE3. Those co-localized with PDE2 in the lipid raft/caveola fraction act for the non-genomic pathway of sex steroid hormones.

2. Atrial fibrillation

a. AF associated gene polymorphism

Recent clinical data indicate the presence of genetic background for development of AF. In collaboration with Dr. Nakamura Y in The Institute of Medical Science, The University of Tokyo, and Tanaka T. in RIKEN, we performed GWAS (genome-wide association study) to search for SNPs associated with AF. In the 1st project, we performed typing of 230,000 Tag SNPs, and found 9 SNPs statistically significantly associated with AF after the Bonferroni's correction, and 10 SNPs with borderline significance (p-value; 10^{-5} – 10^{-8}). In the 2nd project, we performed typing of 610,000 Tag SNPs, and found 9 SNPs statistically significantly associated with AF after the Bonferroni's correction, and 82 SNPs with borderline significance (p-value; 10^{-5} – 10^{-8}). Nine significant SNPs in the 1st project, and 9 SNPs in the 2nd project are in the same chromosome region. Thus, we identified SNPs associated with AF in Japanese population.

b. Inflammatory and immunological mechanism in AF pathogenesis

Inflammatory and immunological mechanism has been implicated in the development of AF. In fibrillated atrium, inflammatory cells, such as T-lymphocytes, macrophages, and mast cells, invaded. We examined the mechanism of macrophage invasion induced by atrial stretch, which is the most consistently found macroscopic phenomenon in AF. In Boyden chamber, in which atrial myocytes are cultured in the lower chamber and macrophages in the upper chamber, stretch of atrial myocytes by 20% induced migration of macrophages into the lower chamber, suggesting a role of some humoral factor. Among several candidate humoral factors, we found that ATP is responsible because in the presence of ATP secretion inhibitor, ATP hydrolyzing enzyme or ATP receptor inhibitor, stretch-induced migration of macrophages was abolished (Fig. 1). Thus, ATP is an autocrine/paracrine factor causing migration of macrophages in response to atrial stretch.

3. Ventricular tachyarrhythmias and sudden cardiac death

a. Analysis of *NOS1AP* KO mice

NOS1AP was found to be most closely related to the QT interval and sudden cardiac death in European and American GWAS. Infection of adenovirus carrying NOS1AP to guinea-pig ventricular myocytes suppressed L-type Ca^{2+} channels via NOS1-dependent manner. We used *NOS1AP* KO mice to examine its role in *in vivo*. *NOS1AP*$^{-/-}$ mice showed prolonged QT interval, which was abolished by a NOS1 inhibitor, L-NAME. In optical mapping system under Langendorff perfused hearts, APD was prolonged and electrical stimulation more easily induced conduction block and ventricular tachyarrhythmias. Thus, in *in vivo NOS1AP* is related to the QT interval and arrhythmia development in a NOS1-dependent manner.

b. Development and analysis of Brugada syndrome mouse model

Brugada syndrome is a familiar sudden death syndrome found frequently in East Asia; it causes nocturnal sudden death in the prime of manhood, and is referred as "pokkuri disease" in Japan. Furthermore, because of the lack of suitable animal model, the underlying pathogenesis is poorly understood. We found that homozygous and heterozygous deletion of a gene regulating SCN5A expression induced mice, whose phenotypes are closed related to Brugada syndrome. Brugada syndrome is characterized by a unique ECG including bundle branch block and ST elevation. This KO mouse also showed unique ECG finding including bundle branch block and ST elevation. Electrical stimulation induced both atrial and ventricular fibrillation. Thus, we successfully developed mouse model for Brugada syndrome.

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Technical Assistant	**Rie TAGUCHI, Ahmed RAMADAN, Yuhei YAMAGUCHI, Yasuhiro KOKUBU**

Our research is aimed to elucidate mechanisms by which multicellular organs, in particular the central nervous and hematopoietic systems, are developed. We have mostly focused on molecular regulation of neural stem cells and hematopoietic stem cells in view of cell-external cues such as cytokines as well as cell-intrinsic programs including chromatin modification. These projects have been performed, for instance by analyzing cross-interactions of transcriptional regulatory signaling pathways, which lead to spatio-temporally coordinated gene expression. Our major research subjects are as follows:

1) Molecular basis for the maintenance of neural stem cells
2) Regulation of the neural stem cell fate
3) Characterization of hematopoietic stem cells in fetal hematopoietic organs
4) Characterization of cancer stem cells
5) Epigenetic regulation of neural development

1. Regulation of neural stem cell self renewal

Fibroblast growth factor 2 (FGF2) is used widely to generate neurospheres and expand neural precursor cells in basic and translational research. We previously reported that FGF2 induced cortical neural precursor cells to express Olig2 transcription factor, which is essential for oligodendrocyte formation. FGF2-treated cortical precursors were adapted for generating numerous O4-positive oligodendrocytes but not GABA neurons (Abematsu et al. J Neurosci Res 2006; 83 731-743). Another significant aspect of FGF2 signaling in neural precursor cells is to promote their proliferation and maintain them in an undifferentiated state. Similarly, Wnt signaling promotes the proliferation of neural precursor cells and inhibits their differentiation (Shimizu et al. Dev Biol 2005; 282 397-410). Thus, we explore whether any of intracellular components common to the FGF2 and Wnt signaling pathways are involved in both the promotion of proliferation and the inhibition of differentiation. We demonstrate that FGF2 signaling through phosphatidylinositol 3-kinase activation inactivates glycogen synthase kinase 3 beta (GSK3beta) and leads to the accumulation of beta-catenin in a manner different from that in the Wnt canonical pathway. The nuclear accumulated beta-catenin leads to cell proliferation by activating LEF/TCF transcription factors and concurrently potentiates the Notch1 signaling pathway, which inhibits both neuron and oligodendrocyte differentiation. Of note, beta-catenin and the Notch1 intracellular domain form a molecular complex with the promoter region of the *hes1* gene, allowing its expression. Thus, the GSK3beta/beta-catenin signaling axis regulated by FGF2 and Wnt signals plays a pivotal role in the maintenance of neural stem/precursor cells by linking the cell proliferation to the inhibition of differentiation, providing new insights into the emerging research field of regenerative medicine.

2. Inhibition of astrogliogenesis by a signaling pathway that induces neural stem cell growth

Proliferation of neural stem/progenitor cells (NSCs/NPCs) is intimately linked to the inhibition of neural and glial differentiation. However, the molecular link between induction of cell cycle progression and inhibition of cell differentiation in NSCs/NPCs has been poorly understood. We recently reported that FGF2 and Wnts promoted the proliferation of NSCs/NPCs through the common signaling pathway, GSK3beta, beta-catenin, LEF/TCFs, and cyclin D1 (Shimizu, T. et al., MCB 2008). Astrocyte differentiation from NSCs/NPCs is synergistically induced by combination of LIF and BMP2, which activate transcriptional factors, STAT3 and Smad1 (Nakashima, K et al. Science 1999), respectively. We here found that the cells expressing cyclin D1, a common target for FGF2 and Wnt signaling, rarely co-expressed GFAP, an astrocyte marker. Since a recent study using a hepatoma cell line demonstrated that cyclin D1, a key regulator of G1 to S phase progression of the cell cycle, repressed LIF-induced transactivation of STAT3 (Bienvenu F, et al. J Biol Chem 2001), we hypothesized that cyclin D1 suppressed astrocyte differentiation via inactivation of STAT3-mediated transcription of astrocyte-specific genes. Forced expression of cyclin D1 in NSCs/NPCs cultured with LIF and BMP2 decreased the number of GFAP expressing astrocytes by approximately 50%. Forced expression of cyclin D1 to some extent increase the number of neurspheres and their sphere-sizes. CDK4 inhibitor hardly recovered the GFAP expressing cells in number, indicating that cyclin D1 inhibited astrocyte differentiation in a manner independent of cell cycle. Co-immunoprecipitation assay demonstrated that cyclin D1 could directly bind to STAT3 and p300. Cyclin D1 suppressed GFAP promoter activation induced by LIF and BMP2. These data suggested that cyclin D1 inhibited astrocyte differentiation by disturbing STAT3/p300-mediated transcription of astrocyte-specific genes.

3. Characterization of tumor stem cells

Tumor stem cells (TSCs) are primarily responsible for tumor maintenance and relapse, and thus considered as a potential target to eradicate tumors. C6 rat glioma cell line also contains a sub-population of TSCs, which is enriched using the Hoechst 33342 side population (SP) technique. SP in C6 is tumorigenic, but a majority of main population (MP) is not. Recently, it has been proposed that disrupting "tumor stem cell niche" could impair TSC self-renewal and thereby significantly inhibits the tumor growth. In this study, we show that MP could function as a microenvironment to support SP. Consistent with previous studies, cultures initiated with only SP cells exhibited more than 80% of MP frequency after 1 week, but thereafter the proportion was retained for at least 3 weeks, suggesting that the existence of MP cells may be helpful to the maintenance of SP cells. To confirm the positive effect of MP cells on SP maintenance, we co-cultured SP cells with different proportions of MP cells, and observed a marked maintenance of SP in a MP-dependent manner. To further determine whether the direct interaction with MP cells is required for SP maintenance, we co-cultured SP cells with pre-fixed MP cells, and detected highly maintained SP, indicating that SP maintenance is mediated by direct contact with cell surface molecules in MP cells. cDNA microarray analysis identified the upregulation of molecules in MP, which are known to function in cell communication. Our findings suggest that TSC-derived non-tumorigenic progeny could be a specific candidate for TSC-targeting therapy.

4. Characterization of hematopoietic stem cells in placenta

The discovery of a major hematopoietic stem cell (HSC) pool in midgestation mouse embryo has defined the placenta as an important anatomical site that participates in HSC development. In this study, we examined the flow cytometric pattern of mouse placenta cells on embryonic days (E) 10.5 to E15.5, in view of CD45 and c-Kit expression. We also determined which population of these cells has the highest hematopoietic property and exhibits differentiation potential towards multiple lineages by performing (1) co-culture with OP9 stromal cells and (2) colony forming assay in methylcellulose. When co-cultured with OP9 stromal cells and cultured in methyl-cellulose, only CD45+c-Kit+ population showed the ability to form hematopoietic colonies including multiple lineages. To distinguish which fraction of placenta cells (the fetal part or the maternal part) have the hematopoietic activity, we used placentas from heterogeneous crossing of GFP transgenic male and non transgenic female mice in which the fetal part of placenta is GFP positive (GFP+) and the maternal part is GFP negative (GFP-). E11.5 and E13.5 CD45+c-Kit+ placenta cells that have ability to form hematopoietic colonies are the fetal GFP positive (GFP+) placenta cells. E11.5 and E13.5 CD45+c-Kit+ placenta cells that have an ability to form hematopoietic colonies mainly reside in Hoechst dye-effluxing side population area (SP) when co-cultured with OP9 stromal cells and cultured in methyl-cellulose. Taken together, in the placenta of mouse embryo, we conclude that, SP cells in the $CD45^{+}c\text{-}Kit^{+}$ fetal placental cells have the ability to form hematopoietic colonies.

Publications

[Original Article]

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2. Namihira M, Kohyama J, Semi K, Sanosaka T, Deneen B, Taga T, Nakashima K. Committed neuronal precursors confer astrocytic potential on residual neural precursor cells. Dev Cell 16:245-255, 2009.

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】

- Identification of a novel candidate drug for treatment of PQBP1-linked mental retardation
- Identification of a DNA repair protein, Ku70 as a novel mediator of Huntington's disease pathology

【Pathological Biochemistry】

- Establishment of an method for ***ex utero*** transplantation of hepatic progenitor cells into the mouse fetal liver and successful application to ES cell-derived hepatoblasts
- Proteasome inhibition suppresses DNA-dependent protein kinase activation caused by a DNA-damaging agent, camptothecin

【Pathological Cell Biology】

- Discovery of Atg5/Atg7-independent alternative macroautophagy.
- Involvement of JNK in regulation of autophagic cell death.

【Developmental and Regenerative Biology】

- Discovery of a molecular mechanism of liver specification using medaka.
- Generation of a novel and accurate model for human nonalcoholic steatohepatitis using medaka.

【Stem Cell Bilogyy】

- Discovery of a mechanism of hair graying by genotoxic stress.
- Identification of an essential signaling pathway for melanocyte stem cell maintenance.

【Immunology】

- Elucidation of mechanisms for augmented immune responses of IgG-positive memory B lymphocytes.
- Development of synthetic sialosides that binds to CD22 and regulates B lymphocyte activation.

【Molecular Pathogenesis】

- Identification of phosphoglucomutase-1 as a binding protein to ZASP/Cypher and loss of their binding due to ZASP/Cypher mutations causing dilated cardiomyopathy.
- Identification or rare TRIM5alpha variants, G110R and G176del, associated with susceptibility and resistance, respectively, to HIV-1 infection and demonstration of their functional changes.

【Virus Research Unit】

- Development of new drugs with EBV related diseases by the chronic EBV infection animal model using the NOG mice reconstituted with human cord blood-derived hematopoietic stem cells.
- Development of an exhaustive and quantitative pathogen microbes screening system capable of screening dozens of virus, bacteria and protozoa simultaneously.

Department of Neuropathology

Professor	Hitoshi Okazawa
Associate professor	Yasushi Enokido
MTT fellow	Masaki Sone
Assistant professor	Takuya Tamura
Assistant professor	Akihiko Komuro
Research scientist	Shigeki Marubuchi
Graduate students	Hikaru Ito, Hiroki Shiwaku, Chan Li, Min Xu, Yoshie Yuki, Zi-Hyang Chin, Risa Shiraishi
Research trainees	Sainawer Maimaiti,

Research contents

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

Research Projects

1) Impairment of DNA-repair in polyglutamine diseases

Polyglutamine diseases are hereditary neurodegenerative disorders whose population is the 3rd largest among various neurodegenerative diseases. Expansion of polyglutamine tract in multiple proteins creates structurally abnormal proteins that lead to formation of inclusion body (huge aggregates of mutant protein) and finally induce neuronal cell death. Polyglutamine diseases have been classified into 9 types depending on the mutated that has gene polyglutamine tract. In vivo and in vitro experiments have demonstrated that mutant huntingtin and ataxin-1 proteins have dominant roles in inducing cell toxicity by their translocation to the nuclei.

We previously reported a physical interaction of mutant nuclear huntingtin or ataxin-1 with high-mobility-group B protein (HMGB). Our data indicated that mutant polyglutamine disease proteins sequester HMGB protein resulting in impairment of HMGB's function such as transcription and DNA repair (Qi et al., Nat. Cell Biol., 2007). This year, we reported another aspect of nuclear dysfunction induced by mutant huntigntin. Interactome data from our collaborative research group indicated interaction between huntingtin and Ku70, a molecule involved in the repair of DNA double strand break (DDB). We confirmed predominant interaction of Ku70 to mutant than normal huntingtin, dysfunction of Ku70 in the DSB repair by the abnormal interaction, and DNA damage response induced in primary neurons as well as in mouse models. Mutant huntingtin x Ku70 double transgenic mice show a remarkable improvement of their lifespan in comparison to the mutant huntingtin transgenic mice.

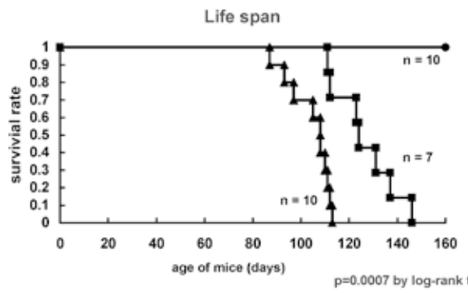


Fig.1 Elongation of the life span of Ku70/mutant huntingtin double transgenic mice.

2) A new therapeutic drug for mental retardation

We previously found a novel gene, PQBP1 whose mutations cause mental retardation (MR) syndromes including Renpenning syndrome, Golabi-Ito syndrome, Sutherland-Haan syndrome and so on. PQBP1 is also causative for non-syndromic MR. PQBP1 locates dominantly in the nucleus, and interacts with transcription and splicing related proteins. Most of the reported mutations in MR families cause frame shift of the codon and reduction of PQBP1 mRNA due to non-sense RNA decay. Therefore, the reduction of PQBP1 is suspected to be the underlying mechanism. We generated PQBP1-knock down mice and analyzed their behavioral changes. The KD mice showed abnormalities in anxiety-related cognition and memory. PBA, an HDAC inhibitor, ameliorated the abnormal phenotype. These results suggested abnormal gene expression of certain target genes following the reduction of PQBP1 underlies the symptoms of PQBP1-KD mice, and the mechanism might be homologous to the human pathology in PQBP1-linked MR. Furthermore, our results suggested that HDAC inhibitors could be useful for the treatment of MR patients caused by gene expression abnormalities.

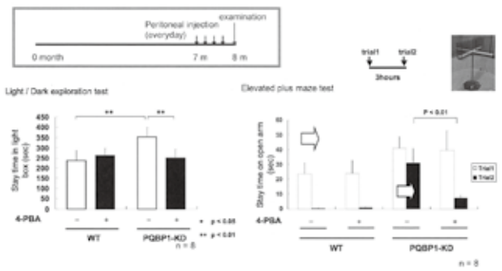
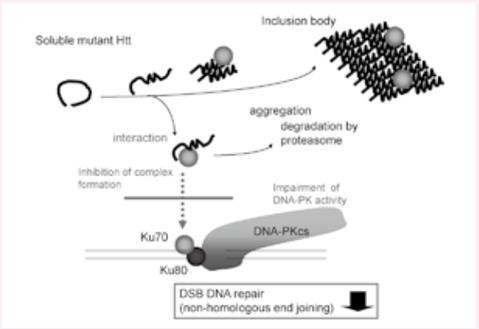


Fig.2. Light-dark box and elevated plus maze tests unraveled decline of anxiety-related cognition and memory in PQBP1-KD mice. PBA rescued the abnormalities.

Highlight

Ku70-mediated DNA repair dysfunction in the Huntington's disease pathology.



Highlight 1:
A hypothetical scheme of Ku70-mediated impairment of DNA double strand break repair in Huntington's disease pathology.

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1. Takahashi K, Yoshina S, Maekawa M, Ito W, Inoue T, Shiwaku H, Arai H, Mitani S, and Okazawa H (2009) Nematode Homologue of PQBP1, a Mental Retardation Causative Gene, Is Involved in Lipid Metabolism. PLoS One 4, e4104 .
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4. Takahashi M, Mizuguchi M, Shinoda H, Aizawa T, Demura M, Okazawa H, Kawano K (2009) Polyglutamine tract binding protein-1 is an intrinsically unstructured protein. Biochimica et Biophysica Acta. 1794(6):936-43.
5. Ito H, Yoshimura N, Kurosawa M, Ishii S, Nukina N, Okazawa H (2009) Knock down of PQBP1 impairs anxiety-related cognition in mouse. Human Molecular Genetics. 18:4539-54
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Division of Pathophysiology
Department of Pathological Biochemistry

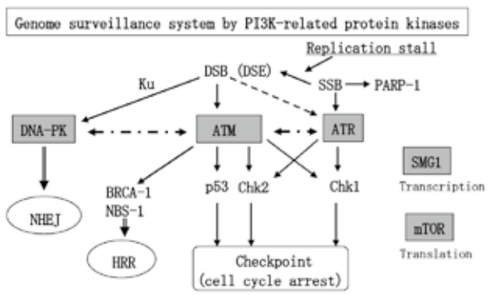
Professor **Hirobumi Teraoka, Ph.D.**
Assistant Professor **Ryo Sakasai, Ph.D.**

In relationship between cell fate and DNA metabolism, we have mainly investigated molecular mechanisms of non-homologous end-joining (NHEJ) in repair of DNA double-strand breaks (DSBs), and of hepatocyte differentiation from extrahepatic origins including pluripotent stem cells for preclinical application. Our study will contribute to improving therapy for human diseases including cancer and serious liver failure as well as to understanding molecular mechanisms of DSB repair and liver development.

Research Projects

1. DNA damage response and DSB repair

DSBs are caused by a variety of exogenous and endogenous genotoxic agents and may lead to genomic instability. DSBs are repaired by NHEJ and homologous recombination (HR) pathways. The NHEJ pathway is mediated by DNA ligase IV/XRCC4, Ku (p70/p86) and DNA-dependent protein kinase (DNA-PK) in a cell-cycle-independent manner, whereas the HR pathway preferentially occurs during S/G2 phase and depends on Rad51 and Rad51 paralogs. Molecular mechanism of NHEJ, DSB signal transduction and functional analysis of PI3K-related protein kinases (PIKPK) have been investigated.



In response to replication-mediated DSBs possessing only one DNA double-strand end (DSE), DNA-PK was responsible for the RPA2 hyperphosphorylation following ATR-dependent RPA2 focus formation in treatment with camptothecine (CPT) inducing directly DSEs. The ubiquitin–proteasome pathway plays an important role in DNA damage signaling and repair by facilitating the recruitment and activation of DNA repair factors and signaling proteins at sites of damaged chromatin. Proteasome activity is generally not thought to be required for activation of apical signaling kinases including the PIKPK family that orchestrates downstream signaling cascades in response to diverse genotoxic stimuli. We found that proteasome inhibition suppresses DNA-PK activation caused by the topoisomerase I (Top I) poison CPT, implying that CPT-dependent replication fork collapse activates DNA-PK signaling through a proteasome dependent, Top I degradation-independent pathway.

H2AX, a variant of histone H2A, is well-known to be phosphorylated by ATM in response to DSBs and the phosphoryalted form (γ -H2AX) shows foci at the cleavage sites in the nucleus. We revealed that H2AX is phosphorylated at M phase without DNA damage response. We have recently found that replication stress-induced DNA lesion carryover into the M phase triggers genomic instability followed by tumorigenesis.

2. Genome integrity in ES cells

Since pluripotent stem cells including ES cells and iPS cells can proliferate indefinitely in an undifferentiated state and differentiate into various cell types, pluripotent stem cells are expected to be useful for cell replacement therapy and basic research on early embryogenesis. Although molecular mechanisms of ES cell self-renewal have been studied, many uncharacterized genes expressed in ES cells remain to be clarified. Developmental pluripotency-associated 4 (Dppa4) is such a gene highly expressed in both ES cells and early embryos. We previously reported that DPPA4 is a nuclear factor associated with active chromatin and that it regulates differentiation of ES cells into a primitive ectoderm (definitive endoderm) lineage. Recently we have found that DPPA4 exhibits mobility similar to histone H1 in chromatin through direct binding to DNA and histone H3 via the N-terminal and the C-terminal regions, respectively. The binding to both DNA and histone H3 seems to be necessary for the unique chromatin structure in ES cell nuclei.

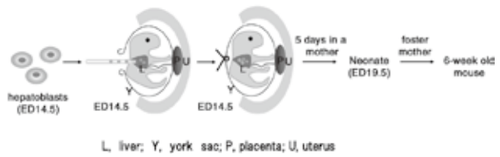
The rapid proliferation of mouse ES cells depends on the activation of the effector molecule ERas. However, the molecular mechanisms that regulate the expression of the ERas gene is poorly understood. We demonstrated the involvement of Nanog in the regulation of ERas transcription in mouse ES cells: the region between positions –173 and +107, to which Nanog binds, was essential for ERas promoter activity; an enhancer region found within intron 1 of the ERas gene markedly elevated its proximal promoter activity in a Nanog-dependent manner.

3. Hepatocyte differentiation from extrahepatic origins

Liver transplantation is the only successful treatment for end-stage liver diseases. Hepatocyte transplantation is an attractive alternative to liver transplantation as a potential treatment for liver diseases and a bridge use for patients awaiting liver transplantation. Because of liver shortage, we have examined hepatocyte differentiation from extrahepatic origins including mouse and monkey ES cells and cord blood cells. We have successfully obtained hepatocytes or hepatocyte-like cells both in vitro and in vivo from mouse and monkey ES cells and human cord blood cells (2003). Hepatocyte-like cells derived from cord blood cells were differentiated much more efficiently and functionally in the chronically damaged liver of ALB-uPA/SCID mice than in the transiently damaged liver of SCID mice. We found that Cyp7a1 as a liver specifc gene is not expressed in york sac tissues expressing many liver-related genes, and revealed that ES cells can differentiate into the hepatocyte lineage derived from definitive endoderm. For improvement of differentiation efficiency of ES cells into hepatocyte-like cells, VEGF was found to be important through hepatocyte-endothelial cell interaction.

4. Establishment of a novel method for mouse ex utero transplantation of hepatic progenitor cells into the fetal liver

Experimental transplantation models using adult injured liver have been developed to evaluate not only mature hepatocytes but also hepatic progenitor cells, including hepatoblasts. However, there is no available way to transplant those hepatic immature cells along with embryology. Avoiding the limitations of the adult liver niche, transplantation of hepatic stem/progenitor cells into fetal liver is desirable to analyze immature cells in a hepatic developmental environment. Recently, we have established a novel method for the ex utero transplantation of hepatic progenitor cells into the mouse fetal liver on embryonic day 14.5 based on reduction in litter size of recipient mice and limitation of injection volume into the fetal liver. When GFP-expressing hepatoblasts isolated from ED14.5 liver were injected into the ED14.5 fetal liver, expanded GFP-positive cells in the neonatal (ED19.5) liver expressed albumin abundantly or α -fetoprotein weakly, and contained glycogen, indicating that transplanted hepatoblasts can proliferate and differentiate in concord with surrounding recipient parenchymal cells. In the liver of 6-week old mice grown from the neonates, GFP-positive cells expressed albumin, but not α -fetoprotein. This unique technique was successfully applied to transplantation of hepatoblast-like cells induced from ES cells and will provide a new in vivo experimental system for studying cell fate of hepatic stem/progenitor cells and liver organogenesis.



L, liver; Y, york sac; P, placenta; U, uterus

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3. Sakasai R , Teraoka H, Tibbetts RS: Proteasome inhibition suppresses DNA-dependent protein kinase activation caused by camptothecin. ***DNA Repair*** (in press)
4. Ichijima Y, Yoshioka K, Yoshioka Y, Shinohe K, Fujimori H, Unno J, Takagi M, Goto H, Inagaki M, Mizutani S, Teraoka H: DNA lesions induced by replication stress trigger mitotic aberration and tetraploidy development. ***PLoS ONE*** (in press)
5. Masaki H, Nishida T, Sakasai R, Teraoka H: DPPA4 modulates chromatin structure via association with core histone H3 and DNA in mouse embryonic stem cells. ***Genes Cells*** (in press)
6. Nishida, T., Masaki H, Yoshioka K, Sakasai R, Teraoka H: Nanog-dependent transcriptional regulation of ERas gene in mouse embryonic stem cells. ***Biochem. Biophys. Res. Commun.*** (in press)

Department of Pathological Cell Biology

Professor Shigeomi Shimizu
Assistant professor Satoko Arakawa, Tatsushi Yoshida
Post-doctoral fellows Reishuku Li, Ikuko Nakanomyo, Michiko Murohashi

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 provision of nutrients when cells face starvation, as well as contributing to the turnover of cytoplasmic components. Studies of yeasts have identified a number of genes, designated ATG, that are required for the formation of autophagosomes. Many mammalian homologues of the yeast ATG genes have also been identified, and studies of mice lacking certain ATG genes, including ATG5, ATG6 (also called Beclin-1), and ATG7, have confirmed that these genes are essential for induction of macroautophagy. However, recently we found that cells lacking ATG5 or ATG7 can still form autophagosomes/autolysosomes and perform autophagy-mediated protein degradation 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 process of macroautophagy. We also found that this alternative process of macroautophagy was regulated by several autophagic proteins, including ULK1 and Beclin-1. In vivo, ATG5-independent alternate macroautophagy was detected in several embryonic tissues. It was also found to play a role even in the clearance of mitochondria during erythroid maturation. 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.

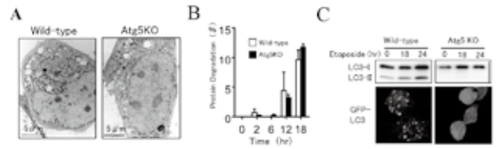

Fig.1 Induction of macroautophagy in Atg5-/- MEFs by etoposide.
(A) Induction of macroautophagy in wild type (WT) and Atg5–/– MEFs by exposure to etoposide. (B) Time-course analysis of long-lived protein degradation in etoposide-treated WT and Atg5–/– MEFs. (C) Absence of LC3 modifications. Both LC3-II formation and punctate GFP-LC3 fluorescence were observed in WT but not Atg5–/– MEFs.

2, Molecular mechanisms of programmed cell death

Programmed 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. 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.

A, Analysis of apoptosis mechanism

Recently, we found that Bcl-2 independent alternative apoptotic pore is present in mitochondria. We are currently investigating its molecular mechanisms.

B, Analysis of autophagic cell death

Bax/Bak-deficient mouse embryonic fibroblasts (MEFs) are resistant to apoptosis induced by various stimuli. Instead, we found that these cells still die by autophagy in response to various death stimuli. Recently, we found that JNK was activated in etoposide- and staurosporine-treated, but not serum-starved, Bax/Bak DKO cells, and that autophagic cell death was suppressed by addition of a JNK inhibitor and by a dominant-negative mutant of JNK. Studies with sek1$^{-/-}$mkk7$^{-/-}$ cells revealed that disruption of the JNK prevented the induction of autophagic cell death. Co-activation of JNK and autophagy induced autophagic cell death. Activation of JNK occurred downstream of the induction of autophagy, and was dependent on the autophagic process. These results indicate that JNK activation is crucial for the autophagic death of Bax/Bak DKO cells.

C, Physiological role of cell death in mammals

We are analyzing physiological role of cell death using apoptosis-resistant mice (Bax/Bak doble KO mice, Bcl-2 Tg mice), autophagy-deficient mice, PT-deficient mice (CyPD KO mice).

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.

Highlight

ATG5 and ATG7 are considered as essential molecules for induction of macroautophagy. However, we found that cells lacking ATG5 or ATG7 can still form autophagosomes/autolysosomes and perform autophagy-mediated protein degradation when subjected to certain stresses. Although lipidation of LC3 is accepted to be a good indicator of macroautophagy, it did not occur during the ATG5/ATG7-independent alternative macroautophagy. Unlike conventional macroautophagy, autophagosomes seemed to be generated in a Rab9-dependent manner by the fusion of the phagophore with vesicles derived from the *trans*-Golgi and late endosomes. Mammalian macroautophagy can occur via at least two different pathways, which are an ATG5/ATG7-dependent conventional pathway and an ATG5/ATG7-independent alternative pathway.

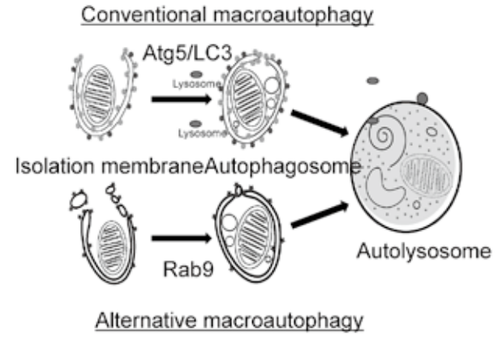

Fig.2. Hypothetical model of macroautophagy.
There are at least two modes of macroautophagy, i.e. conventional and alternative macroautophagy. Conventional macroautophagy depends on Atg5 and Atg7, is associated with LC3 modification. In contrast, alternative macroautophagy occurs independent of Atg5 or Atg7 expression and LC3 modification. The generation of autophagic vacuoles in this type of macroautophagy might be mediated by the fusion of isolation membranes with vesicles derived from the trans-Golgi and late endosomes in a Rab9-dependent manner.

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Department of Developmental and Regenerative Biology

Professor	**Hiroshi Nishina, Ph.D.**
Assistant Professor	**Yoichi Asaoka, Ph.D.**
Assistant Professor	**Takashi Nakamura, Ph.D.**
Assistant Professor	**Takahiro Negishi, Ph.D.**

Our goal is to define the molecular basis for the mechanism of organ formation and regeneration using knockout mice and mutant fishes. To accomplish this goal, we have focused on defining signaling molecules and pathways that regulate liver formation and stress responses. Moreover, we are trying to establish a cell therapy for intractable diseases such as liver failures using self-bone marrow cells. Our study will provide new insights into understanding the precise molecular mechanisms that underlie organ failures found in human disease and will lead to the development of new rational therapy for the diseases (Fig. 1).

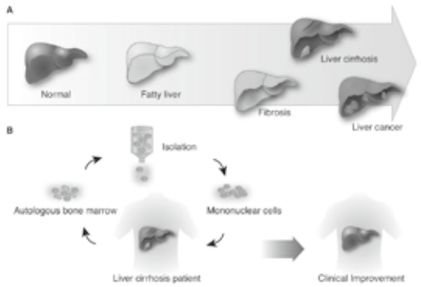

Fig.1. A novel approach to treatment of human liver diseases. (A) Continuum of liver disease development from the normal organ to fatty liver, fibrosis, liver cirrhosis, and liver cancer. (B) Principle of autologous bone marrow cell infusion (ABMI) therapy.

1. Activation mechanism and physiological roles of stress-activated MAP kinase in mouse development

Stress-activated protein kinase/c-Jun NH_2-terminal kinase (SAPK/JNK) is activated by many types of cellular stresses and extracellular signals. We showed that two SAPK/JNK activators, SEK1 and MKK7, are required for fetal liver formation and full activation of SAPK/JNK, which responds to various stimuli in an all-or-none manner. *Sek1*$^{-/-}$ embryos die between E10.5 and E12.5 with impaired liver formation and massive apoptosis. *Mkk7*$^{-/-}$ embryos die between E11.5 and E12.5 with similar defects in liver formation. These results indicate that SEK1 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 SAPK/JNK are sequentially phosphorylated by SEK1 and MKK7, respectively, in mammalian cells. We propose the hypothesis that the strong or weak activation of SAPK/JNK is induced by SEK1 and MKK7 to regulate the cell fate (Fig. 2).

2. Mutations affecting liver development and function in Medaka, Oryzias Latipes

The liver is an organ with vital functions, including processing and storage of nutrients, maintenance of serum composition, detoxification and bile production. Recently, several genes that are crucial for liver formation and function have been isolated in mice and confirmed by reverse genetics. Although a reverse genetic approach is powerful in characterizing function of known genes, knowledge of genes in liver formation and disease is still limited. Therefore, identifying mutations affecting these aspects will uncover genes required for these processes. Systematic forward genetic screens for mutations affecting liver formation and function such as hepatic bud formation, liver morphogenesis, bile color in the gall bladder, lipid metabolism, and liver laterality have been carried out in Medaka, *Oryzias latipes* (Fig. 3). To isolate mutants that model human liver diseases, we are analyzing these mutations. Among them, *kendama* (*ken*) mutation was isolated as a gene that affects the laterality of the liver. *ken* mutant was viable and fertile with inverted positions of liver and heart, and with inverted spiral of gut. Interestingly, the spleen was almost lost in *ken* mutant. This phenotype is very similar to human genetic disease 'asplenia' whose gene mutation is still unknown. Furthermore, white livers consisting of bloated and Oil red O-positive hepatocytes were observed in *ken* mutants. Thus, *ken* mutation models human disease asplenia and Non-alcoholic fatty liver disease (NAFLD) or non-alcoholic steatohepatitis (NASH). Especially, NAFLD and NASH are serious human diseases in the modern world, so *ken* mutation may shed a new light on the molecular mechanisms of these diseases and the preventive medicine.

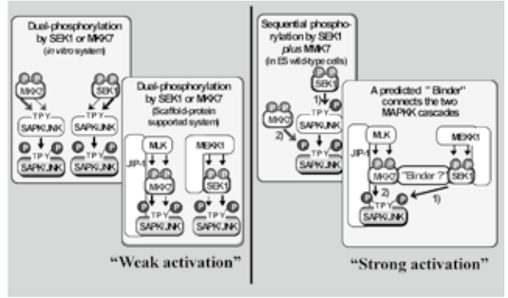

Fig.2. A proposed model for "weak" and "strong" activation of SAPK/JNK.

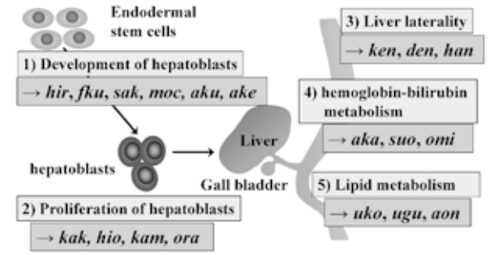

Fig.3. Mutations affecting liver development and function in medaka. The recessive mutations illustrated were identified using multiple criteria and classified into five phenotypic groups: Group 1, impaired hepatoblast development; Group2, impaired hepatoblast proliferation; Group 3, impaired liver laterality; Group 4, impaired hemoglobin-bilirubin metabolism; Group 5, impaired lipid metabolism.

Highlight

During vertebrate embryogenesis, the liver develops at a precise location along the endodermal primitive gut tube due to signaling delivered by adjacent mesodermal tissues. Although several signaling molecules have been associated with liver formation, the molecular mechanism that regulates liver specification is still unclear. We previously performed a screen in medaka to isolate mutants with impaired liver development. The medaka *hio* mutants exhibit a profound (but transient) defect in liver specification that resembles the liver formation defect found in the zebrafish prometheus (*prt*) mutants, whose mutation occurs in the *wnt2bb* gene. In addition to their liver abnormality, *hio* mutants lack pectoral fins and die after hatching. Positional cloning revealed that the *hio* mutation affects the *raldh2* gene encoding retinaldehyde dehydrogenase type2 (RALDH2), the enzyme principally responsible for retinoic acid (RA) biosynthesis. Mutations of *raldh2* in zebrafish preclude the development of pectoral fins. Interestingly, in *hio* mutants, expression of *wnt2bb* in the lateral plate mesoderm (LPM) directly adjacent to the liver-forming endoderm was completely lost. *Conclusion:* Our data reveal the unexpected finding that RA signaling positively regulates the *wnt2bb* gene expression required for liver specification in medaka. These results suggest that a common molecular mechanism may underlie liver and pectoral fin specification during piscine embryogenesis (Fig. 4).

Fig.4. Schematic model of RA signaling during liver and pectoral fin formation in a WT medaka embryo. (Left) During liver formation in medaka, RALDH2 expression in the somites results in the production of RA that induces wnt2bb expression. This *Wnt2bb* then induces *prox1* expression in the liver bud, which in turn drives hepatocyte migration. (Right) During fin formation, RALDH2 expression in the somites results in the production of RA that induces *wnt2ba* and *tbx5* expression in the fin bud that drives fin cell differentiation.

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Department of Stem Cell Biology

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Assistant professor **Takahiro Aoto, D.V.M., Ph.D.**

Stem cell systems play fundamental roles in tissue turnover and homeostasis. Our goal is to understand the mechanisms of tissue homeostasis driven by stem cell systems and to apply the knowledge to better understand the mechanisms underlying the tissue decline, cancer development and other diseases associated with ageing. We further aim to apply those knowledges gained to regenerative medicine using somatic stem cells or iPS (induced prulipotent stem) cells, treatment of cancer and other 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 new cell populations every hair cycle. We previously identified the source of those melanocytes, "melanocyte stem cells" (MSC), which are located in the hair follicle bulge and supply mature melanocytes required for hair pigmentation (Nishimura EK et al. Nature 2002). We are currently trying to identify melanocyte stem cells in hairless skin areas.

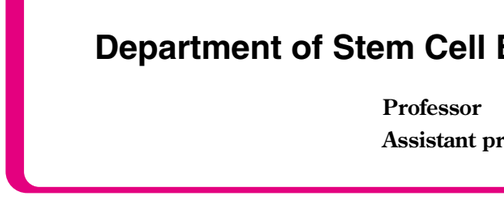

Fig.1

2) Mechanisms of stem cell maintenance

To understand the mechanisms underlying MSC maintenance, we hypothesized that hair graying phenotype is caused by incomplete maintenance of MCSs. To test this, we took advantage of *Bcl2* deficient mice and *Mitf-vit* mutant mice, both of which show irreversible hair graying phenotypes. *Mitf* encodes a transcription factor of bHLH Zip type and is known as a master regulator of melnaocyte development. We found that Bcl2 is one of the target genes of MITF and both *Mitf* and *Bcl2* are essential for MSC maintenance to prevent hair graying (Nishimura EK et al. Science 2005).

We previously showed that the niche microenvironment plays dominant role in MSC fate determination (Nishimura EK et al. 2002), while the identity of the niche cells, niche-derived factors and the underlying molecular mechanisms are still largely unknown. We have searched for niche-derived key factors and found that Transforming Growth Factor β is an essential niche factor for MSC maintenance. We are currently trying to identify the functional "niche cells" which provide the most appropriate environment for MSCs (Nishimura EK et al. Cell Stem Cell, 2010). Through those approaches, we aim to understand the underlying mechanisms for tissue homeostasis, ageing and cancer development.

Highlight 1

Key Roles for Transforming Growth Factor β in Melanocyte Stem Cell Maintenance.

Melanocyte stem cells (MSC) in the bulge area of hair follicles are responsible for hair pigmentation and when defective, result in hair graying. We analyzed the process of MSC entry into the quiescent state and showed that niche-derived transforming growth factor b (TGFb) signaling plays important roles in this process. TGFb1/2 not only induces reversible cell cycle arrest, but promotes melanocyte immaturity by down-regulating MITF, the master regulator of melanocyte differentiation, and its downstream melanogenic genes, *in vitro*. TGFb signaling is activated in MSCs when they reenter the quiescent non-cycling state during hair cycles and this process is Bcl2-dependent for MSC survival *in vivo*. Furthermore, targeted TGFb type II receptor (*TGFbRII*) deficiency in the melanocyte lineage causes incomplete maintenance of MSC immaturity and resultant mild hair graying. These data demonstrate that the TGFb signaling pathway is a key niche factor for melanocyte stem cell quiescence and immaturity (Nishimura EK et al. Cell Stem Cell, 2010)

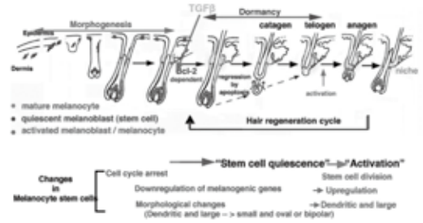

Fig.2

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

Physiological hair graying is the most obvious outward sign of aging even in normal mammals. We previously demonstrated that physiological hair graying is caused by incomplete self-renewal/maintenance of MSCs (Nishimura EK et al. 2005). However, it was still not known what causes the self-renewal of MSCs to become defective during the course of ageing. We recently found that genotoxic stress abrogates renewal of melanocyte stem cells by triggering their differentiation. Stem cell differentiation but not stem cell apoptosis nor senescence turned out to be the major fate of MSCs under irrepairable/excessive genotoxic stress or with ageing (see Highlight). Our findings indicated the existence of "stem cell renewal checkpoint" which maintain the quality of melanocyte stem cell pools.

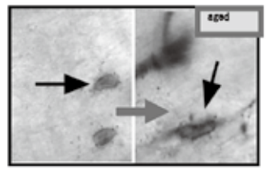

Fig.3

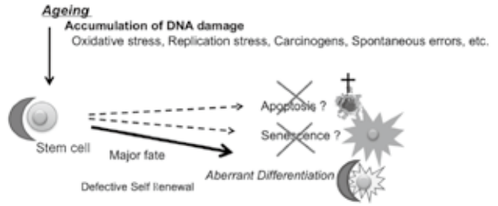

Fig.4

Highlight 2

STRESSED-OUT DNA TURNS BLACK HAIR GRAY?

"Genotoxic stress abrogates renewal of melanocyte stem cells by triggering their differentiation."

Somatic stem cell depletion due to the accumulation of DNA damage has been implicated in the appearance of aging-related phenotypes. However, little is known about the fate of stem cells under the genotoxic stress and its outcome. Hair graying, a typical sign of aging in mammals, is caused by the incomplete maintenance of melanocyte stem cells (MSCs) with age. Here we report that excessive/unrepairable genotoxic stress, such as by ionizing radiation, in mice abrogates renewal of MSCs by triggering their differentiation into mature melanocytes ectopically in the niche, rather than inducing their apoptosis or senescence. The resulting MSC depletion leads to irreversible hair graying. Furthermore, deficiency of Ataxia-telangiectasia mutated (ATM), a central transducer of the DNA damage response, sensitizes MSCs to ectopic differentiation, demonstrating that the DNA damage checkpoint kinase protects MSCs from their premature differentiation by functioning as a "stemness checkpoint" to prevent an aging phenotype, hair graying. (Inomata K., Aoto T. et al. Cell 2009)

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Presentation at international meetings

1. Emi Nishimura: "Why does our hair turn gray?: JSPS/JHU/NIA-sponsored symposium: (Baltimore,U.S.A) Ageing vs. Regenerative Medicine: How Much Can Stem Cell Do? (NIA, Baltimore, USA) Feb. 19th 2010

2. Shintaro Tanimura, Yuko Tadokoro, Ken Inomata1, Wataru Nishie, James R. McMillan, Daisuke Sawamura, Hiroshi Shimizu, Emi K. Nishimura: Hair follicle stem cells provide a COL17A1-dependent niche for melanocyte stem cells, The 7th Stem Cell Research Symposium , May 15th, 2009

Laboratory of Immunology School of Biomedical Science
Department of Immunology Medical Research Institute

Professor	Takeshi Tsubata, M.D., Ph.D.
Associate Professor	Takahiro Adachi, Ph.D.
Assistant Professor	Kozo Watanabe, Ph.D.
Junior Associate Professor	Makoto Tsuiji, Ph.D.
Assistant Professor	Naoko Matsubara, Ph.D.
	Yusuke Kishi
	Man Rong Yong

We are currently conducting the following four projects. These projects are closely related each other.

1) Elucidation of the mechanisms for rapid activation of memory B cells, and drug development for host defense against infection by inducing rapid immune responses.
2) Elucidation of the mechanisms for selection of self-reactive B cells, and development of control methods for self-reactive B cells.
3) Elucidation of stress responses of B cells.
4) Elucidation of the mechanisms for the regulation of acquired immunity by glycan ligands, and development of novel methods to control acquired immunity by modulated glycan ligands.

1. Study on the mechanisms for rapid antibody production during memory responses

During memory responses, immune responses are rapidly induced resulting in rapid elimination of pathogens. The rapid immune responses are induced as a consequence of rapid activation of memory lymphocytes that are generated during primary responses and stay in the body for a long period. Thus rapid activation of memory lymphocytes play a central role in vaccine-mediated host defense against pathogens. However, how memory B lymphocytes (B cells) are rapidly activated is not yet known.

Memory B cells mostly express membrane form of IgG as B a cell antigen receptor (BCR), whereas naïve B cells express both membrane form of IgM and IgD as BCR. Using B cell lines, we have demonstrated that the signaling through IgG-BCR is augmented compared to that through IgM or IgD (Wakabayashi et al. Science 2002, Sato et al. J. Immunol. 2007). Moreover, Goodnow's group demonstrated augmented antibody production in IgG transgenic mice in which naïve B cells express IgG instead of IgM and IgD, in agreement with augmented IgG-BCR signaling. However, they demonstrated later that BCR signaling is rather reduced in IgG-transgenic B cells, suggesting that IgG-BCR signaling in primary B cells is different from that in B cell lines.

To solve this apparent controversy, we analyzed IgG-transgenic mice, and proposed the "idling model" that explains hyperactivation of IgG-expressing B cells with reduced BCR signaling. (Fig. 1) (Man et al. PLoS ONE, 2010) (See Highlight).

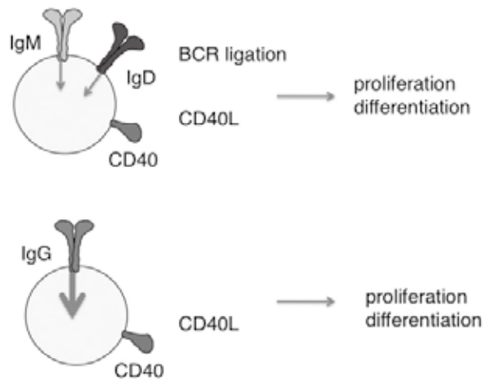

Fig.1. "Idling" model of rapid activation of IgG-producing B cells.
Naïve B cells that express both IgM and IgD as B cell antigen receptors (BCR), require both BCR ligation and interaction with CD40L on T cells for activation. In contrast, IgG-producing B cells are in the "ideling" situation as a consequence of high tonic BCR signaling, and are rapidly activated by interaction with CD40L alone.

2. Regulation of B cell activation by membrane-bound lectins.

Various lectins are expressed on immune cells, suggesting that glycans play crucial roles in the regulation of immune responses. However, the roles of glycans are not well understood in acquired immune responses. We are assessing the roles of glycans in antibody responses by focusing on two membrane-bound lectins CD22/Siglec2 and CD72. Our studies have demonstrated that these molecules are crucial regulators of antibody responses.

3. Regulation of B lymphocyte apoptosis and autoimmunity.

Antigen stimulation induces apoptosis in mature B cells. This apoptosis is blocked by signaling through the CD40 molecule on B cells. In the presence of CD40 signaling, antigen-stimulated B cells undergo activation and proliferation. Thus, CD40 functions as a molecular switch determining whether antigen-stimulated B cells undergo activation or apoptosis as is the case for CD22 and CD72. The CD40 ligand (CD40L) is expressed mainly on activated T cells. CD40L is overexpressed on lymphocytes in patients with systemic lupus erythematosus (SLE). Previously, we demonstrated that transgenic mice overexpressing CD40L spontaneously produce autoantibodies and develop lupus-like autoimmune disease, clearly demonstrating that CD40L overexpression plays a role in the pathogenesis of autoimmune disease. Overexpression of CD40L may induce autoimmunity by blocking apoptosis of self-reactive B cells upon interaction with self-antigens. We are currently analyzing how overexpression of CD40L induces autoimmunity in CD40L-transgenic mice.

Highlight

"Idling" model for rapid activation of memory B cells.

Memory B cells express membrane form of IgG as the antigen receptor (BCR) whereas naïve B cells express IgM and IgD as BCRs. To address how memory B cells generate rapid antibody responses, we established IgG-transgenic mice in which almost all naïve B cells produce IgG instead of IgM or IgD. IgG-transgenic B cells generate rapid antibody responses like memory B cells when immunized in vivo, although IgG BCR does not generate signaling when IgG-BCR is ligated by antigens (Fig. 2). To understand how these conflicting findings are generated, we stimulated IgG-transgenic B cells with various stimuli, we found that these B cells show augmented proliferation upon stimulation with agonistic anti-CD40 antibody alone. Based on this observation, we proposed "ideling" model for activation of IgG-producing B cells (Fig. 1).

B cell activation requires both BCR signaling and CD40 signaling that are generated by interaction of CD40 on B cells with its ligand CD40L (CD154) on activated T cells. Besides antigen-induced BCR signaling, BCR generates a low level signaling in the absence of antigens that is known as tonic signaling and is required for B cell survival. In "ideling" model, IgG-BCR generates an augmented tonic signaling equivalent to antigen-induced signaling through IgM-BCR resulting in an "ideling" situation where B cells can undergo activation by CD40 signaling alone. Such an ideling situation may induces a rapid B cell activation.

Goodnow's group also found reduced IgG-BCR signaling in IgG-transgenic B cells. "Ideling" model is so far the only model that can explain augmented antibody response despite of reduced BCR signaling in IgG-producing B cells, and may provide us with a clue to elucidate mechanisms for rapid antibody production during memory responses.

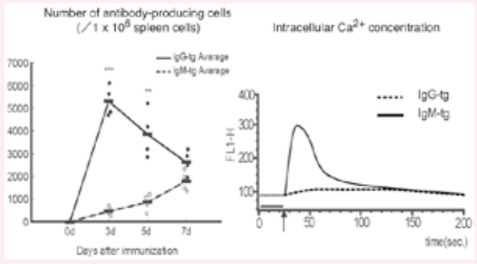

Fig.2. Augmented antibody production in the absence of BCR ligation-induced signaling in IgG-transgenic B cells.
IgG-transgenic B cells undergo rapid differentiation to antibody producing cells upon in vivo immunization (left panel). However, antigen-induced BCR ligation does not generate transmembrane signaling.

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Division of Pathophysiology
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Associate Professor	Toshiaki Nakajima, M.D., Ph.D.
Assistant Professor	Takuro Arimura, D.V.M., Ph.D.
Research Associate	Taeko Naruse, Ph.D.

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

1. Identification of novel mechanisims for hereditary cardiomyopathy

Gene mutations cause hereditary cardiomyopathy including hypertrophic cardiomyopathy (HCM) and dilated cardiomyopathy (DCM). We have identified several disease genes and disease loci for familial HCM or familial DCM. However, not all of the disease-causing gene mutations were revealed and hence the molecular pathogenesis remains unknown. This year we have revealed that PGM1, an enzyme involved in glucose metabolism, binds to a Z-disc protein ZASP and DCM-associated ZASP mutations decreased the binding. We also revealed that ZASP is usually localized in the cytoplasm, but distributes to Z-discs upon under the metabolic stress. (see Highlight)

2. Identification of disease loci for myocardial infarction

A genome-wide screening of loci for myocardial infarction (MI) as well as functional studies and replication studies identified a promoter SNP of MKL1 as the disease gene for coronary artery disease (CAD) in Japanese and Korean populations. On the other hand, extensive studies in Japanese and Korean populations confirmed that BRAP was significantly associated with CAD.

3. Gene analysis of arrhythmia

We identified two KCNQ1 mutations in a autosomal-recessive LQTS family. Functional studies revealed that both mutations cause trafficking abnormality of KvLQT1 channel due to novel mechanisms; the impaired complex formation and retention to endoplasmic reticulum.

4. Analysis of MHC in human and old world monkeys

Linkage disequilibria among the alleles of gene in human MHC, HLA, were investigated in detail in Japanese. We revealed that the evolutional history of HLA-B/Cw haplotypes can be followed by analyzing the structure of a microsatellite C1_2_5, suggesting the usefulness of microsatellite markers in the association studies. In addition, to reveal the molecular mechanism underlying the individual difference in immune response to SIV-vaccine in rhesus monkeys, a well-established animal model of HIV-vaccine, we investigated cDNA sequences of rhesus MHC class I genes, Mamu-A, -B, -I and AG, in 100 rhesus macaques originated from Myanmar and Laos.

5. Genome diversity in association with HIV/AIDS

We are investigating polymorphisms in several immune-related genes in association with the susceptibility/resistance to HIV/AIDS. We found that polymorphisms in TRIM5alpha and TIM1 genes were significantly associated with the susceptibility to HIV infection and AIDS development, respectively. Functional studies revealed that structural changes due to the TRIM5alpha polymorphisms affected the resistance to HIV-1 infection at the cellular level.

Highlight

By using yeast-2-hybrid system, we identified PGM1 as a binding partner to the heart-specific domain of ZASP protein. Biochemical studies revealed that DCM-associate ZASP mutations decreased the binding to PGM1 (Fig.1). In addition, we revealed that localization of PGM1 from cytoplasm to Z-disc was regulated by the metabolic stress to cardiomyocytes (Fig.2).

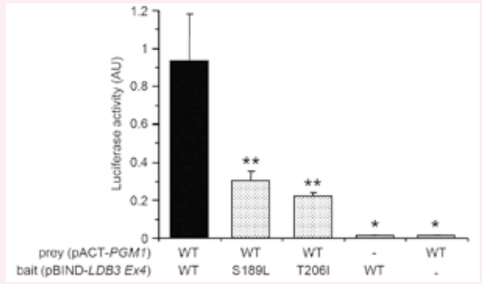

Fig.1. Binding of ZASP and PGM1 was decreased by DCM-associated mutations in ZASP gene (LDB3)

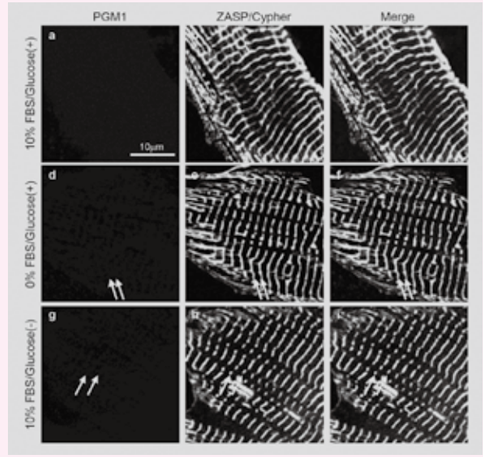

Fig.2. Localization of PGM1 in cultured neonatal rat cardiomyocytes
PGM1 was localized to Z-discs of cardiomyocytes under the stressed culture conditions, elimination of FBS or glucose.

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Frontier Research Unit
Virus Research Unit

Associate Professor **Norio Shimizu, Ph.D.**
Assistant Professor **Masaki Shirakata, Ph.D.**

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

1. Development of novel EBV infection animal models using the hNOG mice

The functional human immune system is reconstituted in NOD/Shi-scid/IL-2Rgamma null(NOG) mice that receive hematopoietic stem cell transplants. We show that these humanized mice can recapitulate key main aspects of EBV infection in humans. The NOG mouse is the most comprehensive small-animal model of EBV infection described to date and should facilitate studies of the pathogenesis, prevention, and treatment of EBV infection.

2. The function of EBNA1 in EBV replication of Epstein-Barr virus

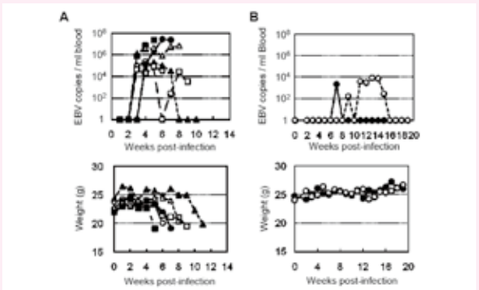
EBV-encoding nuclear factor EBNA1 has been studied to find that EBNA1 is phosphorylated at multiple sites in the Ser-rich region between the Gly-Ala and the DNA-binding domain, and also demonstrated that EBNA1 regulates human replication initiation factors in ORC binding to oriP plasmids in vitro.

3. Development of an exhaustive pathogenic microbe screening system

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

Highlight

Peripheral blood EBV DNA load and body weight in hNOG mice infected with EBV.
A. Infection at a high dose virus. Six mice were inoculated intravenously with 10^3 TD_{50} of EBV. EBV DNA load in the peripheral blood and body weight were then determined weekly. B. Infection at lower doses. Peripheral blood EBV DNA load and body weight of two mice inoculated with low doses of EBV (closed circle, 10^{-1} TD_{50}; open circle, 10^1 TD_{50}) are shown.



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Division of Medical Genomics

【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 department is to understand the molecular basis underlying cancer and genetic diseases including chromosome aberration syndromes. In 2009 we contributed as follows;
1. Identification of novel genes responsible for cancer and unknown genetic diseases.
2. Development of high-resolution in-house CGH arrays and established their applications for detection of cryptic genomic and epigenetic aberrations in cancer and genomic disorders.
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. Regulation of alternate promoters of stress response gene ATF3 was studied. Further, role of p53-ATF3 axis was investigated by genome-wide system biology.
2. Dual role of Elongin A, E3 ligase activity of Rpb1 and transcription elongation, was studied.

【Molecular Genetics】
Our research is directed at understanding the molecular mechanisms of apoptosis in response to DNA damage and genome stability through DNA damage repair in breast cancers.
1. We have demonstrated that BRCA2 complexed with 14-3-3 is required for centrosome positioning.
2. We have identified that ATM augments nuclear stabilization of DYRK2 in the apoptotic response to DNA damage.
3. Analyses of molecular domains of translesion DNA polymerases by introducing a point mutation by homologous recombination in vertebrates.

【Molecular Epidemiology】
1. We identified a rare variant, -856G/A, in the promoter region of the TNF-a, gene and showed that the rare variant allele confers transcriptional enhancement owing to a novel C/EBP binding site.
2. We established a method to quantitate the level of DNA methylation in TNF- a promoter by combining PCR and methylation sensitive restriction enzyme Aci I, and determined methylation status of this region in different tissues from individuals of different genotypes.

【Functional Genomicss】
Our department "Functional Genomics" seeks to resolve how gene expression process is regulated in an individual, and made following progress this year.
1. We found chemical compounds such as SRPIN340 and TG003, which affect mRNA splicing patters and have potentials to be clinically applicable to some incurable diseases including retinopathy of prematurity and viral diseases
2. We established the transgenic reporter worm system to monitor alternative splicing patterns *in vivo* and identified regulatory factors of tissue-specific alternative mRNA splicing in *C.elegans*.

【Epigenetics】
1. We have demonstrated that sushi-ichi retrotransposon-derived genes (*Sirh* genes), such as *Peg10, Peg11/Rtl1* and *Sirh7,* play essential but differential roles in formation of placenta, a unique organ of eutherian mammals.
2. We have demonstrated that *Meg1/Grb10* plays an essential role in pre- and postnatal growth, indicating overexpression of human *GRB10* is responsible for the etiology of Silver-Russell Syndrome caused by trisomy of 7q11-q13.
3. Assisted reproductive technologies, such as *in vitro* fertilization and intracytoplasmic sperm injection, become very popular, however, their effects on human development remain unclear. We have extensively examined pre-and postnatal epigenetic effects caused by such technologies.

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

Division of Medical Genomics
Department of Molecular Cytogenetics

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Assistant Professor	Jun Inoue Ph.D.
Assistant Professor	Shin Hayashi M.D., Ph.D.

The principal aim of Department of Molecular Cytogenetics is to understand the molecular mechanism underlying cancer and genetic diseases including chromosome aberration syndromes. Our research interests are as follows; (1) Identification of genes responsible for cancer and unknown genetic diseases, (2) Development of innovative techniques for detection of genomic and epigenomic aberrations underlying the pathogenesis of cancer and genomic disorders, and (3) Establishment of practically useful tools for diagnosis in Personalized Medicine of cancer and intractable diseases. It is our goal to bridge the gap between basic and clinical research for the benefit of each of the patients.

1. Identification of genes responsible for cancer by CGH

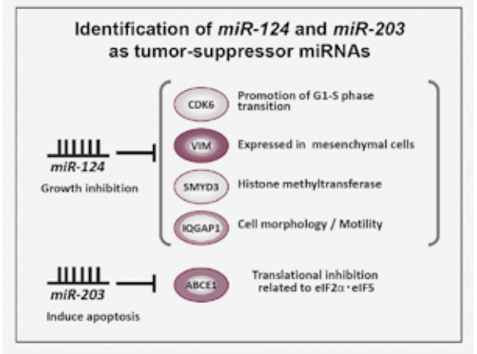
Comparative genomic hybridization (CGH) was developed as a molecular cytogenetic technique to compensate for difficulties presented by conventional methods. CGH analyses of solid tumors have revealed a number of recurrent copy-number aberrations including amplifications that had not been detected previously by any other technique. For the last decade we performed CGH analysis in over 1700 cases of various types of cancer, and we constructed a CGH database that is available through the internet (http://www.cghtmd.jp/cghdatabase/index.html). Through those CGH analyses we detected a number of novel and nonrandom amplifications in various tumors and identified target genes within the amplicons. For example, we identified *GASC1* (Gene Amplified in Squamous Cell Carcinoma 1) and *cIAP1*, as targets for the 9p22-23 amplification or 11q22 amplification in esophageal squamous cell carcinomas (ESCCs), respectively. The former is a demethylase for tri- or dimetylated lysine 9 on histone H3, and the latter is a member of the *IAP* (anti-apoptotic) gene family. We also detected frequent amplifications at 5p12-13 in small and non-small cell lung cancers and at 17q23 in neuroblastoma (NB). Consequently we identified novel target genes of *SKP2* or *PPM1D*, respectively. Some of them are being now focused as the target for the molecular target therapy in collaboration with the Research Institute of Pharmaceutical Companies.

2. Development of innovative techniques for genomics and/or epigenomics in medical science

Standard CGH to metaphase chromosomes can provide only limited resolution: 5-10 Mb for detection of copy-number losses and gains, and 2Mb for amplifications. To circumvent this limitation, we have also constructed different types of BAC-based CGH-arrays (so-called MCG array series) as follows, (1) MCG Whole Genome Array (WGA)-4500 and WGA-15000; those arrays provided a resolution of ~0.7Mb and ~0.2Mb, respectively. (2) MCG Cancer Array (CA)-800 and CA-1500, those two consisted of ~800 or ~1500 BACs harboring known cancer-related genes respectively, intended for diagnosis of cancer-specific copy-number aberrations, (3) MCG 1p36 Contig Array, which contains 212 contiguous BACs spanning a ~20-Mb region at 1p36, (4) MCG X-tiling array, which contains 1003 BACs throughout human X chromosomes without pseudo-autosomal regions, and (5) MCG Genome Disorder (GD) Array, which harbors BACs involved in loci responsible for congenital chromosomal aberrations for their diagnosis. In addition, we recently constructed a new type BAC-array, so called, Genome Variation (GV) array, which enables us to investigate copy number variation (CNV) at 677 loci throughout the human genome. It has been notable that CNV is relevant to the predisposition to common diseases.

3. Identification of cancer-related genes within genomic aberrations detected by array-CGH

We recently identified cancer-related genes upregulated by a gene amplification mechanism as follows, *CDK6* in gastric cancer, *CCND3* in liver metastatic colon cancer, *DUSP26* and *ITCH* in anaplastic thyroid carcinoma, *KLK5* in Bladder cancer, *BCL2L2* in non-small cell lung cancer (NSCLC), *POU2AF1* in multiple myeloma, *SMURF* in pancreatic cancer, and *SMYD2* in ESCC within the novel amplifications, that emerged via in-house BAC-array platform. Moreover, we recently detected submicroscopic homozygous deletions in many types of cancers, and within those as the landmarks for positional cloning of tumor suppressor genes (TSGs) we could identify novel candidate TSGs including *LRP1B* and *PCDH17* in ESCC, *DBC1* and *PCDH20* in NSCLC, *ADAM23* and *VLDLR* in gastric cancer, *RGC32* in malignant glioma, *PRTFDC1 MTNR1A* and *PAK4* in oral squamous cell carcinoma (OSCC), *CTGF* and *ANGPTL2* in ovarian cancer. Some of those genes are also epigenetically silenced due to the promoter CpG island methylation in some GCs. Recently, we successfully identified two tumor-suppressor miRNAs, *miR-137* and *miR-193a*, or *miR-124* and *miR-203* (Figure), epigenetically silenced in OSCC or hepatocellular carcinoma, respectively.



4. Applications of array-CGH

To accomplish genome-wide screening for methylated sites in the whole genome, we have combined array-CGH with MCA, and our preliminary data show that this "BAC array-based MCA (BAMCA)" can discriminate BAC clones that harbor methylated CpGs on an array platform. Our BAMCA method not only allows us to explore methylated CpG sites within spotted BACs, for if we apply BAMCA to a tiling-resolution CGH-array, methylated CpG sites emerge on an array platform throughout the entire genome. Recently, by using this BAMCA method, we identified candidate tumor suppressors, *NR1I2* and *PTGER2* in the progressive type of NB, and *CEABP1* in ESCC. In addition, a combination of chromatin immunoprecipitation (ChIP) assays with hybridization to DNA arrays, the so-called "ChIP-on-chips" technique, has proven to be a powerful way to explore sites of interaction among DNA-binding proteins across the entire genome.

5. Molecular cytogenetic investigation of genomic disorders

Array CGH is also one of the most powerful tools to detect cryptic chromosome aberrations in genomic disorders including multiple congenital anomalies and mental retardation (MCA/MR). In order to identify disease-specific genome alterations in MCA/MR we have extensively performed array-CGH analysis in 536 patients with unknown MCA/MR by suing GD-array and WGA4500 array, and have found genomic alterations closely associated with the pathogenesis in at least 114 cases (21.3%) as of Mar 10. 2010. GD-array has been launched as a practical diagnostic tool to detect cryptic copy number variants (CNVs) responsible for MCA/MR in the clinical setting in September 2009 by Fuji film Co.Ltd.

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Division of Medical Genomics
Department of Molecular Genetics

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Associate Professor	Kiyotsugu Yoshida, MD. Ph.D.
Associate Professor	Akira Nakanishi, Ph.D.
Assistant professor	Katsuya Takenaka, 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. Functional analysis of the BRCA2 gene.

BRCA2 protein interacts via the BRC (breast cancer) domain with RAD51, an essential component of the cellular machinery for the maintenance of genome stability and double strand-breaks repair. Recently, it was reported that this dissociation worked as a trigger to the M phase entry, and it was suggested that BRCA2 had the function to adjust DNA repair and the M phase progression of the cell cycle (figure 1.A). Moreover, BRCA2 is localized, surrounding around centrosome from the G1/S transition to the early M phase of the cell cycle. Centrosome is located in spindle pole at metaphase, and BRCA2 is no longer observed in centrosome at this time. When the cell cycle progresses further, and cytokinesis begins, BRCA2 is localized in the mid-body. However, the physiological role in the centrosome and the mid-body is not well understood. Then, we hope that we will clarify the role of BRCA2 protein, and find out how BRCA2 contributes to incidence of breast cancer.

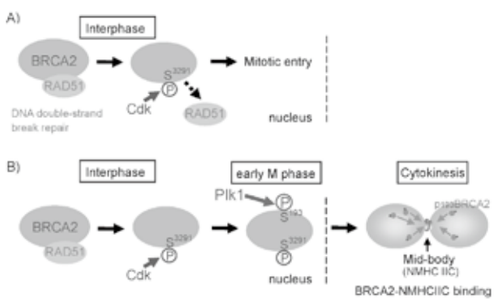

Fig.1. Localization of the BRCA2-myosin IIC complex to the midbody during cytokinesis.

1) BRCA2 complexed with plectin is required for centrosome positioning.

To search novel protein which interacts with BRCA2, HeLa cells extracts were separated by glycerol density gradient centrifugation. The endogenous BRCA2 consisted of a 700-800-kDa complex, and plectin was identified in the complex by analysis of tandem mass spectrometry. We confirmed that endogenous BRCA2 was immunoprecipitated with endogenous plectin. We have used the PLEC repeats (2738-3021 aa.) of plectin Ni(2+)-affinity pull-down assay and showed that PLEC repeats interacts with BRCA2. We further demonstrated that PLEC M1 was essential for the association of BRCA2 and plectin by the pull-down assay using glutathione S-transferase fusion proteins. When HeLa cells were transfected with the HA-PLEC M1, which acts as a dominant negative, centrosome positioning changed and micronucleus were observed by immunofluorescence microscopy. We previously reported that similar changes were also observed with the depletion of BRCA2 by small interfering RNA in HeLa cells. Plectin interacts with actin, intermediate filament and outer nuclear membrane protein. These findings suggest that BRCA2-plectin contributes to maintaining centrosome positioning, thus both proteins are required for connection between centrosomes and intermediate filaments.

2. Regulatory mechanisms of tumor cells in the apoptotic response to DNA damage

1) Protein kinase C delta activates RelA/p65 and NF-kappaB signalling in response to TNF-alpha.

NF-kappaB is tightly modulated by I-kappaB kinases and I-kappaB-alpha in the cytoplasm. Upon stimulation, NF-kappaB translocates into the nucleus to initiate transcription, however, regulation of its transcriptional activity remains obscure. Here we demonstrate that protein kinase C delta (PKC-delta) controls main subunit of NF-kappaB RelA/p65. Upon exposure to TNF-alpha, the expression of RelA/p65-target genes such as I-kappaB-alpha, RelB, and p100/p52 is up-regulated in a PKC-delta-dependent manner. The results also demonstrate that PKC-delta is targeted to the nucleus and forms complex with RelA/p65 following TNF-alpha exposure. Importantly, kinase activity of PKC-delta is required for RelA/p65 transactivation. In concert with these results, PKC-delta activates RelA/p65 for its occupancy to target-gene promoters, including I-kappaB-alpha and p100/p52. Moreover, functional analyses demonstrate that inhibition of PKC-delta is associated with substantial attenuation of NF-kappaB activity in response to TNF-alpha. These findings provide evidence that PKC-delta orchestrates RelA/p65 transactivation, a requisite for NF-kappaB signalling pathway in the nucleus.

2) DNA damage signalling recruits RREB-1 to the p53 tumor suppressor promoter.

Transcriptional regulation of the p53 tumor suppressor gene plays an important role in expression control of various target genes involved in the DNA damage response. However, the molecular basis for this regulation remains obscure. Here we demonstrate that RAS-responsive element-binding protein-1 (RREB-1) efficiently binds to the p53 promoter via the p53 core promoter element and transactivates p53 expression. Silencing of RREB-1 significantly reduces p53 expression at both the mRNA and the protein levels. Notably, disruption of RREB-1-mediated p53 transcription suppresses the expression of the p53 target genes. We also show that, upon exposure to genotoxic stress, RREB-1 controls apoptosis in a p53-dependent manner. These findings provide evidence that RREB-1 participates in modulating p53 transcription in response to DNA damage.

3. Analyses of molecular domains of translesion DNA polymerases by introducing a point mutation by homologous recombination in vertebrates.

The chicken B-lymphocyte line DT40 is characterized by a high efficiency of gene targeting and phenotypic stability. Utilizing this feature, we have established a method to introduce a point mutation on genome to see functions of molecular domains of translesion polymerases in detail. We successfully substituted an aspartate residue in the active site to inactivate the Polκ activity without affecting its expression. Analyzing the *POLK* polymerase-dead cell line demonstrated that the polymerase activity of Polκ is necessary for translesion DNA synthesis across monoalkylation damage, and implies that the catalytically inactive Polκ would be removed from the damaged sites and another responsible polymerase would be recruited efficiently.

Next, we applied this mutation introduction method for analyzing *REV3*, another translesion polymerase. Recently an amino acid on Rev3 responsible for binding to Rev7, another subunit of Polκ, was uncovered. We are introducing a point mutation into this residue. A preliminary result showed that the mutant has sensitivity to an intrastrand crosslinker, cisplatin. Sensitivities to wide variety of mutagens will be examined.

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Department of Molecular Epidemiology

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Many common diseases 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 as well as their interaction with environmental factors. We also pay attention in epigenetic alteration that may contribute to the progression of diseases.

1. CYP3A5 polymorphism and sensitivity of blood pressure to dietary salt.

Background/Aims: The drug metabolizing enzyme, cytochrome P-450 3A5 (CYP3A5) has recently been implicated in blood pressure regulation through metabolism of endogenous steroids. The genetic effect of CYP3A5*1 (expressor) and *3 (non-expressor) variants on blood pressure have been studied in African American and Caucasian but the effect on Asian population has not been addressed. The potential interaction with sodium intake needs to be taken into account.

Methods: A total of 238 unrelated apparently healthy Japanese male workers (20-64 year) were included in the study. The A6986G polymorphism (rs776746) in intron 3 was determined by melting curve analysis. Sodium intake level was inferred from spot urine specimen by calculating 24-h urinary sodium excretion (24HUNaClV).

Results: CYP3A5 *1/*1 genotype group had higher diastolic BP but not systolic BP than *3/*3 genotype group (p=0.038), which remained significant after adjustment with age, BMI and sodium intake. When sodium intake was considered, CYP3A5*1/*3 had a significant interactive effect on both SBP (p=0.046) and DBP (p=0.003) .

Conclusions: Genotypes of CYP3A5 may have a weak effect on blood pressure by interacting with sodium intake in Japanese men. (176 words)

2. Smoking confers a MTHFR 677C>T genotype-dependent risk for systemic atherosclerosis

Objective: To explore interactions between smoking and genetic polymorphism of 24 atherosclerosis-related candidate genes in systemic atherosclerosis.

Methods: The study comprised 1,503 consecutive autopsy cases. The men-to-women ratio was 1.16 and the average age at death was 80.3 years. Seventy point three percent of men and 21.6% of women were current or past smokers. The degree of atherosclerosis in 10 arteries was semi-quantitatively assessed. Thirty-four single nucleotide polymorphisms (SNPs) of 24 genes were analyzed by melting curve analtsis

Results: Twenty-four SNPs did not interact with smoking on atherosclerosis, while 7 SNPs interacted with smoking in one artery and 2 SNPs in two arteries. The genotypes of MTHFR 677C>T and smoking significantly interacted in four arteries: the common carotid artery, common and external iliac arteries, and femoral artery. The odds ratios of smoking on atherosclerosis were high (3.03 - 4.63) in MTHFR TT homozygotes, intermediate (1.75 - 5.24) in heterozygotes, and low (1.75 - 2.63) in CC homozygotes in systemic arteries except for the cerebral and coronary arteries.

Conclusions: MTHFR 677 TT homozygotes are more likely to develop atherosclerosis than heterozygotes or CC homozygotes, if they smoke. Thus, smoking cessation is more important in the prevention of atherosclerosis in MTHFR 677 TT homozygotes.

3. TNF α promoter -856 G→A rare variant confers novel C/EBP binding and enhances transcription

Tumor necrosis factor alpha (TNF α) is a pro-inflammatory cytokine involved in various biological and pathological processes. In a previous study, we identified a rare variant (RV), which involves a G to A substitution at the position -856 in the TNF promoter region. The current study was conducted to determine the function of this RV in vitro, since motif search predicted appearance of a novel C/EBP binding site in the RV allele. Both C/EBP α and C/EBP β efficiently bound to the RV allele but not to the wild type (WT) allele. Transfection of luciferase constructs in U937 cells revealed that RV allele confer higher transcriptional activity than the WT allele after over expression with C/EBP α and C/EBP β. Stimulation with LPA and PMA in U937 cells augmented endogenous C/EBP β expression in the nucleus and enhanced transcription activation from the RV allele more efficiently than the WT allele. These results suggest that the -856G→A can alter promoter activity of TNF through C/EBP binding. This RV may be a good candidate to study its relation with diseases and clinical phenotypes that relate to the expression of TNF α.

4. Clique-based data mining for related genes in a biomedical database.

Progress in the life sciences cannot be made without integrating biomedical knowledge on numerous genes in order to help formulate hypotheses on the genetic mechanisms behind various biological phenomena, including diseases. There is thus a strong need for a way to automatically and comprehensively search from biomedical databases for related genes, such as genes in the same families and genes encoding components of the same pathways. Here we address the extraction of related genes by searching for densely-connected subgraphs, which are modeled as cliques, in a biomedical relational graph. RESULTS: We constructed a graph whose nodes were gene or disease pages, and edges were the hyperlink connections between those pages in the Online Mendelian Inheritance in Man (OMIM) database. We obtained over 20,000 sets of related genes (called 'gene modules') by enumerating cliques computationally. The modules included genes in the same family, genes for proteins that form a complex, and genes for components of the same signaling pathway. The results of experiments using 'metabolic syndrome'-related gene modules show that the gene modules can be used to get a coherent holistic picture helpful for interpreting relations among genes. CONCLUSION: We presented a data mining approach extracting related genes by enumerating cliques. The extracted gene sets provide a holistic picture useful for comprehending complex disease mechanisms.

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Scope of research :

Transcriptional regulation is one of the most important processes by which genome information is expressed from DNA to mRNA to protein. The faithful synthesis of mRNA is achieved by transcriptional machinery comprised of RNA polymerase II, basal factors and many other protein factors, whose dysfunction is implicated in various human diseases. Our research interest is focused on the basic mechanism of transcription cycle and implication of early response transcription factors in determining cell fate in stress response. We are also studying on the mechanism of cell cycle arrest of terminally differentiated cardiac cells and its re-activation to provide novel regeneration therapy.

Key words

・To provide novel paradigm of transcriptional regulation
・To understand role of transcription factor in cell fate determination
・To innovate novel in situ regeneration therapy of heart

Research 1 : Transcription

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

1-1 Elongin A plays dual roles in stress response

Elongin (Elongin ABC complex) is considered to have at least dual functions, one is activation of trasncriptional elongation by RNA polymerase II (PolII), and another is the control of protein degradation since Elongin BC is a component of E3 ligase. By scanning stress response genes by chip analysis, Elongin A is recruited to the HSP70 and ATF3 gene from their promoter through 3' -downstream region, showing it can associate with elongating PolII. More importantly, Elongin A forms E3-ligase complex that target Pol II into ubiquitin-mediated degradation upon DNA damage. This is novel and finding and is the first report to assign Elongin A as DNA-damage-inducible Pol II degradation gene. Elongin A may function as one of safety net mechanism in mammalian cells.

1-2 Crosstalk between nucleoplasm and nucleolar

The rRNA is transcribed by RNA polymerase I in nucleolar and provides a place for protein synthesis as a center of ribosome activity. Only recently, a couple of transcription factors have been reported as important players for both in Pol I and Pol II transcriptions, suggesting that the information is dynamically shared between nucleoplasm and nucleolar. The aim of this project is to elucidate the detail of this shuttle regulation between Pol I and Pol II transcriptions. We are focusing on NF-kB and FCP1 as key molecules of this crosstalk mechanism.

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

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

2-1 Novel alternate promoter of ATF3

ATF3 has an upstream promoter P1 ~40kb apart from the putative P2 promoter. We showed that it is conserved between human and mouse, and showed that various stress strimuli activate the P1 promoter, indicating that ATF3 expression is controlled by at least two promoters. mRNAs derived from these two promoters encodes the protein with the same coding sequence, however, their 5' -UTR has significant difference of translational efficiency. In human cancer cells, prostatic and Hidgkin Reed-Sternberg cells that express high level of ATF3, the transcription from the P1 promoter was selectively activated and associated with altered configuration of chromatin.

2-2 Genome-wide screen of the role of ATF3 in stress response and human cancer

ATF3 funstions as both oncogene and tumor suppressor. For example, in prostate cancer and Hodgkin disease, ATF3 expression is positively correlated with cell proliferation and also enhanced metastasis. Conversely, ATF3 inhibits p53 degradation and stabilizes its expression level. As a first step, we are trying to decipher genetic pathway of these biological phenomenon using ChIP-chip and expression profile analysis of 1) cells after DNA-damage, 2) Prostate cancer cells, 3) Hodgkin Reed-Sternberg cells. Comparative study would provide a clue to role of ATF3. In human colorectal cancer cells, ATF3 binds over 6,000 gene promoters in stress response to DNA damage (MMS), while it binds to ~1,300 gene promoters in ATF3-expressing prostate cancer cells. We also performed the genome-wide expression analysis after ATF3 knockdown followed by expression microarray analysis. The results show ATF3 does regulate approximately 40% of p53 target genes, demonstrating that ATF3, a target gene of p53, functions as co-regulator of p53. ATF3 may play diverse regulstory role in oncogenesis.

2-3 ATF3 complex; transcriptional repressor or activator

According to our result from ChIP-chip analysis combined with expression array, ATF3 apparently works as not only traditional transcriptional repressor but as activator. In order to reveal the molecular mechanism for dual function of ATF3, we started to purify ATF3 complexes from Hodgkin' s cell line, and identify the component of each complex. We anticipate ATF3 constitutes different complex as an oncogenic protein from as a stress-induced repressor.

Research 3: Mechanism of cell cycle arrest in terminal differentiation of cardiac cells and its re-activation both in vivo and in vitro

Cardiac cells cease to proliferate after birth and enter the terminal differentiation,-thus can not regenerate when they are damaged by cardiac infarction or cardiomyopathy. We have succeeded in re-activating cell cycle of cardiac cells by nuclear expression of cyclin D1 (D1NLS), an accelerator, and forced degradation of p27, a brake of cell cycle by Skp2.

3-1 Factors to prevent the nuclear expression of cyclin D1

Sole over-expression of wt Cyclin D1 cannot push cardiomyocyte cell-cycle forward efficiently. One of the reasons is the deterrent effect against nuclear import of cyclinc D1. To elucidate this mechanism, we are trying to identify the factor which retains Cyclin D1 at cardiomyocyte cytoplasm. Several ubiquitin ligases are expected to play roles for cyclin D1 localization and turn-over. Furthermore, we are investigating the possibility that peptide-decoy which traps this deterrent works as a cell-cycle accelerator.

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Research Interests

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 splicing, through which a single gene can generate structurally and functionally distinct protein isoforms. Based on genome wide analysis, 75% of human genes are thought to encode at least two alternatively spliced isoforms. The regulation of splice site usage, so called "splicing code" provides a versatile mechanism for controlling gene expression and for the generation of proteome diversity. Thus splicing code may play essential roles in many biological processes, such as embryonic development, cell growth, and apoptosis.

A Transgenic Reporter Worm System Offers a Path to Alternative Splicing Codes *in vivo*.

More than 90% of human genes have alternative mRNA isoforms. What is a spatiotemporal expression profile of each isoform? How are so many genes regulated *in vivo*? Regulation mechanisms of alternative splicing, however, have been studied mostly *in vitro* or in cultured cells.

We have recently developed a transgenic alternative splicing reporter system that visualizes expression profiles of mutually exclusive alternative exons of a nematode C. *elegans* at a single cell level *in vivo* (Nature Methods, 2006). With this reporter system, we have visualized spatiotemporal profiles of tissue-specific and developmentally regulated alternative splicing events in living worms. 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). Through these studies, we are coming to realize that molecular mechanisms of the alternative splicing regulation are conserved throughout metazoan evolution. Our reporter system will further elucidate expression profiles and regulation mechanisms of alternative splicing *in vivo*.

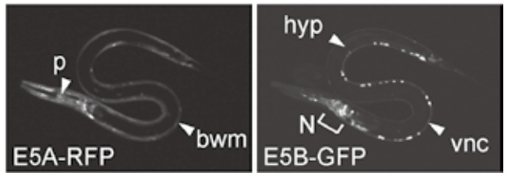


Fig1. An *egl-15* alternative splicing reporter worm with tissue-specific expression of exon 5A-RFP and exon 5B-GFP.

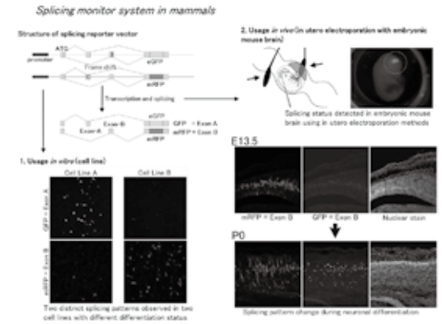
Regulating mechanism of alternative splicing and its physiological function during the development of mouse brain

Alternative splicing generates a large number of mRNA and protein isoforms from a limited number of gene subsets. In the brain, many important molecules are regulated their structures and functions with this system, like signaling receptors, cell adhesion molecules, transcription factors, and so on. This mechanism contributes to the functional complexity and high performance of the mammalian brain through making divergent protein subsets from small number of genes. Also many neuronal diseases are caused by the mis-regulation of alternative splicing, i.e.; neurodegenerative disease, mental disorder, neuronmuscular dystrophy, or brain tumors. However, the mechanism of gene-, cell-, organ-, developmental stage-, and brain structure-specific regulation of alternative splicing is still mostly unknown.

We are developing a monitoring system of alternative splicing during the development of the brain using a fluorescent-based reporter vector system, and revealing their dynamism in single cell resolution. With this system, we screen and select molecules that are essential for regulating alternative splicing, and exam their biological function during development. This project will reveal the regulating mechanism of alternative splicing and its important roles from *in vitro* to *in vivo*.

mRNA splicing regulation and virus infection.

Although the viral genome is often quite small, it encodes a broad series of proteins. The virus takes advantage of



the host-RNA-processing machinery to provide the alternative splicing capability necessary for the expression of this proteomic diversity. Serine-arginine-rich (SR) proteins and the kinases that activate them are central to this alternative splicing machinery. We originally developed SR protein phosphorylation inhibitor 340 (SRPIN340), which preferentially inhibits SRPK1 and SRPK2. SRPIN340 suppressed propagation of HIV, herpes simplex virus (HSV) type 1 and 2, Sindbis virus, SARS virus, and cytomegalovirus. These observations have led us to apply SRPIN340 to an antiviral drug. We are also going forward synthesis of series of SRRPIN derivatives and testing the effect of these compounds on viral replication. Some SRPIN compounds dramatically inhibit the replication of hepatitis C virus, influenza virus, and Dengue virus.

Furthermore, we showed herpesvirus protein ICP27 changes the alternative splicing of *promyelocytic leukemia protein* (PML) pre-mRNA to affect virus replication (*NAR*, 2009). Our paper is first report showing the relationship between virus replication and host alternative splicing in detail.

Development of Novel Specific Inhibitors of "PSYCHIK" Family Kinases and their Potentials as Pharmaceutical Drugs

We have previously reported the development of SRPIN340, a specific inhibitor of SR protein kinase (SRPK) family, and, TG003, a specific inhibitor of cdc2-like kinase (Clk) family. To date, they are the only specific inhibitors for SRPK and Clk, respectively. Significantly, we also proved that SRPIN340 is a potent anti-viral agent.

We are further developing novel inhibitors of other protein kinase families that are phylogenetically related to SRPK and Clk. SRPK family and Clk family are closely related protein kinase families, and they constitute a larger family of phylogenetically related protein kinases together with dual-specificity tyrosine-(Y)-phosphorylation regulated kinase (DYRK) family, Homeodomain interacting protein kinase (HIPK) family, and human pre-messenger RNA processing 4 protein kinase (PRP4). We propose to call the entire family as PSYCHIK family (PRP4, SRPK, DYRK, Clk, HIPK Family). These kinases are suggested to play important physiological roles including development and normal functioning of central nervous system, regulation of apoptosis, and pre-mRNA splicing.

We focus on PSYCHIK family members as targets for discovery of specific inhibitors, and indeed some novel specific inhibitors have been obtained.

We have characterized the new compounds through 1) in vitro assays, 2) in cell functional assays, 3) X-ray crystallography, and 4) whole embryo development assay. The findings demonstrated that the compounds are not only useful biological tools, but are potential drug seeds for hitherto untreatable diseases.

Splicing regulation and stress response

Cells are often exposed to circumstance changes and various kinds of stresses such as canceration, infection, hypoxia condition, heat shock or radical toxicity. In cells, there are mechanisms to protect the cell function and survival from these stresses by regulating expression levels and functions of related stress-responsive genes. As reported by numerous researchers, some stress-responsive genes are regulated the expressions and the functions by stress-induced splicing change. However, the mechanisms underlying the stress-associated splicing regulations are not clear. Then, to reveal the splicing regulation dependent stress response system, we analyze the mechanism of splicing regulation of a model gene, of which splicing is actually changed by some kind of stress or circumstance change, using our techniques for splicing reporter system and molecular/cellular biology techniques.

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Division of Medical Genomics
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Introduction of Department of Epigenetics

"Epigenetics" coupled with "Genetics" enables us to elucidate several 'genomic functions' in inheritance, development and evolution of organisms including our human beings. Genomic imprinting is one of the mammalian specific gene regulation mechanisms 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. Our department focuses on these mammalian specific genomic functions to elucidate how these genomic functions work and how new genomic functions have been evolved during evolution. Our final goal is to contribute to the establishment of 21th's medicine and human biology by understanding of such genomic functions.

Latest researches

1. How does embryo manipulation technologies affect on transcriptome of embryos and neonates?

Embryo manipulation technologies, such as *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI), are widely and successfully applied not only to fields of livestock and laboratory animal production but also to human assisted reproductive technologies (ART). However, long-term influence of these technologies remains elusive. Many cohort studies on the children conceived by ICSI and/or *in vitro* fertilization (IVF) have been established in attempts to investigate the risks of congenital malformations, developmental delays in organs, growth retardation, and mental impairments. Some studies reported mild delays in organ developments, growth, and psychosocial activities in these groups, while others found no difference. Epidemiological approach has its limits in distinguishing the causes of phenotypic variances; whether they are really caused by the technology of sperm injection or they originate from genetic abnormalities of the sperms used. Obviously, other factors, such as genetic background and environment surrounding of the children including family structure, education and training available should also be considered for accurate evaluation. Thus, focusing the effects in the fertilization step of ICSI or IVF, we have been carrying out systematic experiment using genetically defined mouse model.

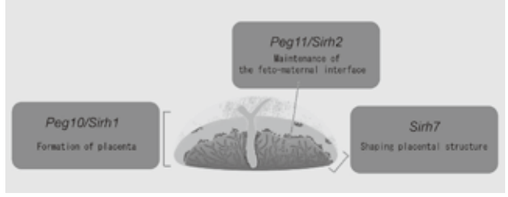

Fig1. ***Peg10, Peg11/Rtl1* and *Sirh7* genes play essential but different functions in placenta formation in mice.** Gene targeting experiments of *Peg10*, *Peg11/Rtl1* and *Sirh7* demonstrate that these genes are essential for placenta formation, maintenace of the feto-maternal interface exchanging nutrients and gases between fetuses and mother, and formation of proper placental structure, repectively. All these three genes are derived from a certain kind of retrotoransposons, indicating integrated retrotransposons turned to essential endogenous genes during mammalian evolution.

2. Roles of Mammalian-specific genes in the evolution of viviparity

Viviparity is one of the most familiar characteristics of therian mammals, eutherians (including human beings) and marsupials. Placenta is a novel evolutionary organ essential for viviparous reproductive systems: it supplies nutrients and oxygen to fetuses growing in the uterus. The eutherians and the marsupials adopt different reproductive strategies: the former usually give birth to fully matured pups after a long gestation period while the latter deliver relatively tiny and immature pups after a short gestation period, and feed them in the mother's pouch by lactation for a very long period. Interestingly, the eutherians and the marsupials use different types of placenta: a chorioallantoic placenta and a choriovitelline placenta (yolk sac placenta), respectively. It is reasonable to hypothesize that the difference in placental performance (or efficiencies of nutrient and gas exchanges) led to the diversification of the two viviparous mammals during evolution.

To address this question, we have analyzed three retrotransposon-derived genes, *PEG10, PEG11/RTL1* and *SIRH7*. The former exists only in the therian mammals and the latter two in the eutherian mammals. Gene targeting experiments in mice demonstrated that these genes plays essential but different functions in formation of placenta, respectively, suggesting that they have contributed to the establishment and diversification of mammals via placental formation in crucial ways (Fig. 1 and 2).

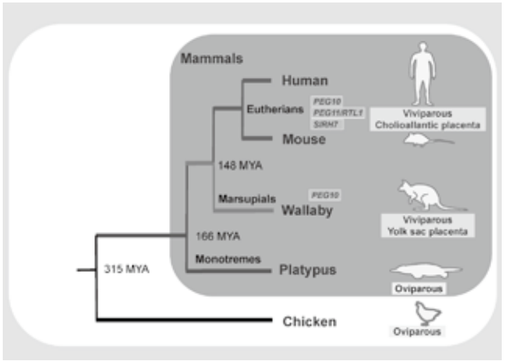

Fig2. **Genetic origins of *PEG10, PEG11/RTL1* and *SIRH7***
Comparative genomics study clarified that *PEG10* exists in both eutherians and marsupials, however, *PEG11/RTL1* and *SIRH7* in only eutherian mammals, supporting our hypothesis that the difference in placental performance (or efficiencies of nutrient and gas exchanges) led to the diversification of the two viviparous mammals during evolution.

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Presentation at International meetings

1) Fumitoshi Ishino, Ryuichi Ono, Shunsuke Suzuki, Yoichi Sekita, Mie Naruse, Takashi Kohda and Tomoko Kaneko-Ishino. Retrotramnspopsons and Evolution of Genomic Imprinting and Placentation in Mammals. Symposium: Epigenetic impacts for differentiation and patterning. The 42nd Annual Meeting for the Japanese Society of Developmental Biologists. May 28-31, 2009 (Toki Messe, Niigata).
2) Fumitoshi Ishino, Ryuichi Ono, Shunsuke Suzuki, Yoichi Sekita, Mie Naruse, Takashi Kohda and Tomoko Kaneko-Ishino. Contribution of Retrotransposons to the Evolution of Genomic Imprinting and Placentation in Mammals The 24th NAITO Conference on Nuclear Dynamics and RNA (II) June 23-26, 2009 (Chateraise Gateaux Kingdom, Sapporo, Hokkaido).
3) Fumitoshi Ishino. Retorotransposons and trophoblast biology. Trophoblast Day Meeting. July 14-15, 2009 (University of Cambridge, UK).
4) Fumitoshi Ishino. Retorotransposon-derived imprinted genes, *Peg10* and *Peg11/Rtl1* and their relation to the origin of viviparity in mammals. From Imprinting to the Epigenome in 25 years. September 4-6, 2009 (University of Cambridge, UK).

Laboratory of Systems Biology, School of Biomedical Sciences
Department of Bioinformatics, Medical Research Institute

Professor	Hiroshi Tanaka
Associate Professor	Yoshihito Niimura
Assistant Professor	Soichi Ogishima
Visiting Professor	Hiroki Nogawa (-July), Hiroshi Mizushima (Oct-)
Visiting Assistant Professor	Isao Yamaguchi (-Oct), Yasen Mahmut (Oct-)
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Project Assistant Professor	Takeshi Hase, Naoki Hasegawa, Kaei Hiroi, Keisuke Ido, Kaoru Mogushi, Satoshi Shoji, Masaki Morioka

In our laboratory, we conduct biological and medical researches from the viewpoint of Systems Biology.

Biological sciences: Recently, the whole genome sequences of diverse organisms have become available. Moreover, various "omix" information such as a proteome, transcriptome, and metabolome are currently accumulating. Our goal is to establish a grand-theory of biological sciences from the viewpoint of "evolving networks composed of biological molecules" by integrating omix information.

Medical sciences – Genomic and omix data are also utilized in the field of medicine. It has been revealed that most diseases are caused by the interaction among abnormalities of multiple genes, those at the tissue level, and environments. It is therefore possible to consider diseases as a system. From this standpoint, we try to establish the omix-based medicine.

Omics-based study of disease mechanisms

Due to recent advances in life science research, comprehensive data such as genome, transcriptome, and proteome can be routinely obtained. In order to interpret such genome-wide data in clinical research, we need to apply bioinformatics analysis such as data mining, statistical analysis and machine learning in combination with existing biological and medical knowledge.

We focus on development and application of bioinformatics methodology and have been conducting collaborative works with several research laboratories including following topics: (1) identification of gene sets and their interaction networks associated with phenotypes and prognosis of hepatocellular carcinoma (HCC) patients, (2) expression analysis of Aurora kinase B and alternative variant forms in HCC, (3) analysis of HCV-associated gene expression and cell signaling pathways, (4) identification of IQGAP1 and vimentin as a key regulator genes in naturally occurring hepatotumorigenesis induced by oxidative stress, and (5) identification of MUC12 as a prognosis marker in colorectal cancer.

Evolution of olfactory receptor gene families

Olfaction is essential for the survival of animals. Versatile odor molecules in the environments are received by olfactory receptors (ORs), which form the largest multigene family in vertebrates. Identification of the entire repertories of OR genes from the whole genome sequences revealed that the numbers of OR genes vary enormously, ranging from ~1,200 in rats and ~400 in humans to ~150 in zebrafish and ~15 in pufferfish. Extensive phylogenetic analyses suggested that the numbers of gene gains and losses are extremely large in the OR gene family. It appears that OR gene repertoires dynamically changed depending on each organism's living environment. For example, higher primates equipped with a well-developed vision system have lost a large number of OR genes. Moreover, two groups of OR genes for detecting airborne odorants have greatly expanded after the time of terrestrial adaption in the tetrapod lineage, whereas fishes retain diverse repertoires of genes that were present in aquatic ancestral species. The origin of vertebrate OR genes can be traced back to the common ancestor of all chordate species, but insects, nematodes, or echinoderms utilize distinctive families of chemoreceptors, suggesting that chemoreceptor genes had evolved many times independently in animal evolution.

Systems evolutionary biology

Our mission is to understand both (1) evolution and (2) dynamics of biological systems based on omics data from the point of view of "systems evolutionary biology".

(1) Evolutionary studies on biological systems are to understand evolution of life not only as gene evolution but also as systems evolution. We are focusing on evolution of both transcriptional networks of development and large-scale protein interaction networks. The former is to analyze evolution of Hox transcriptional networks reconstructed by our novel promoter analysis, while the latter is to reveal functional modularity in protein network evolution.

(2) Dynamical studies of biological systems are studies for revealing mechanism of transcriptional regulation by developing novel algorithm for trend analysis on time-series microarray data and by developing novel 3D visualization application of hierarchical molecular network based on the central dogma.

Transdisease Omics analysis of cancer by SAGE

Recently, comprehensive information on various biomolecules such as genes and proteins (Omics information) can be obtained easily by rapid advance of the molecular biological experimental technique. Therefore, drawing out of clinical useful information is becoming possible by comparing these molecular information of various human diseases and revaluing the similarity between the diseases. We have analyzed comprehensive gene expression data of 11 human diseases obtained from GEO (Gene Expression Omnibus). In this research, SAGE(serial analysis of gene expression) data was chosen to analyze gene expression data, and the comparison analysis was performed among several cancer samples. As a result, we have found that the combination of gene expression pattern of Breast cancer and Prostate cancer are similar in the 11 diseases samples. We were able to find that there is a similar character for the malignant alteration, and it has a similar treatment method, when we focus on the common feature of these carcinoma.

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15. Suzuki A, Takai-igarashi T, Numabe Y, Tanak H: Development of a database and ontology for pathogenic pathways in periodontitis, In Silico Biol, 9:1-11, 2009
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[Reviews]

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Frontier Research Unit Redox Response Cell Biology

Associate Professor Shun-Ichi Kurata

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

Induction of p63 by the keratinocyte-specific TGF-β I signal with SMAD2 and IKK α

Unlike *p53* which is ubiquitously expressed to exert the tumor-suppressing function, *p63* (*TP63, p51*) is required for development of epithelia including the skin and oral tissues. High-level expression of p63 occurs not only in keratinocyte stem cells of normal stratified epithelia but also in squamous cell carcinomas (SCCs) of head-and-neck and skin as well as in carcinomas of cervix, urothelia, breast, etc. After the intensification in lower-grade carcinomas, however, *p63* expression diminishes during the malignant progression. Although various genes induced by p63 have been reported (14-19), it remains obscure how transcription of *p63* is enhanced at the limited stages of the specific lineages in epithelial development and cancer progression. We report here that the major promoter of p63, which drives transcription of ΔNp63, is activated by a recently identified keratinocyte-specific TGF-β signal pathway, where IκB kinase α (IKK α) instead of Smad4 serves as a cofactor of Smad2. This activation is suppressed by IKK α-silencing and by Smad7, an antagonist of Smad2. TGF-β1 induces endogenous *p63* expression in SCC line A431. Furthermore, both p63 and IKK α were essential to maintain the non-migratory phenotype of A431 cells. p63 may be a key mediator of the tumor suppressing function of the keratinocyte-specific TGF-β signal. We propose a mutual amplification mechanism between p63 and IKK α.

Lung-lung interaction in isolated perfused unilateral hyperventilated rat lungs

High tidal volume (TV) ventilation-induced lung inflammation including remote organs has still been open to discussion, and our aim is to determine this issue in isolated ventilated rat lungs perfused with salt solution. Selective right lung (RL) hyperventilation ZEEP or PEEP and left lung (LL) on CPAP for 60 min was realized after 30-min both lung ventilation by occluding the left main bronchus, and allocated to five groups: hyperventilation under ZEEP and 3 under PEEP with re- or non-recirculation, Control; (recirculation means the same perfusate recirculates the system throughout the procedure). Wet dry ratio and protein content of bronchoalveolar lavage fluid (Prot-BALF), cytokine mRNAs, localization of TNF-α and its concentration in the perfusate and BALF in each lung were measured and compared between groups. Lung injury was shown in the hyperventilated RLs with ZEEP compared to their corresponding CPAP LLs, and PEEP prevented these injury. Lung injury was also proved in the recirculated LL compared to the non-recirculated LL. Unilateral hyperventilated lungs with ZEEP induced TNF-α, permeability increase, and injured the control lung via perfusion.

Publications (Original papers)

Lung-lunginteraction in isolated perfused unilateral hyperventilated rat lungs
Asan B, Kurata, S., Mitaka, C., and Imai, T. ***Translational Research*** 154:228-237, 2009

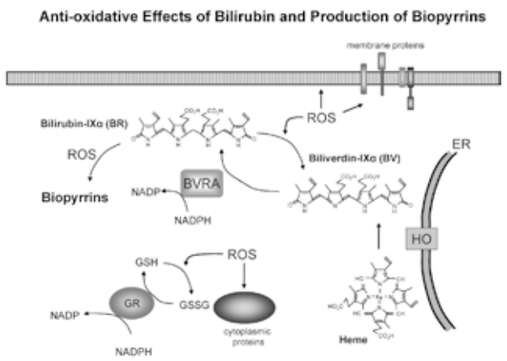
Salvage of non-ischemic control lung from injury by unilateral ischemic lung with apocynin, an NADPH (nicotinamide adenine dinucleotide phosphate) oxidase inhibitor, in isolated perfused rat lung. Chenting, Z., Kurata, S., Mitaka, C., and *Imai, T. ***Translational Research*** 152:273-282, 2009

Project Research Unit

Project Research Unit

(Associate Professor; Tokio Yamaguchi)

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



Research projects

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

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Pathophysiology

(Associate Professor Saburo Horikawa)

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

Publications

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2. Noda Y, Eto K, Horikawa S, Sasaki S. Water transport in the renal tubules. JIN to TOSEKI. 67(3): 333-338, 2009.

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Annual report 2009: Project Research Unit: Medical Genomics

(Associate Professor Shinobu Sakamoto, MD, PhD)

(Assistant Professor Shuji Sassa)

Damage to the proximal tubular epithelial cells of the kidney and a loss of connectivity of cancellous bone in the epiphysis and of trabecular bone in the metaphysis of the distal femur were observed in iron-overloaded rats with a reduction of femoral bone mineral density, i.e. reabsorption of calcium from the proximal tubular epithelial cells of the kidney might be affected and urinary discharge of calcium might be elevated. Gender differences were observed in the bone loss, i.e. the bone mineral density was more affected in male rats than the females by the iron-overload. Daily walking for more than one hour and fish intake reduce plasma levels of lipids in postmenopausal Japanese healthy women. Supplement with eicosapentaenoic acid reduces levels of lipids in plasma and liver in mice fed a high-fat diet.

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Medical Genomics

(Associate professor Michinori Kubota)

The spatiotemporal neural responses to center stimuli were a little weaker than those to contralateral stimuli in the left and right core fields of the auditory cortex. Especially, in fields P and VP, responses to center or ipsilateral stimuli were much weaker than those to contralateral stimuli. These results suggest that caudal fields in the belt regions of the auditory cortex are related to processing of azimuthal information.

International Meeting

Hosokawa Y, Kubota M, Horikawa J. Optical imaging of azimuthal activities in multi-fields of the left and right guinea-pig auditory cortices.
J Physiol Sci, Vol 59, Suppl 1, 193. The XXXVI International Congress of Physiological Sciences, 2009.

Affiliated Institutes

Department of Biosystem Generation

Professor **Takehiko Sasazuki, M. D., Ph. D.**
Associate Professor **Koji Furukawa, Ph. D.**

1. Analysis on function and structure of cells (Fig. 1)

Cell-based 3D culture systems provide a suitable approach bridging the gap between 2D culture and animal models. KRAS mutations are found at high frequencies in human colorectal cancer (CRC), however, KRAS-targeted cancer therapy has not been developed. We have established 3D cell culture model resembling the colonic crypt by use of HKe3 cells, human CRC HCT116 cells disrupted at activated KRAS. In this 3D colonic crypt model, HKe3 cells showed the features of time course-dependent transit amplifying and terminal differentiated stages, but not HCT116 cells, suggesting that activated KRAS inhibited normal cell polarity and apoptosis in 3D culture. The expression of DNA repair-related tumor suppressor genes was markedly suppressed by activated KRAS in 3D culture, but not in 2D culture. These results together suggest that activated KRAS plays critical roles in accumulation of genetic alterations through inhibition of DNA-repair genes and apoptosis and that this 3D culture model will provide a useful tool for investigating the molecular mechanisms of CRC development.

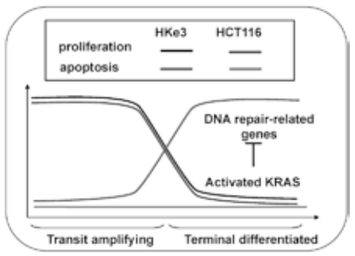

Fig.1 Mutant KRAS inhibits apoptosis and DNA repair in microenvironment.

2. Development of neutralizing monoclonal antibody (human type) against SARS coronavirus

As a safe immunotherapeutic approach against SARS, hIgG neutralizing SARS-CoV should be effective in clearing the viruses from affected patients. Transgenic mouse and cow producing hIgGs constructed by using the transchromosomic (TC) technology allowed us to prepare such hIgG. To immunize those animals, recombinant SARS antigens was designed based on the genome information of SARS-CoV, which made it possible to avoid handling of the dangerous SARS-CoV, were used to immunize the transgenic animals. The recombinant antigens successfully induced antibodies showing neutralizing activity against SARS-CoV in vitro. As the results, we obtained the fully human SARS-CoV-neutralizing monoclonal antibody 5H10. 5H10 recognized an epitope, which is found dominantly in the convalescent sera from SARS patients and a cleavage site critical for the entry of SARS-CoV into host cells. 5H10 inhibited the fusion process between virus envelope and host cell membrane. Evaluation of the efficacy of 5H10 in an in vivo SARS model in Rhesus macaques showed that it inhibited propagation of the virus as well as pathological changes found in infected animals. This study also represents a platform to produce fully human antibodies against emerging infectious diseases in a timely and safe manner.

3. A crucial role of Ly49Q in neutrophil polarization and migration by regulating raft trafficking. (Fig.2)

Blood neutrophils become polarized and move quickly to immediately infiltrate inflammatory sites. To do this successfully, neutrophils possess a particular competence for polarization and directional movement. However, the molecular basis for the specific behaviors of neutrophils is still largely unknown.

Ly49Q is an ITIM-bearing receptor belonging to NK receptor family and recognizes classical MHC class I molecules, and is preferentially expressed on Gr-1+ cells, including neutrophils and plasmacytoid dendritic cells. We found, using Ly49Q-deficient mice, that a MHC class I receptor, Ly49Q, plays a critical role in the ability of neutrophils to become polarized and infiltrate extravascular tissues. In the presence of inflammatory stimuli, Ly49Q mediated rapid neutrophil polarization and tissue infiltration in an ITIM-domain-dependent manner. However, in the steady-state, Ly49Q inhibited neutrophil adhesion by preventing focal-complex formation, likely by inhibiting Src and PI3 kinases. Ly49Q associated with the inhibitory phosphatase SHP-1 in the steady-state, but it recruited SHP-2, which plays a largely positive role in cell activation, adhesion, and migration, in the presence of inflammatory stimuli. Therefore, these apparently opposite functions of Ly49Q in the steady-state and the inflammatory state appeared to be mediated by recruiting an additional associated effector phosphatase. We also demonstrated that Ly49Q was responsible for the organization of a certain type of raft and for the correct partitioning of Src to the rafts, with the correct timing. We propose that Ly49Q is pivotal in switching neutrophils to their polarized morphology and rapid migration upon inflammation, through its spatiotemporal regulation of membrane rafts and raft-associated signaling molecules. These switching functions of Ly49Q permit the rapid reorganization of neutrophils in the presence of inflammatory signals, and maintain neutrophil homeostasis in the steady-state.

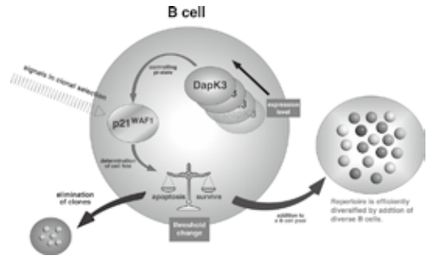

Fig.2 A crucial role of Ly49Q in neutrophil polarization and migration by regulating raft trafficking

4. A molecule important for controlling diversity of antibodies. (Fig.3)

Recently, we found augmented expression of DapK3 in IgG1+ B cells whose immunoglobulin repertoire was relatively convergent in comparison with those bearing the other IgG subclasses; e.g., the expression level of DapK3 in IgG2b+ B cells was relatively low. This molecule was known as a kinase for $p21^{WAF1}$, a key molecule determining fate of cells (*i.e.,* survive or apoptosis). In this study, to confirm whether DapK3 is involved in diversity control of antibodies, we examined influence of DapK3 expression upon apoptosis induced by ligation of B cell antigen receptors (BCRs) using a cell line, WEHI-231. At first, we constructed lentivirus expression vector allowing for inducible expression of DapK3, in which flip-flop Cre-loxP recombination was used for strict control of the gene expression. Using this vector, we could easily obtained stable transfectants of WEHI-231 cells harboring an inversed DapK3 gene flanked by loxP sequences. When DapK3 was induced by introducing Cre into these cells, apoptosis provoked by BCR-ligation increased by 100 percent. In contrast, induction of mutant DapK3 lacking its kinase activity resulted in half efficiency of the BCR-ligation to the apoptosis (dominant negative effect). These results suggested that expression level of DapK3 was critical for threshold determining survival or apoptosis of cells in antigen-based B cell selection.

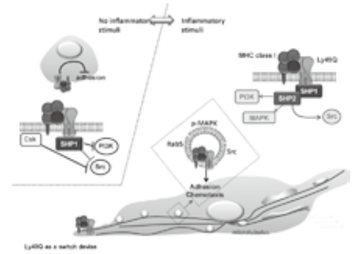

Fig.3 DapK3 plays an important for controlling diversity of antibodies.

List of Publication

1. Sasawatari S, Yoshizaki M, Taya C, Tazawa A, Furuyama-Tanaka K, Yonekawa H, Dohi T, Makrigiannis AP, Sasazuki T, Inaba K, Toyama-Sorimachi N. The Ly49Q Receptor Plays a Crucial Role in Neutrophil Polarization and Migration by Regulating Raft Trafficking. **Immunity,** in press, 2010
2. Haga S, Nagata N, Okamura T, Yamamoto N, Sata T, Yamamoto N, Sasazuki T, Ishizaka TACE antagonists blocking ACE2 shedding caused by the spike protein of SARS-CoV are candidate antiviral compounds. **Antiviral Res.** in press, 2009
3. Patrick MS, Oda H, Hayakawa K, Sato Y, Eshima K, Kirikae T, Iemura S, Shirai M, Abe T, Natsume T, Sasazuki T, Suzuki H. Gasp, a Grb2-associating protein, is critical for positive selection of thymocytes. **Proc Natl Acad Sci USA.** 106(38):16345-50, 2009
4. Yoo BH, Woo X, Li Y, Haniff M, Sasazuki T, Shirasawa S, Eskelinen EL, Rosen KV. Oncogenic ras-induced downregulation of autophagy mediator Beclin-1 is required for malignant transformation of intestinal epithelial cells. **J Biol Chem,** 285(8):5438-49, 2009
5. Nakabayashi K, Komaki G, Tajima A, Ando T, Ishikawa M, Nomoto J, Hata K, Oka A, Inoko H, Sasazuki T; Japanese Genetic Research Group for Eating Disorders (JGRED), Shirasawa S. Identification of novel candidate loci for anorexia nervosa at 1q41 and 11q22 in Japanese by a genome-wide association analysis with microsatellite markers. **J Hum Genet,** 54(9):531-537, 2009
6. Palmioli A, Sacco E, Airoldi C, Di Nicolantonio F, D'Urzo A, Shirasawa S, Sasazuki T, Di Domizio A, De Gioia L, Martegani E, Bardelli A, Peri F, Vanoni M. Selective cytotoxicity of a bicyclic Ras inhibitor in cancer cells expressing K-Ras(G13D). **Biochem Biophys Res Commun,** 386(4):593-597, 2009
7. Trobridge P, Knoblaugh S, Washington MK, Munoz NM, Tsuchiya KD, Rojas A, Song X, Ulrich CM, Sasazuki T, Shirasawa S, Grady WM. TGF-beta Receptor Inactivation and Mutant Kras Induce Intestinal Neoplasms in Mice via a beta-Catenin-Independent Pathway. **Gastroenterology,** 136(5):1680-1688, 2009
8. Fujimoto T, Doi K, Koyanagi M, Tsunoda T, Takashima Y, Yoshida Y, Sasazuki T, Shirasawa S. ZFAT is an antiapoptotic molecule and critical for cell survival in MOLT-4 cells. **FEBS Lett.,** 583(3):568-572, 2009
9. Fujimoto T, Miyasaka K, Koyanagi M, Tsunoda T, Baba I, Doi K, Ohta M, Kato N, Sasazuki T, Shirasawa S. Altered energy homeostasis and resistance to diet-induced obesity in KRAP-deficient mice. **PLoS ONE,** 4(1):e4240, 2009
10. Tsunoda T, Takashima Y, Fujimoto T, Koyanagi M, Yoshida Y, Doi K, Tanaka Y, Kuroki M, Sasazuki T, Shirasawa S. 3D-specific inhibition of DNA repair-related genes by activated KRAS in colon crypt model. **Neoplasia,** in press
11. Kuba, H., Furukawa K. : An optimized procedure for efficient phage display of antibody fragments with a low folding efficiency. ***Protein Expr. Purif.*** 65, 148-153, 2009

Integrative Research: Pathogenetic Regulation

Splicing variants are produced from more than 80% genes of human genome. We predicted whether the putative protein products by alternative splicing make stable conformations or not. We discussed the effect of splicing variants on protein net-works on the bases of the conformations of the protein variants. Also we study the post-transcriptional regulation of gene expression during development and differentiation.

1. Masafumi Shionyu, Akihiro Yamaguchi, Kazuki Shinoda, Ken-ichi Takahashi, and Mitiko Go, AS-ALPS: a database for analyzing the effects of alternative splicing on protein structure, interaction and network in human and mouse. Nucl. Acids Res. 37: D305-D309 (2009). http://nar.oxfordjournals.org/cgi/content/abstract/37/suppl_1/D305

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3. Kei Yura and Mitiko Go, Roles of RNA Editing in Land Plant Organelles, Biophysics (Seibutsubutsuri), 49(5), 244-255 (2009).

4. Mitiko Go, "Events during Information Flow from Genomic Sequence to Protein Structure/Function in Biological System", The 4th Global COE International Symposium 2009 Joint with the 19th Hot Spring Harbor Symposium "Molecular Evolution and Bioinformatics", 2009.11.1.

5. Mitiko Go, "Information Flow from Genes to Proteins", "International Symposium: Fifty Years of Biophysics Research at Nagoya University" March 12-14, 2010.

6. Fukumura, K., Taniguchi, I., Sakamoto, H., Ohno, M., & Inoue, K. U1-independent pre-mRNA splicing contributes to the regulation of alternative splicing. Nucl. Acids Res. 37, 1907-1914. (2009).

7. Fukumura, K., & Inoue, K. Role and mechanism of U1-independent pre-mRNA splicing in the regulation of alternative splicing. RNA Biol. 6, 395-398 (2009).

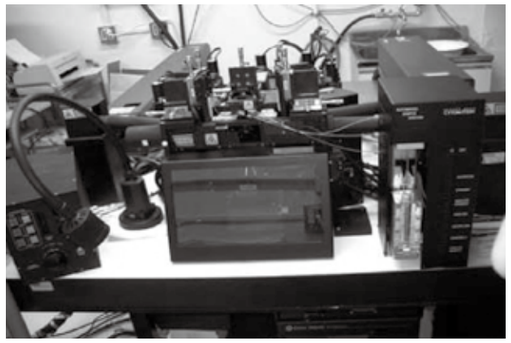
8. Takeda, Y., Mishima, Y., Fujiwara, T., Sakamoto, H., & Inoue, K. DAZL relieves miRNA-mediated repression of germline mRNAs by controlling poly(A) tail length in zebrafish. PLoS ONE 4, e7513 (2009).

Medical Research Institute
Advanced Technology Laboratories

Laboratory of Cytometry and Proteome Research

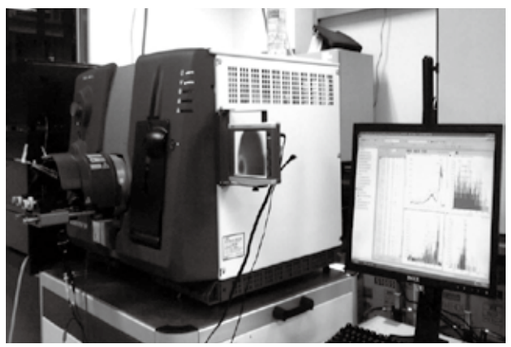
This laboratory will help researchers to separate specific cells and analyze the proteome expressed in the cells. To this end, we set up equipments written below.

MOFLO cell sorter can rapidly refine specific cells with high efficiency.



MOFLO cell sorter

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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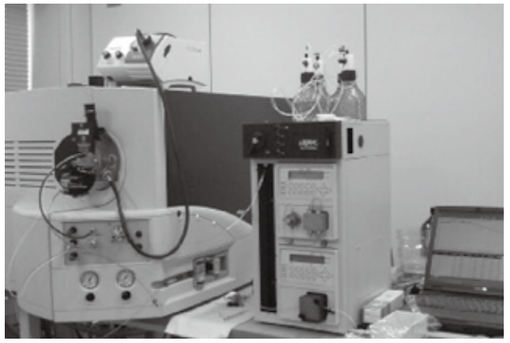


Q-Tofmicro

Laboratory for Structure Analysis

The Laboratory for Structure Analysis is the latest member of the Facilities and equipped with a high-brilliancy X-ray generator and an image plate X-ray detector. The Laboratory became fully operational last year and an introductory practical course was held for researchers from MRI and other departments of the university. Similar practical courses were also held for graduate students of School of Biomedical Science. (The laboratory has moved to the 22nd floor of the M&D Tower in February 2010.)

Medical Top Track (MTT) Program

MTT Fellow: Yoshiko Iwai
Technical staff: Shoko Kuroda
Research Project
The mechanisms of memory CD8 T cell development

Memory CD8 T cells are one of the most important components of protective immunity against viral infection and disease, and understanding their development is the necessary basis for developing effective vaccines. Upon antigen stimulation, most activated T cells differentiate into effector T cells and undergo cell death, but a small fraction of cells become memory T cells. IL-7 has received much attention as a possible mediator of the decision for long-term survival of memory T cells. IL-7R^{high} effector T cells have a greater potential to form memory T cells than IL-7R^{low} effector T cells. Although several transcription factors have been reported to regulate IL-7R expression, the *Il7r* transcriptional regulation during the memory T cell development is not well understood. By generating knock-in mice, we have shown that a novel AP-1 transcription factor plays an important role for memory T cell formation by regulating *Il7r* transcription (in submission). Since its expression is restricted to activated lymphocytes, this transcriptional factor will be a promising therapeutic target to control memory T cell formation in vaccine strategies and immunotherapies.

Publications

1. Okamoto, K., Iwai, Y., Oh-hora, M., Yamamoto, M., Morio, T., Aoki, K., Ohya, K., Jetten, A.M., Akira, S., Muta, T., and Takayanagi, H. IκBζ regulates TH17 development by cooperating with ROR nuclear receptors. Nature (*in press*)

Kou Nakayama
Aim of the Research

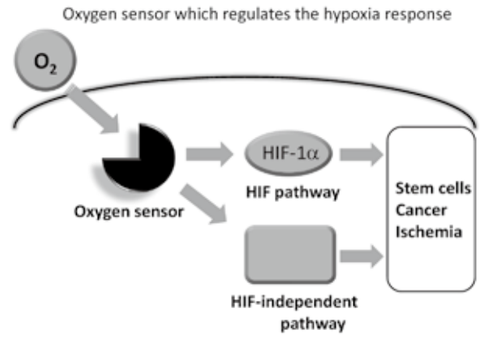
Study in our laboratory aims to understand how changes in the ambient oxygen concentration affects our body function. It is well known that oxygen is required for the efficient energy production in mitochondria. Furthermore, recent studies revealed the role of oxygen, particularly a hypoxic condition,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

Subjects of Research
1. Signal transduction of hypoxia response

Hypoxia-inducible factor (HIF)-1α is a transcription factor which plays a central role during hypoxia response by altering multiple cellular functions including metabolism, respiration, and cell growth. HIF-1α 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 level of HIF-1α We focus on PHD enzymes and study hypoxic cell signaling pathways which are connected to the HIF-dependent and -independent pathways.

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

Our recent study demonstrated the formation of 'hypoxia complex' under hypoxia condition which consists of PHD and other unidentified proteins. We hypothesized that the hypoxia complex contains an oxygen sensory molecule(s) and regulates the complex formation. We work on characterization of the hypoxia complex proteins identified in the proteomics approach to define the oxygen sensor. Ultimately, we challenge to invent a tool which would suppress the progression of hypoxic tumor by modifying the oxygen sensor.



Publications

Nakayama K. (2009) Cellular signal transduction of the hypoxia response. *J. Biochem.* 146, 757-65

MTT Fellow: Masaki Sone
MTT Technical Staff: Tayoko Tajima
Research Project
Study of neural development and neurodegeneration using *Drosophila* genetics

We have identified a novel *Drosophila* gene, *yata*. *yata* is required for neural homeostasis through the regulation of intracellular trafficking of APP and some related proteins. We further identified molecules that interacted with *yata* physically or genetically. Our results suggested that *yata* is involved in the conditional regulation of intracellular trafficking of some specific target proteins.

MTT Fellow: Hiroaki Hemmi
Research assistant: Miwa Hayashida
Research Project
The functional analysis of a new molecule expressed on myeloid lineage cells

As well as T and B lymphocytes, macrophages (Mφs) and dendritic cells (DCs) play crucial roles in the host defense. Recently, we identified a new molecule, Treml like 4, which expressed on a subset of Mφs and DCs. We also found that a soluble form of Treml4 showed an affinity to dead cells. Now, we are analyzing its physiological functions on immune system. Moreover, we are also focusing on bone metabolism, especially on osteoclasts which belong to myeloid lineage cells like DCs/Mφs and are essential for bone resorption and bone remodeling.

MTT Fellow: Atsushi Sato
Technician: Yoko Mitsutomo
Research Project
Characterization of *Drosophila* Corin.

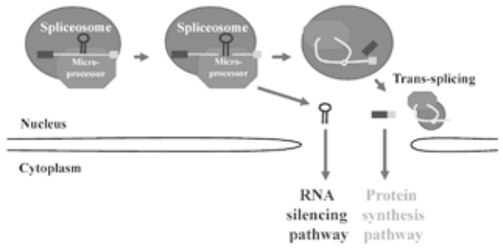
Human Corin functions as a conversion enzyme of ANP hormone, and is involved in the hypertension. I am focusing on the elucidation of function for *Drosophila* Corin (DCorin, DCorin2). *DCorin* mutant could rescue the phenotypes of gain of function mutant of EGFR. This suggests that DCorin is the new component of EGF signaling pathway. The overexpression of DCorin2 induced the loss of wing margin, indicating that DCorin2 had an important role for wing development. Further analyses for DCorin and DCorin2 will facilitate *in vivo* regulation and mechanisms in the development.

Publications

1. Sato, A., Ohnishi, E., Goto, T. (these three author contributed equally), Kim, M-S., Iemura, S., Natsume, T., Ohnishi, J. and Shibuya, H. (2010). Nemo-Like Kinase, an essential effector of anterior formation, functions downstream of p38 mitogen-activated protein kinase. *Mol. Cell Biol.* 30: 675-683.

MTT Fellow: Naoyuki Kataoka, Ph.D.

In higher eukaryotes, most genes in the nucleus are separated by introns. Introns often contain functional RNAs, such as snoRNA and miRNA. miRNAs are small RNAs that cause inhibition of translation or mRNA degradation. Analysis of human genome revealed that about 80% of miRNAs are encoded in introns of other genes. In order to analyze the biogenesis of intronic miRNAs, we have developed in vitro splicing reaction with pre-mRNA containing miRNA in its intron. We show splicing enhances excision of miRNA from intron, whereas the presence of miRNA in intron slows down splicing. Our results provide a molecular basis for the postulating existence of a pathway in which the Microprocessor complex associates with the spliceosome to produce two functional RNA molecules, miRNA and mRNA, from one pre-mRNA molecule. (687 letters)



MTT Fellow: Shingo SUZUKI

Research Summary

Brain-derived neurotrophic factor (BDNF) is a molecule which regulates the development and specific functions of CNS neurons. Although BDNF can control transcription and protein synthesis, it still remains unclear whether BDNF controls neuronal lipid homeostasis in brain. Our previous study indicated that BDNF-induced cholesterol biosynthesis in cultured CNS neurons is essential for synapse development. However its effects on cholesterol metabolism in vivo or on other lipid species are not fully investigated. For a better understanding of the effect of BDNF on lipid homeostasis in brain, we investigated the metabolomic analysis of neuronal lipids using Gas Chromatography-Mass Spectrometry (GC/MS), and profiled the brain lipids in BDNF-KO mice. Our results indicated the impairment of cholesterol biosynthesis and metabolism in BDNF-KO mice brain. This suggested that BDNF not only controlled brain cholesterol synthesis, but also regulated cholesterol metabolism in brain. Alterations of cholesterol homeostasis in brain are observed in neurodegenerative disorders, such as Alzheimer' disease. Therefore, we now focused on the role of cholesterol homeostasis in CNS neurons.

Publications

1. Koshimizu H, Kiyosue K, Hara T, Hazama S, Suzuki S, Uegaki K, Nagappan G, Zaitsev E, Hirokawa T, Tatsu Y, Ogura A, Lu B, Kojima M. Multiple functions of precursor BDNF to CNS neurons: negative regulation of neurite growth, spine formation and cell survival. Molecular Brain 2(1):27

MTT Fellow: Tetsuo Sasano

Research Project

Autocrine mechanism of extracellular nucleotide as a contributor of inflammation in initial process of atrial fibrillation.

Atrial fibrillation (AF) is the most common arrhythmia in the world. Since people with AF have a greater risk of stroke and heart failure, it is important to establish the treatment for AF. Recent findings indicate that atrial inflammation plays a critical role in the initiation and progression of AF. However the mechanism of inflammation is still not well understood.

We pursued to elucidate the mechanism of inflammation under mechanical stretch stimulation of atrium. Infiltration of macrophage was observed in enlarged atrium in animal model. This infiltration of macrophage was evoked by extracellular nucleotide released through gap junction channel on atrial myocyte. Extracellular nucleotides stimulate chemokine expression in myocytes by autocrine fashion, resulting in increased migration and activation of macrophage. This novel mechanism might be a new target for the treatment of AF.

Role of NOS1AP in ventricular repolarization and arrhythmogenicity.

Recent genome-wide association study revealed that single nucleotide polymorphism of NOS1AP (Nitric Oxide synthase 1 Adaptor Protein) was highly associated with QT interval and sudden cardiac death. We previously reported that overexpression of NOS1AP shortened action potential duration by reducing ICaL current in guinea pig ventricular myocyte.

We evaluate the role of NOS1AP in several pathological conditions using NOS1AP knockout mouse. This study may contribute to understanding a part of mechanisms of cardiac sudden death.

Publications

1. Sasano T, Kelemen K, Greener ID, Donahue JK: Ventricular tachycardia from the healed myocardial infarction scar: validation of an animal model and utility of gene therapy. Heart Rhythm 2009; 6: S91-7.
2. Johnston PV, Sasano T (contributed equally), Mills K, Evers R, Lee ST, Smith RR, Lardo AC, Steenbergen C, Gerstenblith G, Lange R, Marbán E. Engraftment, differentiation and functional benefits of autologous cardiosphere-derived cells in porcine ischemic cardiomyopathy. Circulation 2009; 120: 1075-83.
3. Kakusaka S, Asayama M, Kaihara A, Sasano T, Suzuki T, Kurokawa J, Furukawa T. A receptor-independent rffect of estrone sulfate on the hERG channel. J Pharmacol Sci. 2009; 109: 152-6.

MTT Fellow: Yukio Yamamoto

Research Project

Activation balance of nuclear receptors by estrogen regulates hepatic lipid metabolism

The sex difference in lipid metabolism is well documented. However, the underlying molecular mechanism of this symptom remains largely unknown. To elucidate the regulatory mechanism of hepatic lipid metabolism by estrogens, we investigate the roles of hepatic nuclear receptors: ERα (Estrogen Receptor α), which is an authentic estrogen receptor, and LXR (Liver X Receptor), which is regulator of hepatic lipid metabolism. Our current data suggest that the transcriptional crosstalk between LXR, and ERα may explain the suppression mechanism of lipid synthesis by estrogens.

MTT Fellow: Takeshi Matsui

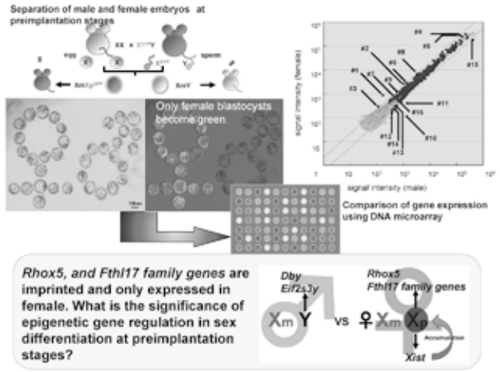
Analysis of Stratified Epithelial Differentiation

The epithelial cell layer is a cell sheet covers each individual tissue, establishing a separate space for the environment of each tissue. Placing particular emphasis on stratified epithelium, intends to uncover the mystery surrounding epithelial tissue. We previously identified a mouse homologue of skin-specific protease, SASPase. We have recently generated SASPase-deficient hairless mice. Biochemical and physiological analysis of epidermis of these mice are now underway.

Epigenetic regulation in male and female at preimplantation stages

Shin Kobayashi

When do sex differences first appear in mammals? To answer the question, our group tried to compare the gene expression patterns by sex before implantation. However, the sex of pre-implantation embryos is difficult to determine morphologically. So, utilizing the sex-determining method based on EGFP transgenic mouse, we collected more than 1000 sexed blastocyst samples. Furthermore, we compared the gene expression patterns of male and female blastocysts using DNA microarray, and found that nearly 900 genes showed differential expression between sexes at the stage! Among these, *Rhox5, Fthl17 family genes, and Xist* showed female specific expression. Using DNA polymorphisms, we successfully verified *Rhox5 and Fthl17 family genes* are imprinted and expressed from paternal X chromosome, like the *Xist* gene (Kobayashi, S. et al. Curr. Biol. 2006, and Kobayashi, S. et al. Nucleic Acids Res., In press). The discovery of imprinted genes only expressed in female suggests that there is epigenetic gene regulation in early sex differences. Now, we are trying to clarify the relationship between epigenetic gene regulation and mammalian sex differentiation and/or diseases.



Publications

Shin Kobayashi*, Yoshitaka Fujihara, Nathan Mise, Kazuhiro Kaseda, Kuniya Abe, Fumitoshi Ishino, Masaru Okabe (2010) The X-linked Imprinted Gene Family *Fthl17* Shows Predominantly Female Expression Following the 2-cell Stage in Mouse Embryos Nucleic Acids Res. In press (*: corresponding author)

Papers presented at meetings

Shin Kobayashi, Yoshitaka Fujihara, Nathan Mise, Kazuhiro Kaseda, Kuniya Abe, Fumitoshi Ishino, Masaru Okabe, Epigenetic regulation in male and female at preimplantation stages
THE 24th NAITO CONFERENCE, June 24th,2009,

Shin Kobayashi, Yoshitaka Fujihara, Nathan Mise, Kazuhiro Kaseda, Kuniya Abe, Fumitoshi Ishino, Masaru Okabe Discovery of X-linked Imprinted *Fat45* Family Genes Showing Female Predominant Expression following 2Cell Stage. 3rd Annual Congress of The Japanese Society for Epigenetics, May 22, 2009

Invitation to be plenary speaker

Shin Kobayashi The identification of female expressed X-linked imprinted genes.
54th Annual Meeting Japan Society for Reproductive Medicine
November 23rd 2009

MTT fellow: Akimitsu Konishi

Research assistant: Kyoko Tsujimura

Subjects of Research

1. Understanding of DNA damage signaling

2. Mechanism of the chromosome end protection

Research Summary

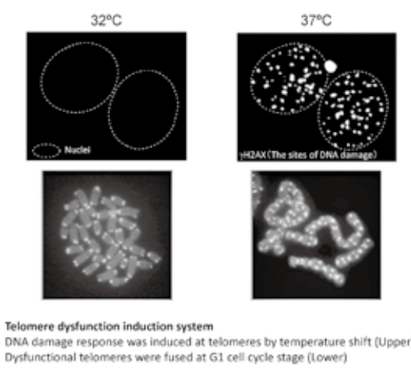
The biological response to DNA damage is one of the central issues in modern biology. The continuity of life depends on the ability of cells to protect their DNA from intrinsic and extrinsic damage and deficiencies in the DNA damage response can contribute to tumorigenesis, aging, hereditary disorder, and fertility problems. Eukaryotic chromosomes end in specialized structures, called telomeres, to cap chromosomal ends, preventing induction of DNA damage response. Our group studies DNA damage signaling using the dysfunctional telomere as a tool.

We have developed the system to control telomere function for chromosome ends protection (Figure, Genes Dev 2008).

Now, we are trying to apply this system to understand the general DNA damage responses. We also study the mechanism of telomere length regulation that is deeply involved in tumorigenesis and aging.

Grant

Takeda Science Foundation (2009)



Telomere dysfunction induction system
DNA damage response was induced at telomeres by temperature shift (Upper)
Dysfunctional telomeres were fused at G1 cell cycle stage (Lower)

Annual Report (MTT fellow) Jun Hirayama

Summary of Research

Circadian clocks are intrinsic, time-tracking systems that endow organisms with a survival advantage. Studies of animal models and human tumor samples have revealed that the disruption of circadian rhythms is an important endogenous factor that can contribute to mammalian cancer development. Recent studies have implicated the core circadian components in the regulation of both the cell cycle and DNA damage response. We are working on a mechanistic insight into the intricate interaction between genotoxic stress response and the circadian system.

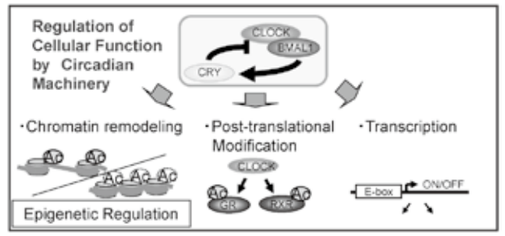


Fig.1 Circadian Control of Cellular Physiologies

Publications

1. Miyamura N*, **Hirayama J*#**, , Sawanobori K, Tamaru T, Asaoka Y, Honda R, Yamamoto T, Uno H, Takamatsu K, Nishina H. CLOCK:BMAL-independent circadian oscillation of zebrafish Cryptochrome1a gene. ***Biol. Pharm. Bull***. 32:1183-1189, 2009.
(*Contributed equally; #Corresponding author)

2. **Hirayama J#**, Miyamura N, Uchida Y, Asaoka Y, Honda R, Sawanobori K, Todo T, Yamamoto T, Sassone-Corsi# P, and Nishina H.
Common light signaling pathways controlling DNA repair and circadian clock entrainment in zebrafish.
Cell Cycle 8:2794-2801, 2009. **(#Corresponding author)**

3. Uchida Y, **Hirayama J#**, and Nishina H.
A common origin: signaling similarities in the regulation of the circadian clock and DNA damage responses
Biol. Pharm. Bull. In Press **(#Corresponding author)**

Chemical Biology Screening Center

Chemical biology is a new study to clarify the mechanism of biological phenomena by chemical method. Chemical Biology Screening Center (CBSC) was set up as the support facility to promote the chemical biology. Main aim of CBSC is 1) storage and distribution of small chemical compounds for screening, 2) support of the screening of chemical compounds and 3) preparing the platform for sharing the information about the chemicals which CBSC hold.

Currently, about 18,000 chemical compounds are available from CBSC. All compounds soluble in DMSO are distributed as the plate format or independently, according to the request. Equipments in CBSC are open to all staff and students in TMDU. Information about CBSC is available at the web site (http://www.tmd.ac.jp/mri/SBS/cbsc/index.html, currently only Japanese version is available). The TMDU Chemical Biology Database (CBDB, http://bsmdb.tmd.ac.jp/) has also been available to share the research information from the chemical compounds provided by CBSC.

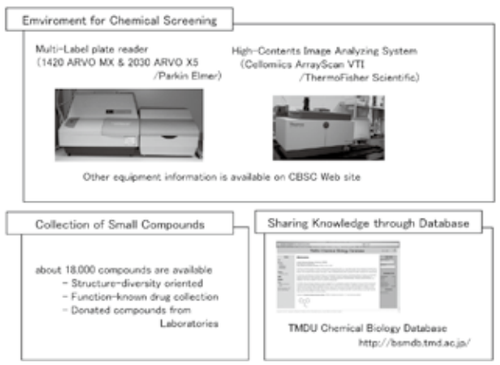


Fig.1: Overview of CBSC

School of Biomedical Science

Laboratory of Structural Biology

Professor	Nobutoshi Ito
Associate Professor	Teikichi Ikura
Adjunct Assistant Professor	Makoto Nakabayashi
Postdoctoral Fellow	Yuko Ogawa (Tsuchiya)
Postgraduate Students	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. Structural analyses of potential drug targets

Collaborations have been set up with other laboratories within and without the School for structural analyses of potential drug targets and their interaction with various compounds. Among them is the vitamin D receptor (VDR), which is described below as an example.

Hereditary vitamin D-resistant rickets (HVDRR) is caused by mutations in VDR. One of such mutations, W286R, fatally weakens the ligand binding ability of VDR and causes to serious defects of the function of VDR. Although a major therapeutic approach to HVDRR is intravenous injection of calcium, it must be continued for long period and fails to improve patients' quality-of-life. So development of other therapeutic methods for the mutant VDRs is desirable.

We determined the structure of the mutant VDR to elucidate why the point mutation inactivated the VDR and to obtain clues for novel therapeutic compounds for HVDRR patients. The W282R mutant of the ligand binding domain of rat VDR (R282 rVDR-LBD), which was corresponding to the W286R mutant of human VDR-LBD, was crystallized as a ternary complex with a natural hormone and a short peptide derived from the coactivator DRIP205. Its crystal structure was determined at 1.65 Å. To our surprise, the central β-strand, including Arg282, of the anti-parallel β-sheet obviously 'disappeared' in the W282R mutant (Fig.1A), suggesting it was highly disordered. It should be noted that despite such a drastic disorder in the secondary structure, the overall folds of the mutant remains very similar to that of the wild-type VDR (W282 rVDR-VDR) (Fig.1B). A novel compound which stabilizes the conformation of the central β-strand might rescue the function of the mutant VDR.

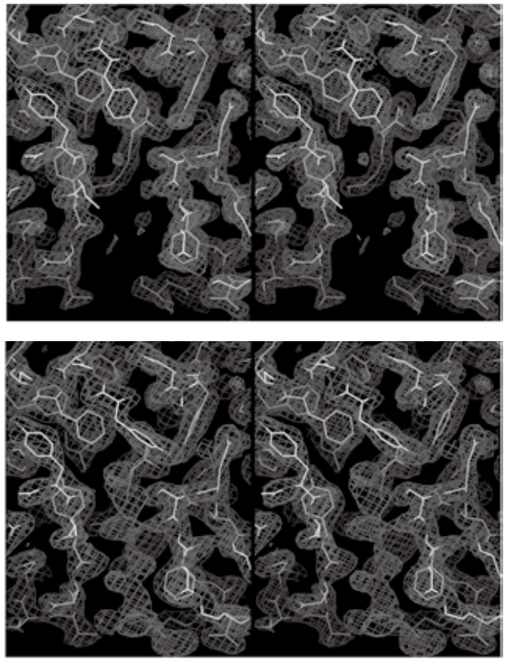


Fig1. Stereo views of electron density of the W282R mutant rVDR-LBD (A) and wild-type rVDR-LBD (B). $2F_{obs}$-F_{calc} maps (contoured at 1.5σ) around the anti-parallel β-sheet are shown. The W282R mutant and wild-type rVDR-LBD correspond to the HVDRR-mutant (W286R) and normal human VDR, respectively. The protein atoms are drawn as lines except residues from Asp278 to Gln287. The electron density of the region in the mutant (A) is missing whereas that of the wild-type (B) is clearly visible. This large change was caused by the single point mutation W282R.

2. Physicochemical analysis on the mechanism of the signal transduction for activation of T cells

T cells play a central role in cell-mediated immunity. Activation of T cells occurs through the engagement of both the T cell receptor (TCR) and CD28. Both are required for production of an effective immune response. The first signal for activation is provided by binding of the TCR to a short peptide presented by the major histocompatibility complex (MHC) on another cell (APC). The second signal comes from CD28, which is co-stimulated by CD86 (B7) of the APC. The cytoplasmic region of CD28 contains one SH2 and two SH3 binding motifs. The SH2 binding motif is critical for the recruitment of SH2-domain containing proteins, especially PI3K, Grb2 and Gads. These proteins include SH3 domains as well as SH2 domains. The SH2 binding to CD28 at its pYMNM motif is critical, while the SH3 binding to the PXXP site(s) in CD28 is still controversial.

The cytoplasmic region of CD28 was prepared by the peptide synthesis to specifically phosphorylate Tyr at the SH2 binding site and biotinylate at the N-terminus, and immobilized on the Biacore streptoavidin sensor chip to analyze the interaction with the three proteins. The binding affinities of their SH2-domains were different from one another; PI3K C-terminal SH2 > PI3K N-terminal SH2 > Grb2 SH2 ≅ Gads SH2. The binding affinities of intact Grb2 and Gads were about three-times and twenty-times higher than those of Grb2 SH2 and Gads SH2, respectively, indicating that their SH3s also contribute to the CD28 binding. However, unexpectedly, the binding affinity of intact Gads to the mutant CD28, in which Pro residues in the SH3 binding sites were mutated to Ala, was similar to that to the wild-type CD28.One of the possible explanations is that Gads SH3 might be able to bind the mutated sites in CD28. To confirm this possibility, we will prepare another CD28 mutant, in which the C-terminal residues containing the SH3 binding sites are deficient, and analyze its interaction with Gads.

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.

Research Papers

1. Inaba Y, Yoshimoto N, Sakamaki Y, Nakabayashi M, Ikura T, Tamamura H, Ito N, Shimizu M., Yamamoto K; A new class of vitamin D analogues that induce structural rearrangement of the ligand-binding pocket of the receptor. J. Med. Chem., 52, 1438-1449 (2009).

International conferences

1. Masuno H, Fujii S, Nakabayashi M, Ikura T, Ito N, Shimizu M, Kagechika H: Crystal structures of non-secosteroid ligands bound to vitamin D nuclear receptor. 14th Workshop on Vitamin D, Brugge (Belgium), October 2009.
2. Yamada S, Nakabayashi M, Ikura T, Ito N, Yoshimoto N, Shimizu M, Yamagishi K, Kudo T, Tokiwa H, Ogura M, Chuma M, Makishima M: X-ray crystal structures, biological activity and structure-activity relationships of novel vitamin D partial agonists/antagonists. 14th Workshop on Vitamin D, Brugge (Belgium), October 2009.
3. Inaba Y, Nakabayashi M, Itoh T, Yoshimoto N, Ikura T, Ito N, Shimizu M, Yamamoto K: 22S-Butyl-1α,24R-dihydroxyvitamin D3: Recovery of agonistic activity for vitamin D receptor. 14th Workshop on Vitamin D, Brugge (Belgium), October 2009.

Domestic conferences

1. Ikura T, Urakubo Y, Ito N: Co-evolutionary analysis of interactions between barnase and barstar. The 47th Annual Meeting of the Biophysical Society of Japan, Tokushima, 2009.
2. Higo K, Takahashi J, Oda M, Morii H, Ikura T, Ito N, Azuma T, Abe R: Purification of Gads and its interaction with CD28 cytoplasmic domains. The 47th Annual Meeting of the Biophysical Society of Japan, Tokushima, 2009.
3. Ohashi N, Okuda Y, Nomura W, Tsutsumi H, Serizawa Y, Ikura T, Ito N, Yoshida K, Lewin N E, Blumberg P M, Tamamura H: Synthesis and evaluation of fluorescent diacylglycerol-lactone derivatives. The 4th Annual Meeting of Japanese Society for Chemical Biology, Kobe, 2009.
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Laboratory of Chemical Bioscience

Professor Takamitsu Hosoya

Identification of Target Proteins of Bioactive Compounds by a Novel Method of Radioisotope-free Photoaffinity Labeling

Photoaffinity labeling (PAL) is a useful technique to identify target proteins of bioactive compounds including drugs and natural products. A probe utilized in PAL study consist of two functional groups: a photoreactive group to form a new covalent bond between the probe and its target biomolecule, and a detectable tag to distinguish the photolabeled biomolecule from unlabeled ones. These groups are required to be introduced to the original compound without diminishing its bioactivity.

We have established a novel protocol for radioisotope (RI)-free PAL based on the use of small bioorthogonal groups such as azido or ethynyl group (Fig. 1). In this scheme, these groups, relatively photo-stable under the photoreactive conditions of aryl azide and trifluoromethyldiazirine, is used as a post-modifiable group to introduce a detectable tag by azido-targeting reactions such as Staudinger–Bertozzi ligation and click chemistry.

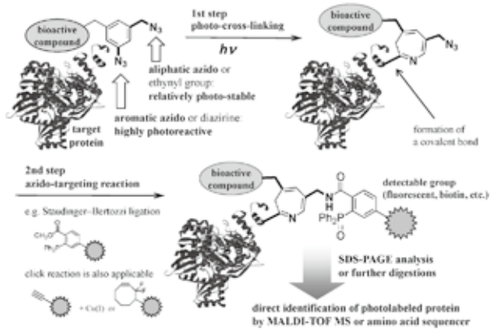

Fig. 1 Principle of RI-free photoaffinity.

This method has enabled the direct analysis of photolabeled proteins and, indeed, diazido-functionalized cerivastatin analogue, photovastatin CAA1 (Fig. 2A), diazidobenzlyated dantrolene derivative, GIF-0430 (Fig. 2B), and diazido-functionalized 1BnTIQ analogue, 1DAzBnTIQ (Fig. 2C) have worked efficiently as photoaffinity probes and resulted in successful fluorescent detection and direct analyses of their target proteins. We recently developed a facile preparative method of biaryl-type PAL probes by Suzuki–Miyaura coupling using 3-azido-5-(azidomethyl)phenylboronic acid pinacol ester as a common intermediate. These results will contribute to drug discovery researches by providing new target biomolecules.

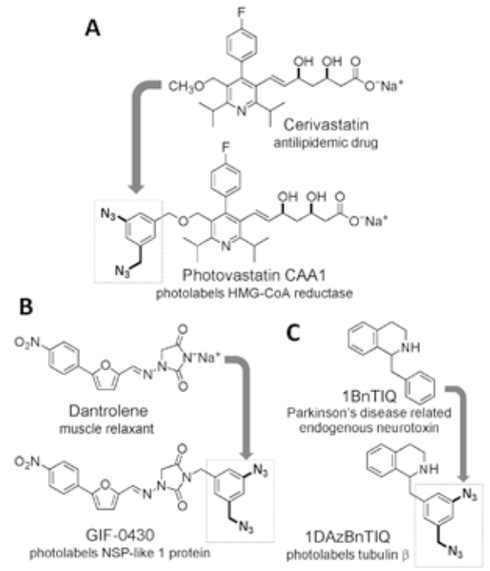

Fig. 2 RI-free PAL probes succeeded in photolabeling the target protein.

Design and Synthesis of Novel Efficient Substrates for Aequorin-related Bioluminescence Systems

Aequorin (AQ) is a Ca^{2+}-binding photoprotein, which was isolated from luminous jellyfish *Aequorea aequorea*. AQ consist of apoaequorin (apoAQ), a 21 kDa apoprotein, and 2-hydroperoxide of coelenterazine (CTZ), a small imidazopyrazinone derivative. Treatment of AQ with Ca^{2+} induces conformational change of the protein and causes flash emission of blue light (λ_{max} = 472.5 nm) accompanied by decomposition of CTZ hydroperoxide to coelenteramide (CTMD) and CO_2 (Fig. 3). The resulting complex of Ca^{2+}-bound apoAQ with CTMD is known as a blue fluorescent protein (BFP) (λ_{max} = 475 nm). Removal of Ca^{2+} from BFP affords a complex consist of apoAQ and CTMD, which also shows greenish fluorescence (λ_{max} = 485 nm) thereby referred to as gFP. Incubation of gFP with CTZ under the presence of oxygen regenerates AQ.

We recently found that BFP can be easily reconstituted almost quantitatively from recombinant apoAQ and synthetic CTMD just by mixing them in the presence of Ca^{2+} (Fig. 3). We also found that some semi-synthetic AQs prepared with CTZ analogues modified at the C2-position shows the slow-decay luminescence pattern and less sensitivity to Ca^{2+}. These semi-synthetic AQs were demonstrated to be applicable to G-Protein-coupled receptor (GPCR) assay.

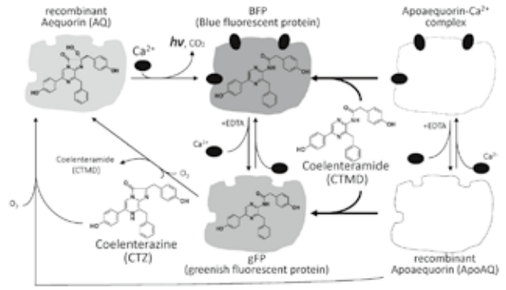

Fig. 3 Aequorin-related bioluminescence system.

Development of New PET Tracers for in vivo Molecular Imaging to Promote Drug Discovery

Positron emission tomography (PET) is an efficient method to image dynamically the disposition of drugs in whole body of humans. A positron-emitting radionuclide such as ^{11}C and ^{18}F with short half-lives of approximately 20 min and 110 min, respectively, is introduced to the structure of a compound of interest to prepare a PET tracer.

Aromatase, also known as cytochrome P450 protein CYP19, is an enzyme that converts androgens to estrogens. It is highly expressed in breast cancer tissues and also expressed in brain, which is considered to be involved in synaptic plasticity, neuroprotection, and emotional behavior. In order to investigate the functional role of aromatase expressed in the brain more precisely, we recently developed [^{11}C]cetrozole as a novel PET tracer, which showed a potent and selective inhibitory activity on aromatase. [^{11}C]Cetrozole possessing a *C*-$^{11}CH_3$ bond was found to be more metabolically stable than [^{11}C]vorozole having an *N*-$^{11}CH_3$ bond, the conventional PET tracer used so far for aromatase imaging. [^{11}C]Cetrozole showed less nonspecific binding in the cortices of monkey brain as compared with [^{11}C]vorozole. Also the significant expression of aromatase at nucleus accumbens was indicated for the first time by using [^{11}C]cetrozole. These results shows that [^{11}C]cetrozole is an efficient PET tracer for quantitative analyses of aromatase expressed in the brain and to assess the pathophysiology of diseases or disorders related to sex steroids in living body.

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School of Biomedical Science, Laboratory of Organic and Medicinal Chemistry

Professor **Hiroyuki Kagechika**
Assistant Professor **Shinya Fujii, Shuichi Mori,**

Research Outline

1. Medicinal Chemistry of Nuclear Receptors

Small hydrophobic molecules such as steroid hormones and activated vitamins A/D control various biological phenomena, including growth, development, metabolism, and homeostasis, by binding to and activating specific nuclear receptors. Nuclear receptors are ligand-inducible transcription factors that regulate the expression of their target genes. Nuclear receptors have become one of the most significant molecular targets for drug discovery in the fields of cancer, metabolic syndrome, autoimmune diseases, and so on. We have developed various agonists and antagonists of retinoid nuclear receptors, retinoic acid receptors (RAR α, β, γ) and retinoid X receptors (RXR α, β, γ) (Fig. 1). Among them, Am80 (tamibarotene, RAR α, β agonist) was approved as a drug for relapsed acute promyelocytic leukemia (APL) in Japan (2005).

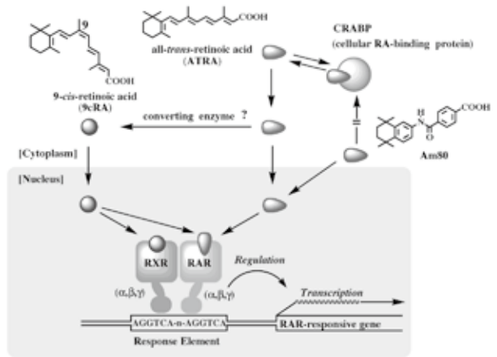

Fig. 1. Action mechanism of retinoic acid and synthetic retinoid, Am80.

We also developed novel vitamin D receptor (VDR) agonists by using carborane as the hydrophobic pharmacophore. Carboranes (dicarba-*closo*-dodecaborane) are icosahedral boron clusters with remarkable thermal and chemical stability. Among synthesized compounds, compound **1** exhibited potent VDR agonistic activity. Further, we succeded in the X ray crystallographical analysis of **1** bound to VDR, which showed the binding feature of **1** with VDR (Fig. 2).

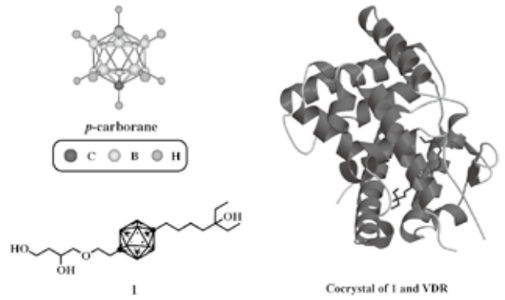

Fig. 2. Novel VDR agonistic carborane derivative and its binding structure to VDR

3. Development of Novel Functional Fluorescent Molecules for Elucidation of Intracellular Signal Transduction Pathways

Functional fluorescent molecules are useful in many fields of scientific research, including analytical chemistry or cell biology. This year, we developed novel fluorescent sensors by control of intramolecular complex formation between a fluorophore and another molecular species (Fig. 3). In this strategy, almost complete quenching of fluorescence was observed in aqueous media, due to the intramolecular heterodimer formation, and the fluorescence is restored by disruption of the heterodimerization in response to change of solvent polarity or protein binding. The design approach is expected to yield superior fluorescence sensors for proteins and for probing proximity relationships and structural transitions.

Fig. 3. Development of fluorescence sensor in response to environmental change

4. Aromatic Architecture Based on the Steric Properties of *N*-Methylated Amides

The amide bond structure of amide derivatives often plays a key role in functions such as molecular recognition events or biological activities. In contrast to the extended trans structures of most secondary amides, such as acetanilide and benzanilide, the corresponding *N*-methylated compounds exist in *cis* form in the crystals and predominantly in *cis* form in various solvents. The *cis* conformational preference is useful as a building block to construct aromatic molecules with unique crystal or solution structures. In this year, the dynamic helical structure of he aromatic multi-layered tetraureas was investigated in details, and the absolute structure of the helix was determined by empirical and theoretical analysis of CD and VCD spectra (Fig. 4).

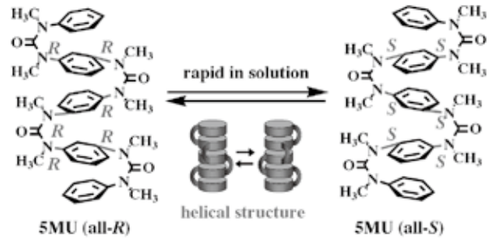

Fig. 4. Aromatic multi-layered urea with dynamic helical behaviors

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6. Kagechika, H. Development of Novel Nuclear Receptor Ligands for Cancer Therapy. 2009 TMD-CMU International Symposium, Tokyo, Oct. 2009.

Laboratory of Biosystem Modeling, School of Biomedical Science

Professor Tadashi Masuda
Associate Professor Yutaka Fukuoka

1. Systems Biology

(1) Molecular dynamics simulation for the docking process of myosin against an actin filament

Myosin is a motor protein that converts the chemical energy of ATP into mechanical work. In spite of the long history of research spanning over 50 years, the fundamental mechanism of this energy conversion is still unclear. There are many theories that try to explain the experimental observations, but none of them succeeded to establish a unified mechanism of motor proteins.

Under these circumstances, I have proposed a theory named "Driven-by-Detachment" mechanism. In this theory, the energy of ATP hydrolysis is mainly used to detach myosin from actin, and not directly for the mechanical power stroke. Myosin in the ADP or nucleotide-free state is attracted to an actin filament. The potential energy associated with this attractive force decreases during the docking process and can be converted into other form of energy such as mechanical work.

As the first step to reproduce the elementary processes assumed in the DbD mechanism, the docking of a myosin molecule against an actin filament is calculated with a molecular dynamics (MD) method (Gromacs code with Gromos96 force field and SPC water model).

The initial configuration was build from a three-dimensional structure of an actomyosin complex (PDB ID: 1M8Q). One molecule of myosin and seven molecules of actin were extracted from the structure. Four actin molecules located at both ends of the filament were fixed to the space. The remaining three actins and one myosin were moved according to the MD simulation. No nucleotide was assumed. The calculation was conducted over 12 ns with a step of 2 fs.

Initially the myosin is located close to the actin molecule on the minus side of the filament but about 1.5 nm away from the second actin on the plus side. After 4 ns, by changing the positions and the configurations, the myosin made a close contact with the second actin as predicted in previous studies.

In addition to muscle contraction, a various kind of cellular functions are supported by myosin family proteins. Therefore, the working mechanism of myosins is a fundamental and important problem of biology, but at the same time, it is based on the principles of physics and chemistry. In the energy conversion process, the related molecules are myosin, actin, ATP and water. Moreover, only the chemical reaction is the hydrolysis of ATP. If all the reactions involved in the energy conversion are computationally reproduced, we can understand the full detail of the working mechanism of myosin. The current molecular dynamics simulation is the first step toward that final goal.

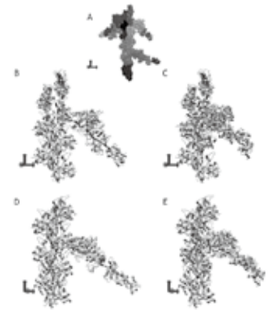

Fig.1. Docking of myosin against an actin filament
(A) Template structure in PDB (1M8Q). The myosin molecule at the lowest position in this figure was used in the MD calculation. (B) Initial configuration. (C) Configuration after 12 ns. (D) Initial configuration from a different view point. (E) Configuration after 12 ns from the same view point as (D). Initially the myosin did not make contact with the second actin in the plus-end direction, but after 4 ns, it made a contact as predicted in a previous study. This configuration was stable and did not change after 12 ns.

2. Bioinformatics

(1) Local effect of miRNAs on expressions of neighboring genes

MicroRNAs (miRNAs), which are non-protein-coding RNA molecules, are increasingly implicated in tissue-specific transcriptional control. Because there is mounting evidence for the localized component of transcriptional control, we investigated if there is a distance-dependent effect of miRNA. We have shown that expression levels of C. elegans genes are lower in the vicinity of 59 of 84 (71%) miRNAs as compared to genes far from such miRNAs and that the lower expression could be, in part, explained by an increased frequency of seed matching near miRNA. Further analyses on mouse and human data revealed that the localized effect in mammalian could be different from that in C. elegans.

To investigate the localized effect of miRNA in cancer, we analyzed gene and miRNA expressions in hepatocellular carcinoma (HCC) and surrounding non-tumor tissues (N=20). First, we analyzed gene expression levels around miRNAs. We then calculated the ensemble mean of gene expressions around the up- and down-regulated miRNAs in HCC (Analysis 1). A percentage of the miRNAs whose expressions correlated positively/negatively with gene expression was calculated in the vicinity of miRNA (Analysis 2.1). Next, the Pearson correlation coefficients were compared between the tumor and non-tumor tissues (Analysis 2.2). This analysis was repeated for intronic and intergenic miRNAs (Analysis 2.3). Finally, the correlation coefficient between miRNAs and their target genes was compared in the tumor and non-tumor tissues (Analysis 2.4).

The results of Analysis 1 indicated that the gene expression levels increased in the vicinity of the miRNAs that were up-regulated in HCC. From Analyses 2.1, 2.2 and 2.3, we found that in HCC, more miRNAs correlated with neighboring genes positively and that the correlation coefficient between the intronic miRNAs and their host genes was higher in HCC. Analysis 2.4 suggested that there was no significant difference between the correlation coefficients in the tumor and non-tumor tissues. These results suggest that the relationship between the intronic miRNAs and their host genes is altered in HCC.

(2) Adaptive threshold for detecting significant changes in microarray data

To detect significant changes in gene expression, a fixed threshold is used in various studies. However, it is not always guaranteed that a threshold which is appropriate for highly expressed genes is suitable for genes with low expression. In this study, aiming at detecting significant changes from a wide range of expression levels, we proposed an adaptive threshold method. The proposed method employs two independent measurements in the same condition to model the total error and divides the data sets into some bins according to the expression level in one data set. Then based on local variance of the data, upper and lower thresholds are calculated in each bin. The minimum requirement of the proposed method is two (or more) independent measurements in the same condition. The method is designed to detect biologically meaningful changes from a small size data set. However, in such a data set, there is a trade-off between suppressing the number of false positives and achieving the perfect detection of all meaningful changes. We focus on suppressing the number of false positives because it would make interpretation of the result easier.

To investigate the performance of the proposed method, we have simulated some data sets in which we know the subsets of genes that are up-regulated, down-regulated and unregulated, respectively. The data sets were constructed as follows. The mean values of 13 experiments from human hippocampus were used as the true values of the control data. Then zero-mean additive and multiplicative noises were added to the control data. The variance of the additive noise was varied in a range between 0.01 and 20 and that of the multiplicative noise was between 0 and 0.1. Figure 2 illustrates a relationship between the sensitivity and the variances of the noises. As the variance increases, the sensitivity decreases, but it is higher than 80% for all the variances tested. Similarly, the specificity is greater than 99% for all variances. These results demonstrate high performance of the proposed method.

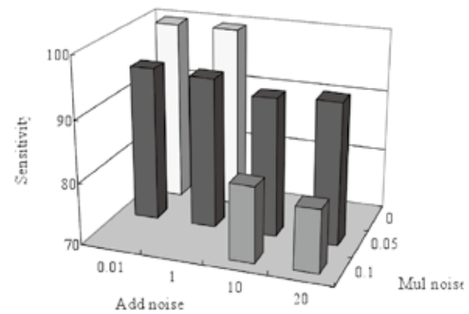

Fig.2. A relationship between the sensitivity and the variances of the additive and multiplicative noises.

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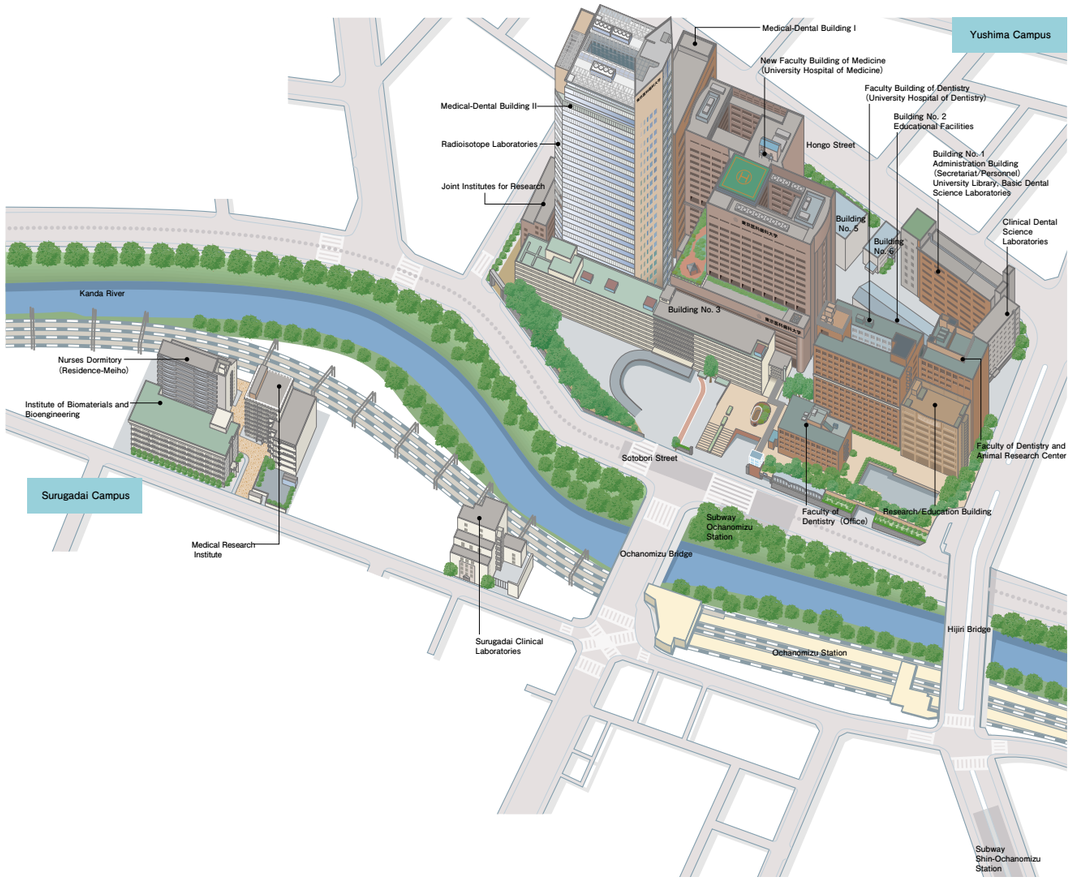

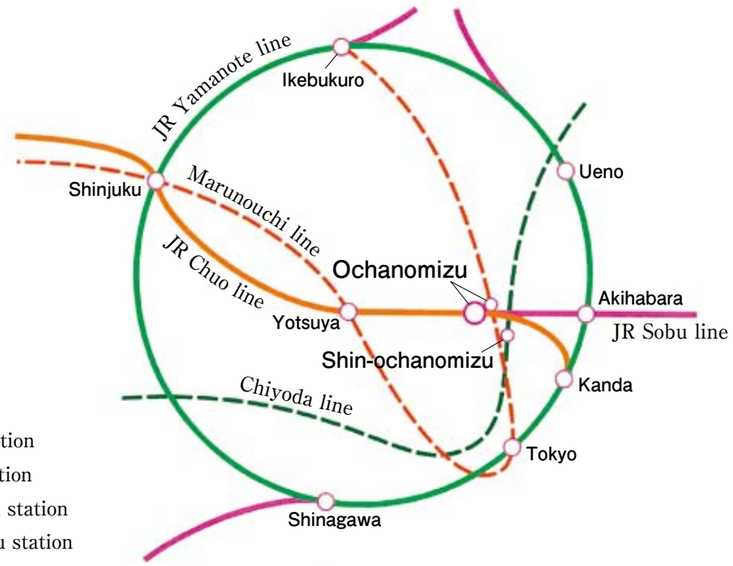

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