Donation Laboratories and Research Units

Division of Stem Cell Regulation (AMGEN)

幹細胞シグナル分子制御(アムジェン)寄付研究部門

Visiting Professor Ryuichi Nishinakamura, M.D., Ph.D. | 客員教授 医学博士 西中村 隆一

Division of Stem Cell Regulation is a donation laboratory supported by Amgen since 1995. Our research interest is to elucidate molecular mechanisms in organogenesis, especially kidney development. We also aim at derivation of kidney progenitors from stem cells, by utilizing knowledge obtained from molecular genetics. The lab was closed in March 2006, and we continue our research at The Institute of Molecular Embryology and Genetics in Kumamoto University, lead by Ryuichi Nishinakamura as a professor (http://www.imeg.kumamoto-u.ac.jp/divisions/integrative_cell_biology/).

Organogenesis-Molecular mechanisms of kidney development

The kidney develops in three stages: pronephros, mesonephros, and metanephros. Many of the genes expressed in the metanephros are also found in the pronephros. Animal caps, a presumptive ectoderm of *Xenopus* embryos at the blastula stage, differentiate into three-dimensional pronephric tubules in three days in chemically defined saline solution upon treatment with activin and retinoic acid. We have used this system to identify molecules expressed in pronephros and potentially in mesonephros and metanephros. One of the genes we isolated was *Xsal-3*, a newly identified *sal* member of *Xenopus*, which was expressed in the pronephros and the brain. We then cloned a member of the murine *sal* family from the developing kidney, which proved to be a mouse homolog of human *SALL1*.

SALL1 is a mammalian homolog of the *Drosophila* region-specific homeotic gene *spalt* (*sal*) and heterozygous mutations in *SALL1* in humans lead to Townes-Brocks syndrome. We isolated a mouse homolog of *SALL1* (*Sall1*) and found that mice deficient in *Sall1* die in the perinatal period and that kidney agenesis or severe dysgenesis are present. *Sall1* is expressed in the metanephric mesenchyme surrounding ureteric bud and homozygous deletion of *Sall1* results in an incomplete ureteric bud outgrowth, a failure of tubule formation in the mesenchyme and an apoptosis of the mesenchyme. This phenotype is likely to be primarily caused by the absence of the inductive signal from the ureter, as the *Sall1* deficient mesenchyme is competent regarding epithelial differentiation. Therefore *Sall1* is essential for ureteric bud invasion, the initial key step for metanephros development.

We are currently examining molecular functions of *Sall1*. In addition, we are trying to establish an induction system of kidney progenitors from a variety of cell sources, and also in vitro and in vivo assays for kidney progenitors. Our final goal is to understand molecular mechanisms of kidney development and to utilize the knowledge for derivation of kidney progenitors for cell therapy.

1. Identification of multipotent progenitors in the embryonic mouse kidney by a novel colony-forming assay

Kenji Osafune and Ryuichi Nishinakamura

Renal stem or progenitor cells with a multilineage differentiation potential remain to be isolated, and the differentiation mechanism of these cell types in kidney development or regeneration processes has been unknown. In an attempt to overcome this issue, we set up an in vitro culture system using NIH3T3 cells stably expressing *Wnt4* (3T3Wnt4) as a feeder layer, in which a single renal progenitor in the metanephric mesenchyme forms colonies consisting of several types of epithelial cells that exist in glomeruli and renal tubules. We found that only cells strongly expressing *Sall1* (Sall1-GFP^{high} cells), a zinc finger nuclear factor essential for kidney development, form colonies, and that they reconstitute a three-dimensional kidney structure in an organ culture setting. We also found that Rac- and c-Jun N-terminal kinase (JNK)-dependent planar cell polarity (PCP) pathways downstream of Wnt4 positively regulate the colony size, and that the JNK pathway is also involved in mesenchymal-to-epithelial transformation of colony-forming progenitors. Thus our colony-forming assay, which identifies multipotent progenitors in the embryonic mouse kidney, can be used for examining mechanisms of renal progenitor differentiation.

2. Mouse homolog of SALL1, a causative gene for Townes-Brocks syndrome, binds to A/T-rich sequences in pericentric heterochromatin via its C-terminal zinc finger domains

Kazunari Yamashita, Akira Sato, and Ryuichi Nishinakamura

The *Spalt* (*sal*) gene family is conserved from *Drosophila* to humans. Mutations of human *SALL1* cause Townes-Brocks syndrome, with features of ear, limb, anal, renal and heart anomalies. *Sall1*, a murine homolog of *SALL1*, is essential for kidney formation, and both Sall1 and SALL1 localize to heterochromatin in the nucleus. Here, we present a molecular mechanism for the heterochromatin localization of Sall 1. Mutation analyses revealed that the 7th-10th C-terminal double zinc finger motifs were required for the localization. A recombinant protein of the most C-terminal double zinc finger (9 th-10th) bound to specific A/T-rich sequences. Furthermore, Sall1 associated with A/T-rich sequences of the major satellite DNA in heterochromatin. Thus, Sall1 may bind to A/T-rich sequences of the major satellite DNA via its C-terminal double zinc fingers, thereby mediating its localization to heterochromatin.

3. The murine homolog of *Sall4*, a causative gene in Okihiro syndrome, is essential for embryonic stem cell proliferation, and cooperates with *Sall1* in anorectal, heart, brain and kidney development.

Masayo Sakaki-Yumoto, Chiyoko Kobayashi[1], Akira Sato, Sayoko Fujimura[1], Yuko Matsumoto, Nobuaki Yoshida[2], and Ryuichi Nishinakamura: [1]Division of Integrative Cell Biology, Institute of Molecular Embryology and Genetics, Kumamoto University, [2]Laboratory of Gene Expression and Regulation, IMSUT

Mutations in *SALL4*, the human homolog of the *Drosophila* homeotic gene *spalt* (*sal*), cause the autosomal dominant disorder known as Okihiro syndrome. We show that a targeted null mutation in *Sall4* lead to lethality during periimplantation. Growth of the inner cell mass from the knockout blastocysts was reduced, and *Sall4*-null embryonic stem cells proliferated poorly with no aberrant differentiation. Furthermore, we demonstrated that anorectal and heart anomalies in Okihiro syndrome are caused by *Sall4* haploinsufficiency and that *Sall4/Sall1* heterozygotes exhibited an increased incidence of anorectal and heart anomalies, exencephaly, and kidney agenesis. Sall4 and Sall1 formed heterodimers, and a truncated Sall1 caused mislocalization of Sall4 in the heterochromatin; thus, some symptoms of Townes-Brocks syndrome caused by SALL1 truncations could result from SALL4 inhibition.

4. *Six1* and *Six4* are essential for *Gdnf* expression in the metanephric mesenchyme and for ureteric bud formation, while *Six1* deficiency alone causes mesonephric tubule defects

Hiroki Kobayashi, Kiyoshi Kawakami[3], and Ryuichi Nishinakamura: [3]Division of Biology, Center for Molecular Medicine, Jichi Medical University

Interaction between the ureteric bud epithelium and metanephric mesenchyme is important for kidney development. *Six1* and *Six4* are the mammalian homologs of *Drosophila sine oculis*, and they are coexpressed in the nephrogenic mesenchyme. *Six1*-deficient mice show varying kidney defects, while *Six4*-deficient mice have no apparent abnormalities. Here, we report the

generation of *Six1/Six4*-deficient mice to elucidate the functions of *Six4* in *Six1*-deficient kidney development. The *Six1/Six4*-deficient mice showed kidney phenotypes more severe than those of the *Six1*-deficient mice; agenesis of kidney and ureters was observed in all neonates examined. The *Six1/Six4*-deficient metanephric mesenchyme cells were specified to the kidney lineage but did not express *Pax2*, *Pax8*, or *Gdnf*, whereas the expression of these genes was partially reduced or unchanged in *Six1* deficiency. Thus, *Six4* cooperates with *Six1* in the metanephric mesenchyme to regulate the level of *Gdnf* expression, which could explain the absence of the ureteric bud in *Six1/Six4* deficiency. In contrast, *Six1* deficiency alone caused defects in mesonephric tubule formation, and no exacerbation was observed in the *Six1/Six4*-deficienct mesonephros. These results highlight the fact that *Six1* and *Six4* have collaborative functions in the metanephros but not in the mesonephros.

5. Synergistic action of Wnt and LIF in maintaining pluripotency of mouse ES cells.

Kazuya Ogawa[4], Ryuichi Nishinakamura, Yuko Iwamatsu[4], Daisuke Shimosato[4], and Hitoshi Niwa[4]: [4]Laboratory for Pluripotent Cell Studies, RIKEN Center for Developmental Biology

Leukemia inhibitory factor (LIF) was the first soluble factor identified as having potential to maintain the pluripotency of mouse embryonic stem (ES) cells. Recently, a second factor, Wnt, with similar activity was found. However, the relationship between these completely different signals mediating the overlapping functions is still unclear. Here, we report that the conditioned medium of L cells expressing Wnt3a maintains ES cells in the undifferentiated state in feeder-free culture, followed by expression of stem cell markers and their ability to generate germline chimaeras. However, although the activity of this conditioned medium is dependent on Wnt3a, recombinant Wnt3a protein cannot maintain ES cells in the undifferentiated state. As supplementation with Wnt3a to the subthreshold level of LIF was sufficient to maintain ES self-renewal, the results of maintenance of the undifferentiated state indicated the synergistic action of Wnt and LIF. Induction of constitutively activated beta-catenin alone is unable to maintain ES self-renewal but shows a synergistic effect with LIF. These observations indicate that the Wnt signal mediated by the canonical pathway is not sufficient but enhances the effect of LIF to maintain self-renewal of mouse ES cells.

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Donation Laboratories and Research Units

Division of Cell Processing (CERES Consortium)

細胞プロセッシング(CERES)寄付研究部門

Visiting Professor	Tsuneo A. Takahashi, D.Sc.		客員教授	理学博士	高 橋 恒 夫
Visiting Research Associate	Koichi Igura, Ph.D.		助 手	食品栄養科学博士	伊 倉 宏 一
Visiting Research Associate	Xiaohong Zhang, MD., Ph.D.		助 手	医学博士	張 暁 紅

Division of Cell Processing was established in 1995 to develop cell-processing technology and support the clinical departments of IMSUT through cell therapy. This division established cord blood bank in 1997 (Tokyo Cord Blood Bank) and has stored 6.000 units and registered more than 4,000 units in Japan Cord Blood Bank Network, International Cord Blood Organization NETCORD, cord blood association AsiaCORD and Bone Marrow Donor Worldwide, and shipped 455 units to transplant centers by the end of 2006. This facility obtained the certification of ISO 9002: 1994 in March 2000 and ISO 9001: 2000 in May 2003. We support the clinical departments through cord blood transplantation for patients with hematological malignancies. We study the expansion of hematopoietic stem cells including CD34＋ cells, NK, NKT progenitor cells and mesenchymal stem cells in cord blood. Using this cord blood banking system, we have started the research on regeneration medicine using placenta-derived and cord blood -derived mesenchymal progenitor and stem cells and those could differentiate into osteoblasts, chondrocytes, adipocytes, cardiomyocytes, neural cells and other type of cells. Those placenta-derived and cord blood derived mesencyhmal cells should be considered as one of the possible allogeneic cell sources for cell therapies and tissue engineering.

1. Cord Blood Banking in IMSUT:

Masako Hirai[1], Tokiko Nagamura-Inoue[1], Koichi Igura, Xiaohong Zhang, Tsuneo A. Takahashi: [1]Department of Cell Processing and Transfusion Medicine, IMSUT

Since 1977 we have started collecting and processing cord blood for clinical use. The collection, processing and cryopreservation are based on the "Guidelines for Umbilical Cord Blood Processing and Transplantation, 2002" developed by Japan Cord Blood Bank Network and the standards of FACT and NETCORD organization. In order to meet the requirements of these standards, we established Tokyo Cord Blood Bank with the Donated Blood Distribution Foundation and Nihon University. Our facility adopted the international quality assurance system, ISO (International Organization for Standardization and Organization) 9002: 1994 first time in Japan as cell processing center and upgraded ISO9001: 2000. We have established NETCORD and AsiaCORD with major banks in

the world. Through these networks, more than 13 CB units were shipped to foreign countries, such as USA, Chile, Vietnam, New Zealand, UK and Australia. As an administrative office of AsiaCord, we exchange information of cord blood banking and transplantation as well as regenerative medicine using cord blood. As it is important to analyze the CBT result as one of the validation of the cord blood units and improvement, we analyzed CBT result with CB processed in Tokyo CBB, in collaboration with Eurocord (European Research on Cord Blood Banking and Use for Transplantation) and JCBN. This cord blood bank will keep the effort to grow with high quality valid to the world by developing new technology for processing and other measures.

2. Chondrogenic differentiation of mesenchymal progenitor cells derived from chorionic villi of human placenta

Xiaohong Zhang, Koichi Igura, Tsuneo A. Takahashi

Human mesenchymal stem cells (MSCs) are currently being studied extensively because of their capability for self-renewal and differentiation to various connective tissues, which makes them attractive as cell sources for regenerative medicine. We report the isolation of human placenta-derived mesenchymal cells (hPDMCs) that have the potential to differentiate into various lineages (Igura et al.: Isolation and characterization of mesenchymal progenitor cells from chorionic villi of human placenta. Cytotherapy 6, 1-11, 2004).

To explore the possibility of using these cells for regeneration of cartilage, we first evaluated the chondrogenesis of hPDMCs in vitro and then embedded the hPDMCs into an atelocollagen gel to make a cartilage-like tissue with chondrogenic induction media. Our results suggested the possibility that hPDMCs might be usable as an alterative cell source for this cellular therapy because the number of chondrocyte isolates from the knee is limited. For in vivo assay, transplantation of the preinduction hPDMC-loaded collagen sponge into node mouse subcutaneous result in production of cartilage tissue with cells within lacunae surrounded by a large amount of metachromatic matrix compared to in vitro culture, indicated that differentiated hPDMCs could produce a substantial cartilage matrix in the in vivo environment. In the osteochondral defect transplantation, the original defect was covered with stiff reparative tissue, which was white, and had a smooth surface at 6 weeks after surgery Histological analysis showed that the cartilage formation within reparative tissue. The results of these in vivo and in vitro studies suggested that hPDMCs can be one of the possible allogeneic cell sources for tissue engineering of cartilage (Zheng et al., BBRC, 2006)

3. Immortalization of mesenchymal progenitor cells derived from human placenta and the differentiation abilities of immortalized cells

Xiaohong Zhang, Yasushi Soda[2], Koichi Igura, Hitoshi Satoh[3], Satoru Yamaguchi[4], Kenzabro Tani[5], Arinobu Tojo[2], Tsuneo A. Takahashi: [2]Division of Molecular Therapy, Advanced Clinical Research Center, IMSUT, [3]Laboratory of Tumor Cell Biology, Department of Medical Genome Sciences, Graduate School of Frontier Sciences, The University of Tokyo, [4]Yamaguchi Hospital, [5]Division of Molecular Genetics, Medical Institute of Bioregulation, Kyushu University

As shown above, we found that mesenchymal progenitor cells derived from chorionic villi of the human placenta could differentiate into 16 osteoblasts, adipocytes, and chondrocytes under proper induction conditions and that these cells should be useful for allogeneic regen17 erative medicine, including cartilage tissue engineering. However, similar to human mesenchymal stem cells (hMSCs), though these pla18 cental cells can be isolated easily, they are difficult to study in detail because of their limited life span in vitro. To overcome this problem, 19 we attempted to prolong the life span of human placenta-derived mesenchymal cells (hPDMCs) by modifying hTERT and Bmi-1, and 20 investigated whether these modified hPDMCs retained their differentiation capability and multipotency. Our results indicated that the 21 combination of hTERT and Bmi-1 was highly efficient in prolonging the life span of hPDMCs with differentiation capability to osteo22 genic, adipogenic, and chondrogenic cells in vitro. Clonal cell lines with directional differentiation ability were established from the 23 immortalized parental hPDMC/hTERT + Bmi-1. Interestingly, hPDMC /Bmi-1 showed extended proliferation after long -term growth 24 arrest and telomerase was activated in the immortal hPDMC/Bmi-1 cells. However, the differentiation potential was lost in these cells. 25 This study reports a method to extend the life span of hPDMCs with hTERT and Bmi-1 that should become a useful tool for the study of 26 mesenchymal stem cells (Zheng et al. BBRC).

4. Isolation of mesencyhmal stem cells from cord blood

Kouichi Igura, Xiaohong Zhang, Tsuneo A. Takahashi

The mesencyhmal stem cells in bone marrow have been studied extensively and now being used for clinical trials to support hematopoietc stem cell transplantation by reducing GVHD, to treat ischemia, and treat myocardial damage etc. Cord blood contains mesencyhmal stem cells but the number of these cells is estimated to be less than 1% of those in the marrow. We have started isolating mesencyhmal stem and progenitor cells from cord blood collected after delivery of neonates. The cord blood was collected, shipped to the facility, mononuclear cells were isolated on Ficoll and these cells were adhered to plates and cultured. The preliminary data indicates that time between delivery and isolation of mononucleated cells is important for the high recovery of these cells as well as the volume of cord blood units. These cells could differentiate to bone and cartilage cells as well as neural type cells.

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Donation Laboratories and Research Units

Division of Genetic Diagnosis (Otsuka)
ゲノム情報応用診断(大塚製薬)寄付研究部門基礎分野

Visiting Associate Professor Ituro Inoue, M.D.
Visiting Research Associate Atsushi Tajima, Ph.D.

客員助教授 医学博士 井ノ上 逸 朗
助 手 理学博士 田 嶋 敦

Our ultimate research goal is to develop personalized therapies for the common metabolic diseases of civilization by direct application of accumulating genomic information to basic and clinical medicine. As a first step, we try to understand complexity of human genome by studying linkage disequilibrium and haplotype structure. Next, we try to map the disease gene loci using non-parametric linkage study and linkage disequilibrium analysis. Accordingly, we try to identify susceptibility genes for common or otherwise clinically relevant diseases of metabolism such as ossification of posterior longitudinal ligament of the spine and intracranial aneurysm and analyze the molecular causality.

1. Genetic susceptibility of intracranial aneurysm

Hiroyuki Akagawa, Boris Krischek, Atsushi Tajima, Ituro Inoue

The rupture of an intracranial aneurysm leads to a subarachnoid hemorrhage (SAH), a sudden onset disease which can lead to severe disability and death. Several risk factors such as smoking, hypertension and excessive alcohol intake are associated with SAH. Intracranial aneurysms, ruptured or unruptured, can be treated either surgically via a craniotomy (through an opening in the skull) or endovascularly by placing coils through a catheter in the femoral artery. Even though the etiology of intracranial aneurysm formation is mostly unknown, several studies support a certain role of genetic factors. In reports so far genome-wide linkage studies suggest several susceptibility loci that may contain one or more predisposing genes. Studies of several candidate genes report association with intracranial aneurysms. To date, no single gene has been identified as responsible for intracranial aneurysm formation or rupture. The identification of susceptible genes may lead to the understanding of the mechanism of formation and rupture and possibly lead to the development of a pharmacological therapy.

Several studies have attempted to identify genes contributing to susceptibility to IA, focusing on candidates for allelic association in genes that encode mostly matrix proteins such as endoglin, lysyl oxidase, and matrix metalloproteases, but did not yield consistent results. Genetic linkage study of IA families represents a more systematic approach. In the first genome-wide linkage study of IA with 104 Japanese affected sib pairs, we identified a significant linkage to chromosome 7q11 (MLS=3.22 near *D7S2472*). Following up on our previous genome-wide linkage study in Japanese population, we extensively analyzed a 4.6 Mb linkage region around *D7S2472* on 7q11 by genotyping 168 SNPs. SNP association and window scan haplotype-based association studies revealed a susceptibility locus for IA on a single LD block covering the 3'UTR of *ELN* and the entire region of *LIMK1*. Association study with 404 IA

patients and 458 non-IA controls revealed that the *ELN* 3′UTR G(+659)C SNP has the strongest association to IA ($P = 0.000002$) and constitutes a tag-SNP for an at-risk haplotype, which contains two functional SNPs, *ELN* 3′UTR (+502) A insertion and *LIMK1* promoter C(−187) T. These allelic and haplotype-based associations were confirmed in a Korean population. *Ex vivo* and *in vitro* analysis demonstrates that the functional impact of both SNPs is decrease of transcript levels, either through accelerated *ELN* mRNA degradation or decreased *LIMK1* promoter activity. Elastin and LIMK1 protein are involved in the same actin depolymerization signaling pathway, therefore, these evidence suggest a combined effect of the SNPs in the at-risk haplotype possibly by weakening the vascular wall and promoting the development of IA.

Lysyl oxidase, encoded by *LOX* located on chromosome 5q31 that showed suggestive linkage to IA, is an extracellular copper-containing enzyme that initiates cross-linking of collagen and elastin by oxidative deamination of lysine residues. It plays an essential role in the formation of the extracellular matrix and connective tissue. *LOX* is considered to enhance the strength of the blood vessel wall, and is therefore a plausible positional and functional candidate for IA. Four SNPs (single nucleotide polymorphisms) of *LOX* were analyzed for allelic and haplotype-based associations in Japanese IA patients, but no association was detected. *LOX* was also screened in Central European IA patients and not associated with IA. Four novel genes encoding LOX-like proteins 1 through 4 (*LOXL1*, *LOXL2*, *LOXL3*, and *LOXL4*), assigned to chromosome 15q22, 8p21.3-2, 2p.13-3, and 10q 24, respectively, are categorized to be the "LOX family". Members of the LOX family proteins have a highly conserved amino acid sequence at the C-terminus and have amine oxidase activity. In transgenic mice lacking *Loxl1*, it was shown that *LOXL1* targets to sites of elastogenesis and is essential to maintain elastic fiber homeostasis. Accordingly, LOX family genes should also be considered to be functional candidates for IA, we evaluated allelic associations between IA and SNPs of the *LOXL1-4*. We found that a synonymous SNP in exon 5 of *LOXL2* was significantly associated with IA, especially with familial cases ($P = 0.0011$) and the association was confirmed in Korean IA patients ($P = 0.034$). These implicate that the polymorphism of *LOXL2* could be a genetic risk to IA and account for a part of the pathogenesis of IA.

3. Genomic approach to Ossification of the posterior longitudinal ligament (OPLL): PLZF and TSG-6 identified by gene expression analysis play roles in the pathogenesis of OPLL

So Tsukahara, Ryuji Ikeda, and Ituro Inoue

Ossification of the posterior longitudinal ligament (OPLL) of the spine is a subset of "bone forming" diseases, characterized by ectopic ossification in the spinal ligaments. OPLL is a common disorder among elderly populations in East Asia, and is the leading cause of spinal myelopathy in Japan. To clarify the molecular pathogenesis of OPLL, we performed cDNA microarray analysis on cultured ligament cells from OPLL patients. We found that tumor necrosis factor-α-stimulated gene-6 gene (TSG-6) is down-regulated during osteoblastic differentiation. Adenovirus vector-mediated overexpression of TSG-6 inhibited osteoblastic differentiation of human mesenchymal stem cells (hMSCs) induced by BMP-2 or osteogenic differentiation medium (OS). TSG-6 suppressed phosphorylation and nuclear accumulation of Smad 1/5 induced by BMP-2, probably by inhibiting binding of the ligand to the receptor, since direct interaction between TSG-6 and BMP-2 was observed *in vitro*. TSG-6 has two functional domains, a Link domain as a HA binding domain and a CUB domain implicated in protein interaction. The inhibitory effect on osteoblastic differentiation was completely lost with exogenously added Link domain-truncated TSG-6, while partial inhibition was retained by the CUB domain-truncated protein. In addition, the inhibitory action of TSG-6 and the *in vitro* interaction of TSG-6 with BMP-2 were abolished by the addition of HA. Thus, TSG-6, identified as a down-regulated gene during osteoblastic differentiation, suppresses osteoblastic differentiation induced by both BMP-2 and OS and is a plausible target for therapeutic intervention in OPLL.

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Donation Laboratories and Research Units

Division of Cellular Proteomics (BML)
細胞ゲノム動態解析(BML)寄付研究部門

Visiting Professor	Seisuke Hattori, Ph.D.	客員教授(委嘱)	理学博士	服 部 成 介
Visiting Research Associate	Hidetaka Kosako, Ph.D.	助 手	理学博士	小 迫 英 尊
Visiting Research Associate	Naoyuki Iida, Ph.D.	助 手	薬学博士	飯 田 直 幸

We analyze intracellular signaling pathways using proteomic approaches. Since resolution power of current proteomic technologies is not sufficient to analyze low abundance proteins such as components of signal transduction, we established the protocols to prefractionate phosphoproteins and proteins in lipid raft. Combining these prefractionation procedures with fluorescence difference two-dimensional gel electrophoresis (2-D DIGE), we identified many novel kinase substrates and raft proteins that are involved in signal transduction.

Proteomic investigation of extracellular signal-regulated kinase (ERK) transduction pathways

Kosako, H., Yamaguchi, N., Machida, M., Han, M., Shirakabe, K., Ushiyama, M[1]., Inagawa, J[1]., Hirano, J[1]., Hattori, S.: Division of Cellular Proteomics (BML), Institute of Medical Science, University of Tokyo, [1]GE Healthcare, KK.

Recently the novel technology to analyze cellular proteome, proteomics, is emerging. This technology greatly depends on the information provided by human genome project. The protein in a single spot or single band on a gel is now identified rapidly by a mass spectrometer, by comparing the molecular weights of the protease-digested peptides to the predicted values from genome database. However, the resolution power of 2-D gel electrophoresis is not enough to resolve total cellular proteins. Especially, the proteins of lower abundance such as the components of signal transduction could not be identified hidden behind the huge spots of housekeeping or cytoskeletal proteins on such analyses.

Suppose there are two samples in front of you, one from cancer and one from the adjacent normal tissue. Did simple 2-D analyses reveal the cause of the cancer? Two-D gel was developed by Dr. O'Farrell in 1975, since then over millions of 2-D gels were performed to compare the protein profiles between cancer and normal tissues. However, these analyses gave rise to essentially no results in regard to the cause of the cancer. Instead, the answer was provided by "transformation assay" described by Dr. Weinberg et al in 1979. The latter approach identified more than 100 so-called "oncogenes." The cancer arises from the malfunction of growth control machinery, the contents of which are not enough to be detected by simple 2-D gel.

To overcome such difficulty, it is necessary to purify and concentrate the components of interest. One approach is to isolate organella such as mitochondria and ribosomes or purify subcellular components biochemically. The second is to immunoprecipitate the protein of interest with associating proteins. The third approach is to purify phosphorylated proteins. We have established a protocol to analyze phosphorylated proteins.

Immobilized metal affinity chromatography

(IMAC) using Fe^{3+} has been shown to be useful to purify phosphopeptides. We improved this method using Ga(III) ion instead of Fe(III). We also optimized the conditions varying pH and ionic strength of the chromatography. By our established procedure, phosphorylated ERK (extracellular signal-regulated kinase) or proteins phosphorylated by Akt kinase are recovered with relatively good yield. Under the same conditions nonphosphorylated proteins such as tubulin was not trapped by the resin at all. By this affinity column procedure, nearly ten-fold purification of these phosphoproteins was achieved. Commercially available phosphoprotein purification column was also found to be as useful as our homemade IMAC, but it cannot be combined with other biochemical fractionation because the conditions are not open.

Phosphoproteins were then analyzed on a two -dimensional fluorescence difference gel electrophoresis (Ettan DIGE, GE Healthcare). By comparing the patterns of ERK-activated and ERK-suppressed samples, we identified more than 70 spots the intensity of which differs between the two samples. Some of them corresponded to the components of ERK signaling cascade, ERK, MEK, and RSK, demonstrating the feasibility of our approach. Known ERK substrates such as nuclear lamin and heterogeneous nuclear ribonucleoprotein K were also identified. We have identified most of the proteins in these spots. We also carried out similar comparison between total cellular extracts from ERK-activated and ERK-suppressed cells. However, due to low abundance of these proteins, such spots were hidden by abundant proteins. The result suggests that the prefractionation procedure may be necessary to get meaningful results. Therefore, the combination of prefractionation of phosphorylated proteins and 2-D Ettan DIGE system is suitable to identify components of signal transduction.

By two-dimensional western blot we confirmed that identified substrates are phosphorylated in living cells. We also showed that these substrates were phosphorylated *in vitro* by activated ERK. Comparing peptide fingerprints of phosphorylated and unphosphorylated substrates, we could identify the phosphorylation sites of these substrates by ERK. Mutagenesis of these sites into unphosphorylatable residue abolished the phosphorylation of these substrates *in vitro* and in living cells, indicating that these sites are indeed phosphorylation sites by ERK. We also made antibodies against these sites and demonstrated that these antibodies specifically stain the cells in which ERK is activated.

Among the substrates identified we further studied the effect of ERK phosphorylation of Nup50 and EPLIN. Nup50 is a member of nuclear pore complex that interacts with importin β. We showed that upon phosphorylation by ERK the affinity of Nup50 to importin β was reduced. The other substrate, EPLIN, is an actin binding protein that bundles actin filaments. Upon phosphorylation by ERK the affinity of EPLIN to actin filament was also reduced. We further demonstrated that nonphosphorylatable mutant of EPLIN but not wild type EPLIN blocked membrane ruffling and cell motility, indicating the important function of ERK in the regulation of cell motility.

Proteomic analyses of p38 MAP kinase pathways

Iida, N., Fujita, M., Saitoh, M., and Seisuke Hattori.:

The p38 MAP kinase cascade is activated by various stresses or cytokines. Downstream of p 38 MAP kinases there are diversification and extensive branching of signaling pathways. Fluorescent two-dimensional difference gel electrophoresis of phosphoprotein-enriched samples from HeLa cells in which p38 MAP kinase activity was either suppressed or activated enabled us to detect many spots unique to p38 MAP kinase-activated cells. Among these candidates, we identified four proteins including Bcl-2 associated athanogene 2 (BAG2) by peptide mass fingerprintings. We also identified other signaling molecules including p50RhoGAP and serine /arginine rich splicing factors. To globally identify p38 MAP kinase substrates more efficiently, we developed an *in vitro* phosphorylation system. In this system cell lysates were first dephosphorylated by potato acid phosphatase, then the proteins were phosphorylated by p38 MAP kinase *in vitro*. By this procedure, only the substrates for p38 MAP kinase were became phosphorylated. We also started a project to determine all cellular phosphorylation sites by a LC-MS (liquid chromatography-mass spectrometry) system. By enrichment of phosphopeptides by IMAC from total cellular protein digests followed by LC-MS, we identified more than 100 phosphorylation sites from anisomycin-treated HeLa cells. The identified sites included a phosphorylation site of Hsp27, which is known to be phosphorylated upon anisomycin treatment.

Identification of T-cell raft proteins involved in T-cell signaling

Kobayashi, M., Katagiri, T., Kosako, H., Iida, N., and Hattori, S.

Recently accumulating evidence shows that various signaling molecules are recruited to cellular lipid raft membranes where they function as signal transducers. We employed Jurkat T-cells as a model system to establish a protocol to analyze raft proteins. In T-cells, it is shown that T-cell receptor forms so-called immune synapse in lipid raft membranes. To identify factors involved in T-cell signaling, raft fractions were isolated by sucrose density gradient centrifugation and proteins were subjected to a fluorescence difference 2-D gel electrophoresis. We found that numerous proteins are recruited to lipid raft fraction upon T-cell receptor stimulation. Among them, we identified tyrosine kinases, regulators for small GTPases, actin binding proteins, and interestingly several factors that posses PH domain with phosphatidylinositol-1,4,5-phosphate (PIP3) biding activity. When the cells were treated by a PI 3-kinase inhibitor, the translocation of these PH domain-containing proteins was blocked, indicating that PI3-kinase activity was essential for the translocation. We are currently studying the function of these factors in T-cell signaling.

Publications

Kobayashi M, Katagiri T, Kosako H, Iida N and Hattori S. Global analysis of dynamic changes in lipid raft proteins during T-cell activation. *Electrophoresis* (in press).

Donation Laboratories and Research Units

Division of Stem Cell Engineering, Tooth Regeneration (Hitach Plant Technorogies, Denics, ArBlast)

幹細胞組織医工学(日立プラントテクノロジー，デニックス，アルブラスト)寄付研究部門

Visiting Professor	Minoru Ueda, D.D.S., Ph.D.		客員教授(委嘱)	医学博士	上　田　　実
Visiting Associate Professor	Hideaki Kagami, D.D.S., Ph.D.		客員助教授	歯学博士	各　務　秀　明
Visiting Research Associate	Masaki Honda, D.D.S., Ph.D.		助　手(担当)	医学博士	本　田　雅　規

Our main project is to regenerate tooth using the methods of tissue engineering. To accomplish this goal, we are focusing on the following subjects; 1) identification and characterization of stem cell in epithelial or mesenchymal tissue from tooth germ, 2) search for molecules to affect the differentiation of the stem cell, 3) assembly of these stem cells on artificial scaffold.

Our division was established in July 2003 to accelerate the research on oral tissue regeneration, especially tooth regeneration, with the support of accumulated knowledge about genomic science and stem cell biology at IMSUT. We are trying to regenerate not only teeth but also the other oral tissue including bone. There are three important elements in tissue regeneration; stem cells, signal molecules, and scaffold. We are focusing on the research of mesenchymal stem cell, bone morphogenetic protein (BMP), and synthetic polymer scaffold as an each element.

1. Clinical study

Mesenchymal stem cells (MSCs) have been widely used for bone tissue engineering. Although bone tissue engineering using MSCs is considered feasible, little is known about the clinical efficacy of this novel treatment. We have developed a clinical trial to test if MSCs will be clinically effective in regenerating bone in patients with severe maxillary and/or mandibular bone absorption who undergo dental implant surgery. So far, ten patients have been enrolled in this phase I clinical trial.

2. Effective Bone Engineering using Periosteum-Derived Cells

Bone engineering has generated significant interest. Bone marrow stromal cells are widely used, although periosteal cells have also been used. For dentists, mandibular periosteum is obtainable during oral surgery and therefore it is preferable. However, the differences in osteogenic potential between them remain unclear. We compared the osteogenic potential of them, and investigated the optimal osteoinductive conditions for periosteal cells. Both cells were induced to differentiate into osteoblasts using

bFGF and BMP-2. Periosteal cells proliferated faster than marrow stromal cells. Alkaline phosphatase activity and the gene expression of osteogenic markers indicated that marrow stromal cells were more osteogenic. However, bFGF pretreatment made periosteal cells more sensitive to BMP-2 and more osteogenic. Transplants of periosteal cells treated with BMP-2 after bFGF pretreatment formed more new bone than marrow stromal cells. These data suggest that the combination use of bFGF and BMP-2 can make periosteum a highly useful source for bone regeneration.

3. Tooth-tissue engineering

3-1. Performance of collagen sponge as a scaffold for tooth-tissue engineering.

Sumita Y, Honda MJ, Tsuchiya S, Sagara H, Kagami H, Ueda M

Tooth structure can be regenerated by seeding dissociated tooth cells onto polyglycolic acid fiber mesh, although the success rate of tooth production is low. The present study is designed to compare the performance of collagen sponge with PGA mesh for tooth-tissue engineering. Porcine third molar tooth cells were seeded onto collagen sponge or the PGA mesh. Scaffolds were then cultured to evaluate cell adhesion and ALP activity *in vitro*. An *in vivo* analysis was performed by implanting the constructs into the rats and evaluating tooth production up to 25 weeks. After 24 h, there were a significantly higher number of cells attached to the collagen sponge scaffold than the polyglycolic acid fiber mesh scaffold. Similarly, the ALP activity was significantly higher for the collagen sponge scaffold was than the PGA mesh after 7 days of culture. The results from *in vivo* experiments show conclusively that a collagen sponge scaffold allows tooth production with a higher degree of success than polyglycolic acid fiber mesh.

3-2. Shear stress facilitate tissue-engineered odontogenesis.

Honda MJ. Shinohara Y. Sumita Y. Kagami H. Ueda M.

Numerous studies have demonstrated the effect of shear stress on osteoblasts, but its effect on the regeneration of odontogenic cells is unclear. In this study, we focused on the effect of shear stress on facilitating tooth regeneration by dissociated odontogenic cells. Cells were harvested from the porcine third molar tooth bud, and the isolated heterogeneous cells were seeded on a biodegradable polyglycolic acid fiber mesh. And then, cell-polymer constructs were exposed to shear stress or not and evaluated them *in vitro* and *in vivo* study. *In vitro* study, the expression of both odontogenic related mRNAs were also significantly enhanced by shear stress for 2hrs. And at 12 hrs after expose the shear stress, the expression of among of amelogenin, BSP and vimentin protein synthesis were significantly enhanced compared with that of control. Moreover, after 7 days, alkaline phosphatase activity exhibited a significant increase without any significant effect on cell proliferation *in vitro*. *In vivo* study, mature enamel and dentin tissues formed after 15 weeks of *in vivo* implantation in constructs exposed to *in vitro* shear stress for 12 hrs, but not in controls. We conclude that shear stress facilitates odontogenic cell differentiation *in vitro* as well as odontogenesis of tissue-engineered teeth *in vivo*.

3-3. Cell proliferation in teeth reconstructed from dispersed cells of embryonic tooth germs in a three-dimensional scaffold.

Iwatsuki S, Honda MJ, Ueda M.

Although recent tissue engineering has been able to reproduce tooth from postnatal tooth cells, even when the enamel-dentin complex structure is reproduced, the crown formation is not accurately reconstituted. Here, we showed that a tissue-engineered (TE) tooth exhibiting morphogenesis according to regular crown-cusp pattern formation was produced by embryonic tooth germ cells in a 3D-scaffold. In addition, to study the developmental process, we examined the growth pattern using 5-bromo-2'-deoxyuridine (BrdU)-labeling analysis.

The heterogeneous cells dissociated from E14 mice tooth germs were seeded on a 3D-scaffold, and implanted under a kidney capsule in adult mice. The developmental process of the implants was examined up to 14 days. At 5 days, the cells formed initial tooth germ, then enamel-covered dentin tissue formed symmetrically. BrdU-labeling analysis showed that the initial cell-proliferation pattern of the TE-tooth was similar to that at the cap and early bell stages in natural tooth. Especially, in the developing cervical loop, the distribution pattern resembled that of BrdU-positive cells. These results suggested in a TE-tooth that even when embryonic tooth germs were dissociated, the single cells have a capacity to reconstitute tooth, and that the enamel organ morphogenesis was present in the same form it developed in natural teeth.

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Donation Laboratories and Research Units

Department of Molecular and Developmental Biology

再生基礎医科学(オリエンタル・トミー・ソフトバンク)寄付研究部門

Visiting Professor	Sumiko Watanabe, Ph.D.	客員教授 医学博士	渡 辺 すみ子
Visiting Research Associate	Shinya Satoh, Ph.D.	助 手 工学博士	佐 藤 伸 哉

Our long-term goal is to understand the molecular mechanisms which coordinately regulate growth and differentiation of stem cells as well as differentiated cells with emphasis on intracellular signal transduction. For this purpose, we are using systems ranging from zebrafish, mouse, monkey and culture cells. The major research areas of interest are on: 1) development and regeneration of eye, 2) roles of cytokines and their receptors in hematopoietic stem cells, 3) development of mammalian tooth. On the basis of these efforts, we intend to develop technologies to manipulate growth and differentiation of various stem cells with high fidelity, which is important for cell and gene therapy.

The neural retina is a part of the central nervous system (CNS), and regeneration of the retina from retinal stem cells or other sources by transplantation is a critical issue from both clinical and neurobiological points of view. Although reports of successful regeneration of the CNS from neural stem cells (NSC) have appeared in the literature, such has not been the case for the vertebrate neural retina. Furthermore, the nature of retinal stem cells has not been clarified, making it difficult to attempt regeneration of the retina. Based on the technique and knowledge that have been accumulated through works of haematopoietic systems in our laboratory, we attempt to identify mammalian retinal stem cells and developmental process. For developmental biological analyses, we use zebrafish system in addition to mouse system as model animals. We are also continue to work on haematological projects, and bidirectional cooperative progress between neurological and haematological works is one of unique features of our laboratory. Projects which gave major findings during 2006 are as follows.

Regulation of mouse retinal development by a network of nuclear receptors

Shinya Satoh, Mariko Inoue, Kotomi Kanzaki, Atsumi Iida, Tatsuhiko Kodama[1], Sumiko Watanabe: [1]Laboratory for Systems Biology and Medicine, Research Center for Advanced Science and Technology, The University of Tokyo.

During retinal development, progenitor cells give rise to a wide variety of neurons and glial cells. It has been shown that nuclear receptors are involved in specification of some retinal subtypes. For example, nuclear orphan receptor NR 2E3 is expressed exclusively in rod and acts as transcriptional repressor to regulate opsin expression. Although importance of some nuclear receptors had been reported, comprehensive

analysis of the role of nuclear receptors in retinal development has not been done. We aimed to reveal the expression patterns of nuclear receptors in retinal development systematically. By using a panel of antibodies for nuclear receptors for immunohistochemistry, we analyzed transition of the expression of nuclear receptors along with mouse retinal development. We found unique spatio-temporal expression patterns of various nuclear receptors during retinal development. Among them, COUP-TF family members are very unique since they were expressed by axis specific manner. COUP-TFI was expressed broadly in embryonic retina with gradient of signal strength along the dorsal/ventral axis. After birth, its expression becomes restricted to be in INL and GCL. COUP-TFII was specifically found in a subset of amacrine cells at ventral side but broadly expressed at dorsal side at all stages examined. Ear2 (COUP-TFγ) was expressed specifically in horizontal cells during retinal development and in adulthood. In addition, Ear2 was expressed in postnatal cone cells at the region from center to ventral side. Gain and loss of functions of COUP-TFs in retinal explant culture using retrovirus-mediated gene expression systems suggested the roles of COUP-TFs in certain retinal cell differentiation. In vivo analyses of retina development are currently undertaken by using COUP-TFII conditional knockout mouse.

Transition of spatio-temporal expression of nuclear receptors in developing mouse retina suggested their roles for retinogenesis. Furthermore, their overlapped expression patterns in retina and the similarity of structure and DNA target sequences suggested that they function coordinately.

Identification of cell surface markers that define temporally and spatially distinct retinal progenitor subsets

1. c-kit was identified as a late progenitor cell marker of neural retina

Hideto Koso, Shinya Satoh, Sumiko Watanabe

Neural retina is an important target organ for regenerative medicine, and isolation and expansion of retinal progenitor cells are critical issues from both scientific and clinical views. However, the characters of the immature retinal cells are not elucidated because of the lack of prospective approach to identify retinal progenitor cells. We aimed to identify cell surface markers of retinal stem or progenitor cells by using cell sorter. For that purpose, we screened the expression pattern of cell surface proteins in mouse immature retina by flow cytometer using a large number of antibodies against different membrane proteins. Among them, 25 antibodies recognized sub-populations of immature retina, and we examined the proliferation and differentiation abilities of purified those sub-populations of retina by various in vitro assay systems.

We found c-kit and SSEA-1 to be spatiotemporal markers of distinct populations of retinal progenitor cells, and these cells dramatically changed their expression profiles of c-kit and SSEA-1 during development. c-kit expression peaked in the retina from P1 mice and decreased drastically thereafter; and c-kit-positive cells expressed various immature retina specific genes. Later onset of rhodopsin expression and stronger proliferation activities were observed for c-kit-positive progenitor cells in in vitro reaggregation cultures, supporting the idea that these cells have an intrinsically immature character. c-kit/SSEA-1 double-positive cells showed stronger proliferation activities and higher differentiation rate into Muller glia cells than c-kit single-positive ones. Only c-kit-positive cells with low SSEA-1 expression responded to a growth factor stimulus. Although the number of SSEA-1-positive cells was augmented by β-catenin signal, c-kit-positive cells were positively regulated by Notch signaling. Therefore, our data suggest that c-kit and SSEA-1 are markers of retinal progenitor populations having intrinsically distinct characters. SCF, a ligand for c-kit is expressed in ganglion cell layer. Prolonged expression of c-kit by a retrovirus resulted in promotion of proliferation and the appearance of nestin-positive cells in response to SCF, suggesting a role for c-kit in retinal development.

Identification of cell surface markers that define temporally and spatially distinct retinal progenitor subsets

2. CD73 is a novel cell surface antigen which defines photoreceptor cell lineage

Mariko Inoue, Tabata Yoko, Shinya Satoh, Sumiko Watanabe

In the screening describing above, we found an antigen which was a possible marker of rod photoreceptor cells. Retinal subpopulations expressing CD73 first appeared in the retina around birth and increased dramatically until up to P5, which corresponds to the period of rod cells differentiation. Then, CD73 continued to be expressed in more than 90% of cells until adult age. CD73 positive cells were postmitotic, and in adult retina, most of them were expressed rhodopsin but not s-opsin. In re-

aggregation culture, CD73 positive cells differentiated into rhodopsin positive cells with faster time course than that of CD73 negative cells, supporting the idea that CD73 is early photoreceptor lineage marker. Results obtained by the ectopic expression of Nrl and Crx, both of which are transcription factor known to be expressed in photoreceptor lineage, suggested that CD73 is genetically in between Crx and Nrl in rod cell differeniation lineage. CD73 is a first step enzyme of adenosine synthesis, and mRNA of adenosine receptors are expressed in CD73 population. Addition of adenosine facilitated the increase rate of rhodopsin positive retinal cells during development, suggesting the roles of adenosine in photoreceptor cell differentiation.

Identification of cell surface markers that define temporally and spatially distinct retinal progenitor subsets

3. Comprehensive proteomic analysis of developing neural retina reveals unique membrane proteins with potential roles in retinal development

Zhao Jing, Tomonori Izumi[2], Kazuto Nunomura[2], Shinya Satoh, and Sumiko Watanabe: [2]Division of Functional Proteomics

The membrane proteins, which are expressed with specific manner in the developing retina can be used not only tools for purification of retinal sub-population by their specific antibodies and cell sorting technique, and they are potentially important for regulation of retinal development by signaling through receptors and cell surface molecules. In this work, we examined the comprehensive expression profile of total membrane proteins of embryonic and adult mouse retina by proteomics approach. We purified retinal membrane fraction by sucrose gradient with ultracentrifugation and analyzed total proteins by shotgun analyses on a nanoflow LC-MS/MS system. A total of 326 proteins in adult murine retinae and 310 proteins in embryonic day 17 retinae were identified, and about half of them are membrane associated proteins. Among them, MARCKS-like protein (MLP), which shares about 50% amino acid identity with myristoylated alanine-rich protein kinase-C (PKC) substrate (MARCKS), was characterized for its function in retinal development. The expression of MLP decreased along with retinal development in mRNA and protein levels. Overexpression of MLP by retrovirus mediated gene transfer enhanced the proliferation of the retinal progenitor cells without affecting differentiation and cell migration in the retinal explant culture system. Clonal assay also showed that the proliferative capability of the virus infected progenitors was higher than that of the control. Mutation analysis of MLP indicated that myristoylation of MLP was necessary to promote the proliferation of retinal progenitors while phosphorylation inhibited its function.

Mouse Rab11-FIP4 regulates proliferation and differentiation of retinal progenitors in a Rab 11-independent manner

Akihiko Muto, Sumiko Watanabe

We identified Rab11-family interacting protein 4 (Rab11-FIP4), which has been thought to be involved in the membrane trafficking, as a gene strongly expressed in developing mouse neural retina. Major transcript of mouse Rab11-FIP4 encoding a full-length protein, Rab11-FIP4A, was expressed predominantly in neural tissues, whereas an alternative transcript encoding an N-terminally truncated protein was expressed ubiquitously as a minor form. Gain of function of mRab11-FIP4A in retina lead to increased sub-population of retinal cells such as bipolar cells and Muller glia, which are localized in inner nuclear layer. In contrast, the number of photoreceptor was decreased and Rab11-FIP4A promoted cell cycle exit was suggested. Reversed phenotype was observed when Rab11-FIP4A was downregulated by using shRNA. Analysis using a truncation mutants revealed that the essential role of the N-terminal region containing a conserved single EF-hand motif for the retinal phenotypes induced by the expression of Rab11-FIP4A. However, binding to Rab11 was dispensable, suggesting the involvement of a Rab11-independent novel mechanism of mRab11-FIP4A was involved in the regulation of the retinal development.

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Donation Laboratories and Research Units

Division of Exploratory Research (Ain Pharmaciez)

探索医療ヒューマンネットワークシステム（アインファーマシーズ）寄付研究部門

Visiting Associate Professor	Masahiro Kami, M.D., Ph.D.		客員助教授	医学博士	上　昌広
Visiting Assistant Professor	Yuji Tanaka, M.D., Ph.D.		助　手	医学博士	田中祐次
Visiting Assistant Professor	Tomoko Matsumura, M.D., Ph.D.		助　手	医学博士	松村有子

The name of our division "Exploratory Research" implies exploration of human networks among medical professionals, among patients, among medical professionals and patients, and beyond the medical field. Our objective is to develop these networks from the center of the medical field. This is a unique research focusing not on critique but on execution.

Actibities in 2006

① Unapproved drugs

We reported that proteasome inhibitor (bortezomib) which had been developed for the treatment of multiple myeloma was individually imported for personal use in Japan before approval (Miyakoshi et al., Blood 2007). We further investigated issues including the health insurance system and remedy for adverse events, and presented our proposal at "Council for medical reform facilitation from the field" on November 25, 2006.

② Criminal arrest of an obstetrician and gynecologist at Fukushima Prefectural Oono Hospital

We have cooperated with the supporting activities for the obstetrician and gynecologist at Fukushima Prefectural Oono Hospital who was arrested on February 18, 2006 by Professor Satoh at Fukushima Prefectural Medical School and continue acting as the secretariat for "Association to Prevent Disruption of Perinatal Care" represented by Professor Satoh.

③ System reform on countermeasures after medical accidents

Following the arrest at Fukushima Prefectural Oono Hospital, we organized a working group for system reform on countermeasures after medical accidents (Chairperson, Dr. Yoshitaka Wada, Professor at Waseda University, Waseda Law School; Vice-chairperson, Dr. Hirotaro Iwase, Professor at Chiba University, Department of Legal Medicine). We proposed introduction of measures for medical mediation, alternative dispute resolution, truth clarification, and forensic autopsy at "Council for medical reform facilitation from the field" on November 26, 2006.

④ Patient dynamics

In "Research on patient dynamics to promote registration necessary for clinical cancer research (18180301)" (Clinical Cancer Grant by the Ministry of Health, Labour, and Welfare 2006), we investigate and analyze population, cancer population, referral patterns to university hospitals

and local central hospitals, and so forth in model areas such as Tokyo, Tokushima, and Yamagata prefectures.

⑤ Cooperation between medical care at home and outpatient treatments for cancer patients

While the current medical system has been developed mainly for the treatments of infectious diseases which are contagious and curable and affect young patients, novel medical system needs to be established for the treatments of cancer which are not contagious or curable and affects older patients. Considering cooperation between medical care at home and outpatient treatments is necessary as part of the novel system, we survey actual situations at Aozora Clinic and Tsukuba Memorial Hospital.

⑥ Patient associations

As the Internet advances, the nature of patient associations has been changing. We support foundation of seven unique style, hospital-based patient associations so far, and will support founding patient associations all over Japan.

⑦ Fungal infections

We investigate the dynamics of importing infectious diseases. Also in clinical field, we studied fungal infections in hematopoietic stem cell transplantation and reported a review article on fungal infections in reduced-intensity stem cell transplantation.

⑧ "Council for medical reform facilitation from the field"

We held a symposium on November 25 and 26 at grand hall of the Institute of Medical Science, the University of Tokyo, gathering members from mass media, government officers, the Diet members, medical and non-medical academic members and so forth. The program was constituted based on the activities in our division. The report on the symposium will be released.

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Donation Laboratories and Research Units

Division of Neural Signal Information (NTT-IMSUT)

神経情報シグナルNTT-IMSUT共同研究ユニット

Project Associate Professor Ichiro Fujimoto, Ph.D.

特任助教授 理学博士 藤 本 一 朗

The sodium-bicarbonate cotransporter 1 (NBC1) is a plasma membrane localized transporter protein, which transports Na^+ and HCO_3^-. Cause HCO_3^- plays important role in the regulation of the pH homeostasis; NBC1 is thought to be a crucial regulator of cellular pH. Recently, the brain type isoform of NBC1 was found in rat brain that binds to IRBIT. We are focusing on Na^+/HCO_3^- co-transporter protein that functioning in brain.

IP_3R binding protein, IRBIT, interacts sodium-bicarbonate cotransporter 1 (NBC1).

Ichiro Fujimoto, Hideomi Yamada[1], George Seki[1], Katsuhiko Mikoshiba[2]: [1]Department of Internal Medicine, Faculty of Medicine, The University of Tokyo. [2]Division of Molecular Neurobiology, Department of Basic Medical Sciences

We proposed a function of IRBIT based on the idea that when IP_3 binds to IP_3Rs, displaced IRBIT might serve to carry a signal to other partners. We searched for proteins that interacted with IRBIT in extracts of mouse cerebellum. A prominent interacting protein from membrane fractions was identified as NBC1, the Na^+/HCO_3^- cotransporter that shuttles HCO_3^- and Na^+ ions across the plasma membrane. Reciprocal immuno-precipitation experiments verified that the endogenous proteins interact. Like its binding to the IP_3R, binding of IRBIT to NBC1 was dependent on phosphorylation of IRBIT. The functional consequence of the interaction was tested in a Xenopus oocyte system. Electrophysiological measurements showed that expression of IRBIT along with NBC1 was necessary to allow full activity of the transporter. IRBIT interacted only with the NBC1 splicing variant called pNBC1, which is present in the pancreas, where it is thought to promote HCO_3^- transport in pancreatic duct cells. Thus, the authors propose that, at least in the pancreas, physiological concentrations of IP_3, which cause dissociation of IRBIT from IP_3Rs, could not only promote release of calcium through the IP_3R channel but also mobilize an IRBIT-mediated signal that modulates acid-base balance.

Functional evidence for NBC1 in HEK293 cells.

Ichiro Fujimoto, Akito Nagayoshi[2]

Intracellular pH was measured in single cells using fluorescence analysis of intracellular trapped BCECF. The steady-state pHi of NBC1 gene stable transfected HEK293 cells in standard HCO_3^- solution was pH 7.34 ± 0.08. After an acid load induced by an NH_4Cl pulse, cells did not show any pHi recovery in Na^+-free HCO_3^- solution. In the presence of Na^+-free solution pHi

continued to progressively fall at a rate of 0.05± 0.01 pH unit/min (n=9), which may represent bicarbonate efflux driven by the outwardly directed Na^+ gradient. However, pHi promptly recovered at a rate of 0.18±0.05 pH unit/min after the readdition of Na^+, indicating that the base influx into HEK293 in the presence of HCO_3^-/CO_2 is Na^+ dependent. To test for a possible involvement of NBC1 in the Na^+-dependent pHi recovery, we repeated the NH_4Cl pulse in the absence and presence of DIDS. DIDS completely inhibited the pHi recovery after the acid load. On the other hand, amiloride did not change the rate of pHi recovery. To test for the dependence of pHi recovery on Cl^-, cells were preincubated with Cl^--free HCO_3^- solution for 20-40 min. In addition the pHi recovery in Cl^--free HCO_3^- solution was completely inhibited by DIDS (0.01±0.02, n=5), indicative that it is not dependent on Cl^-. Finally we applied high-K^+ solution to test for electrogenicity. Since NBC1 is thought to carry a net negative charge by transporting more HCO_3^- than Na^+, depolarizing the cell membrane should suddenly shift the net driving force in the direction of bicarbonate influx. In these cells the addition of 0.2 mM DIDS decreased the steady-state pHi by 0.28±0.08 pH unit (n=5, $P<0.01$), and completely inhibited the alkalinizing response to high-K^+ solution (0.01±0.01 pH unit).

IRBIT knock down effected the NBC1 pH regulation.

Ichiro Fujimoto, Katsuhiko Mikoshiba[2]

To determine that IRBIT response to NBC1 in mammalian cells, we utilized RNA interference to suppress the expression of IRBIT in HEK293 cells. Because small interfering RNA (siRNA) can have nonspecific effects, we used IRBIT-siRNA, which are specific to human IRBIT. IRBIT-siRNA suppressed the expression of IRBIT in HEK293 cells but had no effect on expression of NBC1. We analyzed the effect of IRBIT depletion on pNBC function by pH imaging in pNBC1 stably transfected HEK293 cells. Responses after an acid load induced by an NH_4Cl pulse were examined in cells treated with siRNAs. Depletion of IRBIT by siRNA IRBIT resulted in a decrease in the rate of pH recovery from acidification. We identified pNBC1 as a target molecule of IRBIT, indicating that IRBIT has an important role in the regulation of intracellular and extracellular pH through pNBC1. The regulatory mechanism through IRBIT may also be responsible for the characteristic difference in physiological roles of NBC1 variants. Previous report suggested that coupling ratio for Na^+ to HCO_3^- of 1:2 in pancreatic cells and the same ratio when kNBC1 was expressed in an intestinal cell line, whereas it was 1:3 in a renal cell line, suggesting that it is the cell type, not the NBC1 sequence, that determines the coupling ratio. Another report presented evidence suggesting that the Ca^{2+} dependent stimulation status of the cell determined the coupling ratio of NBC both in native proximal tubules and in kNBC1 expressing oocytes, and recently, they presented evidence for a decrease in the coupling ratio between Na^+ and HCO_3^- by PKA-mediated phosphorylation in heterologously expressed kNBC1. It would be highly interesting to study the stoichiometriy of the pNBC1 and a potential stimulation-associated change with or without IRBIT.

Regional expression of NBC isoforms in developmental mouse brain

Ichiro Fujimoto

NBC is expressed in glial cells in the brain and plays an important role in the regulation of both intracellular and extracellular pH. Ionic substitution studies and inhibition of injury suggest that NBC is involved in astrocyte vulnerability to acidic injury. Recently three NBC cDNAs differing in N-terminal and C-terminal coding sequence have been cloned from kidney, pancreas, and brain. Partially, combination of pancreas type and brain type NBC isoforms were determined the developmental and regional expression in the brain by *in situ* hybridization. According from that data, the expression was observed in the spinal cord at embryonic day 17, whereas expression in brain was first seen at approximately postnatal day 0 (P0), increased at P15, and persisted in the adult brain. Expression was widespread throughout the cerebellum, cortex, olfactory bulb, and subcortical structures. The expression profile suggests that this transporter is critical during the later stages of brain development and could be one of the factors contributing to the different patterns of injury seen in perinatal versus adult cerebral ischemia. We are separating the signal between pancreas type and brain type to focus the function of NBC in brain.

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