Donation Laboratories and Research Units

Division of Cell Processing (CERES Consortium)

細胞プロセッシング(CERES)研究部門

Visiting Professor	Tsuneo A. Takahashi, D.Sc.	客員教授	理学博士	高 橋 恒 夫
Visiting Associate Professor	Tadashi Yamashita. D.V.M., Ph.D.	客員助教授	獣医学博士	山 下 匡
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. 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 registered 3,900 units in Japan Cord Blood Bank Network, International Cord Blood Organization NETCORD, cord blood association AsiaCORD and Bone Marrow Donor Worldwide, and shipped more than 390 units to transplant centers by the end of 2005. 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 in cord blood. Using this cord blood banking system, we have started the research on regeneration medicine using placenta-derived mesenchymal progenitor cells and those could differentiate into osteoblasts, chondrocytes, adipocytes and neural cells. The study has extended to mesenchymal stem and progenitor cells in cord blood, and those placenta-derived and cord blood mesencyhmal cells should be considered as one of the possible allogeneic cell sources for cell therapies and tissue engineering.

1. Cord Blood Banking in IMUST:

Tokiko Nagamura-Inoue[1], Yan Cui, Masako Hirai, Kei Takada, 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 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 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. Development of new system for cord blood processing

Mikitomo Yasutake, Tokiko Nagamura-Inoue[1], Eiji Akagawa, Tsuneo A. Takahashi

Cord blood transplantation has been performed to date, and the results suggest that human umbilical cord blood (UCB) is an excellent source of hematopoietic stem cells for transplantation in patients with various malignant and nonmalignant diseases. However, there are still many patients who cannot receive cord blood transplantation owing to the cell dose in cord blood and/or HLA mismatch. Expansion of the cord blood registry with high quality cord blood and HLA library with extension of clinical cord blood banks would therefore be desirable. Cord blood processing requires sophisticated procedures and manpower. Several centrifugation technologies for cord blood processing have been developed and have contributed to establishment of a foundation for clinical cord blood banking worldwide. In contrast, many kinds of filter devices, such as leukoreduction filters, have been developed and used for processing of blood in the field of blood transfusion medicine. We applied the filtration technology to UCB volume reduction and adopted an innovative closed circuit filter system that lends to simple handing and reproducible performance for cord blood processing. We compared the functions of cryopreserved UCB cells processed by the filter and by the hydroxyethyl starch (HES) sedimentation method that has been used worldwide from the aspect of the graft quality. UCB specimens were divided into two portions, processed in parallel by the filter or HES, and then cryopreserved in the clinical setting. The thawed UCB specimens containing 1×10^5 CD34+ cells were injected into nonobese diabetic/ShiSCID mice, and the engraftment capacity in primary and secondary transplants was assessed. The functions of natural killer (NK) cells and monocyte-derived dendritic cells (DCs) were also assayed in vitro. The percentage of recovery of CD34+ cells by both methods was equivalent. In the marrow of the primary transplant recipients, the percentage of hCD45+ cells in the filter group and HES group was 58.2 ± 31.6 and 46.5 ± 28.4 percent, respectively ($p=0.016$). The engraftment capacity and multilineage differentiation in the secondary transplantations were equal in both groups. The cytotoxic activity of the NK cells and phagocytosis activity of the DCs from both the groups were similar. The filter yielded a desirable percentage of recovery of hematopoietic cells with engraftment ability in the clinical setting. Thus, it is considered that the filter system may be useful for UCB banking for cord blood transplantation.

3. Expansion of NKT cells from cord blood mononuclear cells using IL-15, IL-7 and Flt 3-L

Hikaru Okada, Tokiko Nagamura-Inoue[1], Yuka Mori, Tsuneo A. Takahashi

It is important to prevent rejection of cord blood graft and GVHD on cord blood transplantation as bone marrow transplantation. We have studied to expand immunological cells in cord blood for the purpose. Human Va24+Vb11+ NKT cells are a unique T cell population specifically and potently activated by agalactosylceramide (aGalCer; KRN7000) presented by CD1d. We developed a simple and efficient method for expanding Va24+Vb11+NKT cells from human cord blood mononuclear cells (CBMNC) using a GalCer in the presence of interleukin (IL)-15, IL-7 and Flt3-L. The addition of aGalCer fromday 0, compared to its addition from 8 day or day 15, induced a greater expansion of NKT cells. The maximal expansion of NKT cells was observed after 15 days (2300-fold). Thereafter, the number of NKT cells decreased slowly, a decreased that was correlated with the diminution of CD1d-positive cells. NKT cell proliferation induced by a aGalCer was not observed when CD 1d-expressing monocytes were depleted from CBMNC, whereas B cell and dendritic cell depletions had no effect. Expanded NKT cells were CD4+CD8 and secreted both IL4 and IFNc. In this system, CD3+T cells and CD3 CD56+ NK cells were also expanded. However, the expansion of NKT cells had no significant functional effect on T and NK cells. This expansion method of CBMNC-derived NKT cells is simple and may be helpful for clinical use.

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

Xiaohong Zhang, Ayako Mitsuru, Koichi Igura, Kenji Takahashi, and 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.

5. Isolation of mesencyhmal stem cells from cord blood

Kouichi Igura, Xiaohong Zhang, Ayako Mitsuru, Yuko Mizushima, Tadashi Yamashita, and 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 mesenchyhmal 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.

Publications

1. Mikitomo Yasutake, Yizhou Zheng, Tokiko Inoue-Nagamura, Eiji Akagawa, Yasuo Tokushima, Shuji Terashima, and Tsuneo A. Takahashi
   SCID-repopulating activity of human umbilical cord blood derived hematopoietic stem and/or progenitor cells in a no obese diabetic/Shi-SCID mice serial xenotransplantation model and immune cell activities in vitro: a comparative study of the filter method and hydroxyethyl starch method. Transfusion 45, 1899-1908, 2005.
2. Hikaru Okada, Tokiko Nagamura-Inoue, Yuka Mori and Tsuneo A. Takahashi
   Expansion of Va24+Vb 11+NKT cells from cord blood mononuclear cell using IL-15, IL-7 and Flt3-L depends on monocytes. Eur. J. Immunol. 36, 236-244, 2006.
3. Xiaohong Zhang, Ayako Mitsuru, Koichi Igura, Kenji Takahashi, Shizuko Ichinose, Satoru Yamaguchi, Tsuneo A. Takahashi.
   Mesencyhmal progenitor cells from chorionic villi of human placenta for cartilage tissue engineering. Biochem Biophys Res Commun. 340, 944-952, 2006.
4. Yamashita T, Wu YP, Sandhoff R, Werth N, Mizukami H, Ellis JM, Dupree JL, Geyer R, Sandhoff K, Proia RL
   Interruption of ganglioside synthesis produces central nervous system degeneration and altered axon-glial interactions
   Proc. Natl. Acad. Sci. U.S.A., 2005; 102(8): 2725-2730
5. Sandhoff R, Geyer R, Jennemann R, Paret C, Kiss E, Yamashita T, Gorgas K, Sijmonsma TP, Iwamori M, Finaz C, Proia RL, Wiegandt H, Grone HJ.
   Novel class of glycosphingolipid involved in male fertility. J Biol Chem. 2005 Jul 22; 280 (29): 27310-8.
6. Yamashita T, Allende ML, Kalkofen D, Werth N, Sandhoff K, Proia RL.
   Conditional loxP-flanked glucosylceramide synthase allele controlling glycosphingolipid

synthesis. Genesis. 2005 Nov 10; 43(4): 175-180
7. Mizugishi K, Yamashita T, Olivera A, Miller GF, Spiegel S, Proia RL.
Essential role for sphingosine kinases in neural and vascular development. Mol Cell Biol. 2005 Dec; 25(24): 11113-21.
8. Hashiramoto A, Mizukami H, Yamashita T.
Ganglioside GM3 promotes cell migration by regulating MAPK and c-Fos/AP-1. Oncogene *in press*

Donation Laboratories and Research Units

Division of Genetic Diagnosis (Otsuka)
ゲノム情報応用診断(大塚製薬)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 asthma, ossification of posterior longitudinal ligament of the spine, and intracranial aneurysm and analyze the molecular causality.

1. Genetic susceptibility of intracranial aneurysm

Hiroyuki Akagawa, Haruhiko Yamada, Boris Krischek, Atsushi Tajima, Ituro Inoue

Subarachnoid hemorrhage (SAH) is most often the result of a ruptured intracranial aneurysm (IA). Patients who survive the acute phase are often left with a substantial disability and impaired quality of life as a result of major neurological deficits. The annual incidence of SAH due to ruptured IA is 18 to 23 per 100,000, whereas the prevalence of unruptured IA detected by MR angiography, CT angiography, or digital subtraction angiography in the Japanese population has been reported to be much higher, around 6 to 7%. Epidemiological studies demonstrate a strong genetic influence: the prevalence of unruptured IA is significantly higher (10.5% to 13.5%) in a Japanese subgroup with a family history of IA. The risk of SAH is 4 times higher in first degree relatives of patients with SAH than in the general population, and 6 times higher in siblings. 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*). The elastin gene (*ELN*) a positional and functional candidate gene located within the linkage region, has been investigated for allelic association with IA, but the results are divergent.

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 hap-

lotype-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 (*LOXL 1*, *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.

2. Gene-Expression profiles of human nasal polyp tissues from aspirin intolerant asthma patients

Takashi Sekigawa, Atsushi Tajima, and Ituro Inoue

In a subset of asthmatic patients, aspirin and several other non-steroidal anti-inflammatory drugs (NSAID) that inhibit cyclooxygenase (COX) enzymes induce severe asthmatic attack generally termed aspirin intolerant asthma (AIA). In 198 unrelated AIA patients, we identified prostaglandin E2 receptor subtype 2 gene is a susceptibility to AIA, however, the etiology of AIA can not be approached simply by genetic factor. Symptoms of AIA are characterized by aspirin sensitivity, asthma, and nasal polyposis known as Sameter's triad. It is known that rhinosinusitis or nasal polyp is frequently observed before the onset of asthmatic attack. Therefore, hyperplastic rhinosinusitis associated with aspirin intolerance should play important roles in the development of AIA. We determined the gene-expression profiles of nasal polyp tissues from 18 patients including ten aspirin intolerant asthma and five eosinophilic sinusitis and two chronic sinusitis using Agilent Human Oligo 1A ver2 comprising approximately 18,000 unique DNA oligonucleotides for gene-expression detection. We identify 52 genes whose expression were significantly different (2 fold up-regulated 45 genes or 0.5 fold down-regulated 7 genes were defined as differential expression, T-test P-values ware less than 0.05) between AIA and eosinophilic sinusitis. For clustering purpose, nonnegative matrix factorization (NMF) was applied and the expression profile was classified into three groups. One group was particularly rich in genes participating arachidonic acid metabolic cascade and inflammatory response. These groups would help to identify susceptibility and pathophysiolosical pathway underlying AIA. The study will now being proceeded to disclose expression profiles and individual genes related to AIA that would illustrate the biopathway and genetic abnormality of AIA.

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. We performed a genomewide linkage study with 142 affected sib-pairs to identify genetic loci related to OPLL. The best evidence of linkage was detected near *D21S1903* on chromosome 21q22.3 (maximum $Z_{lr} = 3.97$), therefore the linkage region was extensively investigated for linkage disequilibrium analysis with single nucleotide polymorphisms (SNPs) covering 20 Mb. Extensive linkage disequilibrium and association studies of the 4 genes indicated that SNPs in the collagen 6A1 gene (*COL6 A1*) were strongly associated with OPLL ($P = 0.000003$ for SNP in intron32 (-29)). Pinpointing the susceptibility to OPLL by genomewide linkage and linkage disequilibrium studies permits us to investigate the pathogenesis of OPLL, which might lead to the development of novel therapeutic tools.

To understand the molecular pathogenesis of OPLL, we performed cDNA microarray analysis on cultured ligament cells from the OPLL patients to understand the molecular pathogenesis of OPLL. We identified Zinc finger protein 145 (PLZF) as one of up-regulated genes and tumor necrosis factor-α stimulated gene 6 (TSG-6) and one of down-regulated gene during osteoblastic differentiation. We investigated the roles of PLZF in the regulation of osteoblastic differentiation of hMSCs and C2C12 cells. siRNA-mediated gene-silencing of PLZF resulted in a reduction of the

expression of osteoblast-specific genes such as the alkaline phosphatase (ALP), collagen 1A1 (COL1A1), Runx2/cbfa1 (CBFA1), and osteocalcin (OCN) genes in the presence of OS in hMSCs. The overexpression of PLZF can induce CBFA1 induction suggestiong PLZF is an upstream regulator of CBFA1 and thereby might participate in promoting the ossification of spinal ligament cells in OPLL patients. Adenoirus-mediated TSG-6 overexpression in hMSCs resulted in suppression of oseoblastic differentiation induced by either BMP-2 or OS. TSG-6 can bind to BMP-2 directly, thereby could inhibit BMP-2 signaling. Taken together, these findings indicate that PLZF and TSG-6 play important roles in early osteoblastic differentiation.

4. Systematic sequencing approach to identify the pattern of nucleotide polymorphisms in human microRNA genes

Atsushi Tajima and Ituro Inoue

Expression of every gene is mainly regulated at the transcriptional level, whereas the importance of post-transcriptional regulation has been increasingly recognized. MicroRNAs (miRNAs) are one of the most abundant classes of post-transcriptional regulators in the human genome. The small (~22-nucleotide) RNAs are derived from hairpin-shaped precursor transcripts (pre-miRNAs), and can work as post-transcriptional gene silencers by base-paring with the target mRNAs. Recent bioinformatics analyses have predicted multiple protein-coding mRNA targets for each miRNA, suggesting the large portions of the transcriptome (~30% of the human mRNAs) are under the control of cellular miRNA networks and possible associations between miRNA dysregulation and human diseases. Although miRNA biogenesis and miRNA-mediated regulation of target expression are thought to be highly controlled in a sequence-dependent manner, there has been little attempt to characterize miRNA diversities within human populations at the nucleotide level. To obtain the genetic basis for the extent and pattern of DNA polymorphisms in human miRNA genes, we intended to resequence a total of 30 subjects from three human populations (10 each from African American, European American and Japanese populations). We identified a total of 103 SNPs on 30,967-bp comparisons excluding indel polymorphisms by sequencing 10 selected genomic regions, which are composed of 20 types of miRNA genes and their flanking regions. Among the 103 polymorphic sites, three SNPs ('precursor SNPs') were observed in three distinct pre-miRNA sequences, but none in mature ones. Within-diversity estimate for concatenated sequences of 20 pre-miRNA regions was comparable to those of the adjacent surroundings such as 5'- and 3'-flanking regions, whereas between-species comparison revealed that the 20 orthologues were highly evolutionarily conserved between humans and chimpanzees. *FST*-based analysis indicated that all of the precursor SNPs exhibited low levels of genetic differentiation between three human populations. Considered together with additional results regarding the suppressive effect of a particular precursor SNP on *in vitro* miRNA processing and the SNP locations in hairpin structures of pre-miRNAs, the present observations suggest that purifying and/or balancing selection has shaped the pattern of nucleotide polymorphisms in human miRNA genes.

Publications

1. Nakajima T, Wooding S, Satta Y, Jinnai N, Goto S, Hayasaka I, Saitou N, Guan-Jun J, Tokunaga K, Jorde LB, Emi M, Inoue I. Evidence for natural selection in the HAVCR1 gene: high degree of amino-acid variability in the mucin domain of human HAVCR1 protein. *Genes Immun* 6, 398-406, 2005.
2. Ikeda R, Yoshida K, Tsukahara S, Sakamoto Y, Tanaka H, Furukawa-K, Inoue I. PLZF promotes osteoblastic differentiation of human mesenchymal stem cells as an upstream regulator of CBFA1. *J Biol Chem*, 280, 8523-8530, 2005.
3. Tsukahara S, Miyazawa N, Akagawa H, Forejtova S, Pavelka K, Tanaka T, Toh S, Tajima A, Akiyama I, Inoue I. COL6A1, the candidate gene for ossification of posterior longitudinal ligament, is associated with diffuse idiopathic skeletal hyperostosis in Japanese. *Spine*, 30, 2321-2324, 2005.
4. Yamaguchi T, Park S-B, Narita A, Maki K, Inoue I. Genome-wide linkage analysis of mandibular prognathism in Korean and Japanese patients. *J Dent Res*, 84, 255-259, 2005.
5. Akagawa H, Kasuya H, Onda H, Yoneyama T, Choi K-Y, Lee W-J, Hong S-W, Yang T-K, Kim C-J, Hori T, Inoue I. Endothelial nitric oxidase synyhase T-786C single nucleotide polymorphism is not a factor influencing aneurysm size. *J Neurosurgery*, 95, 622-625, 2005.
6. Krischek B, Kasuya H, Inoue I: Collagen Morphology Is Not Associated With the Ala549

Pro Polymorphism of the COL1A2 Gene. Stroke 36:2068-69, 2005 (response to a letter)
7. Inoue I, Tsukahara S. PLZF and TSG-6 identified by gene expression analysis play roles in the pathogenesis of OPLL. *J Pharmacol Sci* 2005 in press.
8. Inoue I. Genetic susceptibility to OPLL. In: Yonenobu K, Nakamura K, Toyama Y (eds) Ossification of the posterior longitudinal ligament. Springer, Tokyo, in press.

Donation Laboratories and Research Units

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

Visiting Professor	Seisuke Hattori, Ph.D.	客員教授	服部成介
Visiting Research Associate	Hidetaka Kosako, Ph.D.	助手	小迫英尊
Visiting Research Associate	Michimoto Kobayashi, Ph.D.	助手	小林道元
Visiting Research Associate	Naoyiki Iida, Ph.D.	助手	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 protocols 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., Ushiyama, M.*, Inagawa, J.*, Hirano, J.*, Hattori, S.: Division of Cellular Proteomics (BML), Institute of Medical Science, University of Tokyo, *Amershambiosciences, 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 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 were recovered with relatively good yield. 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 can not 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, Amersham Biosciences). By comparing the patterns of ERK-activated and ERK-suppressed samples, we identified more than 70 spots the intensity of which differ 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 heterogenous 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 under more 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 when ERK was activated. 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.

Proteomic analyses of p38 MAP kinase pathways

Naoyuki Iida, Masayuki Fujita, and Seisuke Hattori.:

The p38 MAP kinase cascade is activated by various stresses or cytokines. At downstream of p38 MAP kinase 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 last year four proteins including Bcl-2 associated athanogene 2 (BAG2) by peptide mass fingerprintings. We identified other signaling molecules including p50RhoGAP and serine/arginine rich splicing factors. We also started a project to determine all cellular phosphorylation sites. By enrichment of phosphopeptides by IMAC from total cellular protein digests followed by LC-MS (liquid chromatography-mass spectrometry), 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., and Hattori, S.:

Recently accumulating evidence shows that various signaling molecules are recruited to cellular lipid raft fractions where that 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 fractions. 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 several factors that posses PH domain that binds to phosphatidylinositol-1,4,5-phosphate (PIP3). Since PI3-kinase is essential for T-cell signaling, these results show that these factors are recruited to raft fractions by binding to PIP3. We are currently studying the function of these factors in T-cell signaling.

Publications

Ito, S., Ito, Y., Senga, T., Hattori, S., Matsuo, S., Hamaguchi, M. v-Src requires Ras signaling for the suppression of gap junctional intercellular communication. *Oncogene. in press*

Donation Laboratories and Research Units

Division of Stem Cell Engineering, Tooth Regeneration (Denics, Hitachi Medical)

幹細胞組織医工学(歯胚再生学)(デニックス、日立メディコ)研究部門

Visiting Professor	Minoru Ueda, D.D.S., Ph.D.		客員教授㈱	医学博士	上　田　　実
Visiting Associate Professor	Izumi Asahina, D.D.S., Ph.D.		客員助教授	歯学博士	朝比奈　　泉
Visiting Research Associate	Masaki Honda, D.D.S., Ph.D.		助　手	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 and mucous membrane. 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.

We have also started the clinical trial "Alveolar bone regeneration using osteoblastic cells derived from autogenous bone marrow" with the cooperation of Research Hospital of IMSUT this year.

1. Bone regeneration

(1) Mandibular reconstruction using a combination graft of rhBMP-2 with bone marrow cells expanded in vitro.

Seto I, Marukawa E, Asahina I.: Division of Stem Cell Engineering IMSUT, Tokyo Medical and Dental University Graduate School

The aim of the study was to evaluate the efficacy of a combination graft, using recombinant human bone morphogenetic protein-2 (rhBMP-2) with culture-expanded cells derived from bone marrow, on bone regeneration in a non-human primate mandible.

Bone marrow was obtained from the tibia of Japanese monkeys and was plated into culture flasks. Adherent cells were cultured until near confluence, then the proliferated cells were tran-

sferred to a three-dimensional culture system using collagen beads as the cell carrier. After further proliferation on beads, the cells were mixed with collagen sponge that was impregnated with rhBMP-2, and were grafted into surgically created segmental bone defects of the monkey mandibles. The animals were sacrificed 24 weeks after surgery. The combination graft of culture-expanded bone marrow cells with rhBMP-2 in collagen sponge regenerated the mandibular bone completely. By contrast, the graft of culture-expanded cells alone resulted in only a small amount of bone formation, and the implantation of collagen beads alone led to no bone formation.

We therefore conclude that the combination graft of rhBMP-2 and culture-expanded cells, which requires only a small amount of bone marrow, is a reliable method for the reconstruction of segmental bone defects of the mandible.

(2) Evaluation of Human Periosteum-Derived Cells for Bone Regeneration: Comparison with Human Bone Marrow Stromal Cells

Agata H, Asahina I, Yamazaki Y, Watanabe N, Ueda M.: Division of Stem Cell Engineering IMSUT, Kitazato University School of Medicine. Nagoya University Graduate School of Medicine

Human bone marrow stromal cells have osteogenic potential and they are used for bone reconstructive surgery, while cells from mandibular periosteum are also used for bone regeneration. However the difference between bone marrow stromal cells and periosteum-derived cells concerning about osteogenic potential remains unclear. In this study, we evaluated osteogenic potential of periosteum-derived cells, comparing with that of bone marrow stromal cells *in vitro* and bone forming potential of periosteum-derived cells *in vivo.*

Cell proliferation of periosteum-derived cells was faster than bone marrow stromal cells. The expressions of mRNA for osteogenic markers and alkaline phosphatase activity assay indicated that bone marrow stromal cells were more osteogenic than periosteum-derived. However, periosteum-derived cells that were pretreated with bFGF before adding BMP-2 showed more osteogenic activity than bone marrow stromal cells treated with BMP-2.

Adding certain growth factors made periosteum-derived cells more osteogenic than bone marrow stromal cells. The shorter culture period and more osteogenic potential of periosteum-derived cells are preferable in the clinical practice of bone regeneration. These data suggest that an appropriate osteogenic-inducing technique makes periosteum a highly useful source for bone regeneration.

2. Tooth-tissue engineering

(1) Histological and Immunohistochemical Studies of Tissue Engineered Odontogenesis

Honda M, Sumita Y, Kagami Y*, Ueda M.: Division of Stem Cell Engineering IMSUT, *Nagoya University Graduate School of Medicine

We have examined the regeneration process of tissue engineered teeth with histochemistry to determine the cell types that give rise to these engineered tooth structures. Porcine third molar tooth buds were dissociated into single-cell suspensions and seeded onto a biodegradable polyglycolic acid polymer scaffold. Following varying periods of growth in rat hosts, the specimens were evaluated by histology and immunohistochemistry. Aggregates of epithelial cells were first observed 4-6 weeks after implantation. The aggregates formed three different shapes: a natural tooth germ-like shape, circle shape or bilayer- bundle. Based on the structure of the stellate reticulum in the dental epithelium, the circular and bilayer- bundle aggregates could be clearly classified into two types; one with extensively developed stellate reticulum and the other with negligible stellate reticulum. The epithelial cells in the circular aggregates differentiated into ameloblasts. The continuous bilayer bundles eventually formed the epithelial sheath, and dentin tissue was evident at the apex of these bundles. Finally, enamel-covered dentin and cementum-covered dentin formed, and this was probably mediated by epithelial-mesenchymal interaction. These results suggest the development of these engineered teeth closely parallels that of natural odontogenesis derived from the immature epithelial and mesenchymal cells.

(2) A Novel Culture System for Porcine Odontogenic Epithelial Cells Using a Feeder Layer

Honda MJ, Shimodaira T*, Ogaeri T*, Shinohara Y, Hata K*, Ueda M.: Division of Stem Cell Engineering IMSUT, *Nagoya University Graduate School of Medicine

The growth of cells *in vitro* can provide useful models for investigating their behavior and improving our understanding of their function *in vivo*. Although the developmental regulation of

enamel matrix formation has been comprehensively analyzed, the detailed cellular characteristics of ameloblasts remain unclear because of the lack of a system of long-term *in vitro* culture. Therefore, the establishment of odontogenic epithelial cell lines has taken on a new significance. Here we report on a novel porcine odontogenic epithelial cell-culture system, which has permitted serial culture of these cells. Epithelial cells were harvested from third molar tooth buds in the fresh mandibles of 6-month-old pigs, and seeded on dishes in D-MEM containing 10% FBS. Before the cells reached confluence, the medium was changed to LHC-9 to select the epithelial cells. When trypsinized epithelial cells were plated together with 3T3-J2 cells as a feeder layer, the epithelial cells grew from single cells into colonies. The colonies then expanded and became confluent, and could be sub-cultured for up to 20 passages. The established cell line expressed mRNA for amelogenin and ameloblastin, as well as enamelysin (MMP-20), which is a tissue-specific gene product unique to ameloblasts. These results show that the system is capable of sustaining the multiplication of odontogenic epithelial cells with the characteristics of ameloblasts.

Publications

1. Honda MJ, Shimodaira T, Ogaeri T, Shinohara Y, Hata K, Ueda M. A Novel Culture System for Porcine Odontogenic Epithelial Cells Using a Feeder Layer. Archives of Oral Biology. 2006, in press
2. Honda MJ, Hatanaka T, Okazaki Y, Ueda M. Long-term results of osseointegrated implant-retained facial prostheses: A 5-year-retrosopective study. Nagoya J Med Science. 67. 109-116, 2005
3. Honda MJ, Sumita Y, Kagami H, Ueda M. Histological and immunohistochemical studies of tissue engineered odontogenesis. Archives of Histology and Cytology. 68 (2); 89-101, 2005.
4. Ichiro Seto, Eriko Marukawa, Izumi Asahina. Mandibular reconstruction using a combination graft of rhBMP-2 with bone marrow cells expanded in vitro. Plast Reconst Surg in press 2006
5. Izumi Asahina. Mandible Regeneration using Bone Morphogenetic Protein. The Bulletin of Kanagawa Dental Collage, Vol. 33, No 1, March pp. 21-23, 2005

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

Associate Professor Ichiro Fujimoto, Ph.D.
Research Associate Kyoko Shirakabe, Ph.D.

特任助教授 理学博士 藤本一朗
特任助手 理学博士 白壁恭子

Nano scale biology studies of functional and structural changing molecules in neural signaling pathway are our research interest. We are studying the following subjects by introducing molecular imaging, atomic force microscope (AFM), molecular and cellular biology, and glycobiology. We normalized the AFM to observe the membrane protein structure changing without fixation. N-linked sugar chain structures were identified of this protein. The other hand, we are focusing on Na^+/HCO_3^- co-transporter protein that functioning in brain.

A High-speed atomic force microscopy (AFM) observation of calcium-induced conformational change of inositol 1,4,5-trisphosphate receptor (IP_3R) tetramer structure reconstituted into lipid bilayer

Hidetoshi Miyashita, Wakako Suhara, Keiichi Torimitsu[1], Katsuhiko Mikoshiba and Ichiro Fujimoto: [1]Materials Science Laboratory, NTT Basic Research Laboratories, Nippon Telegraph and Telephone Corporation

Inositol 1,4,5-trisphosphate (IP_3) and Ca^{2+} ion are important second messenger in most species. Inositol 1,4,5-trisphosphate receptor (IP_3R) is an IP_3-gated Ca^{2+} release channel localized in the endoplasmic reticulum (ER). The IP_3R forms homotetrameric Ca^{2+} channels and involved in intracellular Ca^{2+} storage. Binding of the two coagonists IP_3 and Ca^{2+} induces the IP_3R channel to open, which results in Ca^{2+} release from the ER into the cytoplasm. The IP_3R plays pivotal roles in neuronal transmission via Ca^{2+} signaling and for many other functions that relate to morphological and physiological processes in living organisms, such as memory, learning, behavior, fertilization, cell proliferation, cell division, development and apoptosis.

In previous studies, IP_3R protein purified from mouse cerebella has been used to reveal its tetrameric structure. By analyzing the negative-stained IP_3R by electron microscopy (EM), three-dimensional structure was reconstituted. It was demonstrated that its tetramer structure strikingly different depending on Ca^{2+} concentration in the cell. IP_3R was square-shape in unliganded state and windmill-shape in the presence of Ca^{2+} induced state. However, these images were constructed by analyzing thousands of them. The significance of two different structures has also been discussed; however, it has not been solved.

Atomic force microscopy (AFM) is a powerful technique to observe structures in nanometer scale. The resolution mainly depends on the interacting forces between a cantilever and sample, and the mechanical stability of the sample. There are two representative modes in AFM operation: contact and tapping mode. In contact mode, a tip simply scans across the surface. On

the other hand the tip in tapping mode is being excited in resonance oscillation while being scanned across the surface. In this way, the lateral force is significantly reduced making it more applicable for observation of protein samples without damage. To confirm that the IP_3R actually change its structure, tapping mode AFM is expected to be a suitable method. Commonly used AFM can take an image for few minutes. But it is too slow to follow up moving of a protein. By High-speed AFM system it need less than one second to take an image.

The IP_3R was purified from cerebella of ddY adult mice. One-step immuno affinity column made with an antibody against C-terminal region of IP_3R was employed. IP_3R protein purity was confirmed by silver-staining of SDS PAGE gels without contaminant protein. We tried to reconstruct the purified IP_3R into liposome that was made of phosphatidyl serine and phosphatidyl choline by dialysis method. For AFM imaging, samples were suspended in cytosol-like imaging buffer. The solution containing purified IP_3R or the liposome-reconstituted IP_3R were placed on mica. It was incubated for more than 30 min at 4 ℃ before images were taken. The high-speed AFM system (NVB500, Olympus Corporation) was used. A cantilever that has about 10 nm radius tip and the spring constant of 0.1 N/m was used at resonance frequency of about 600 kHz in buffer. The images in tapping mode were taken at a rate of up to 10 frames per second. In this study typically the images were taken at a rate of 2 frames per second and the size were 144×192 pixels.

The AFM image of the purified IP_3R revealed many grain-like structures whose size was relatively regular. This was very consistent with the previous data obtained by EM. Patches of lipid bilayer with IP_3R were succeed to observe in the AFM images of the reconstituted IP_3R. In the images of the reconstituted IP_3R, we analyzed the lipid thickness and the height of the protruding domain of IP_3R that we assigned as a cytoplasmic domain.

For the high-speed AFM system, the resolution is usually lower than common AFM. In our study, we adjusted our High-speed AFM system to get enough resolution for single protein imaging. We performed a statistical analysis and compared the data to those obtained with negative staining EM. The calculated size distribution clearly confirmed that the particle heights and the cytoplasmic domain were very consistent with those of side-viewed IP_3R estimated with negative staining EM. This is the first time that a reliable single channel image of liposome-reconstituted IP_3R has been obtained using a high-speed AFM system.

Interaction between sodium-bicarbonate cotransporter 1 (NBC1) and IP_3R interacting proteins

Kyoko Shirakabe, Hideomi Yamada[2], George Seki[2], Katsuhiko Mikoshiba, and Ichiro Fujimoto: [2]Department of Internal Medicine, Faculty of Medicine, The University of Tokyo.

The sodium-bicarbonate cotransporter 1 (NBC 1) is a plasma membrane localized transporter protein which transports Na^+ and HCO_3^- with stoichiometric ratio of 1 Na^+: 2 HCO_3^- (extracellular->intracellular) or 1 Na^+: 3 HCO_3^- (intracellular->extracellular). Cause HCO_3^- plays important role in the regulation of the pH homeostasis, NBC1 is thought to be a crucial regulator of pH. Actually, several mutations in NBC 1 gene (*SLC4A4*) have been identified in the patients of proximal renal tubular acidosis. This fact strongly suggests that NBC1 is indispensable for pH homeostasis of serum.

Recently we found that a particular IP_3R interacting protein interacts with NBC1 in mouse brain membrane fraction. This IP_3R interacting protein is a cytoplasmic protein thus we thought that this IP_3R interacting protein could regulate NBC1 activity through the interaction with cytoplasmic region of NBC1. NBC1 has two major splicing variants (kNBC1 and pNBC1) which have the differences only in their small N-terminal region which is exposed to cytoplasm. kNBC1 expresses mainly in kidney and pNBC1 expresses relatively many organs including pancreas, brain, liver, and colon. We first tried to identify which NBC1 could interact with the IP_3 R interacting protein using recombinant proteins of cytoplasmic region of pNBC1 and kNBC1. We found that p-type specific region of NBC1 (85 amino acid) is required and sufficient for the interaction with the IP_3R interacting protein. Furthermore we found that the phosphorylation of the IP_3R interacting protein is required for the interaction with pNBC1.

We next examined the effect of the IP_3R interacting protein on NBC1 activity using *Xenopus* oocytes. We first injected pNBC1 or kNBC1 cRNA in *Xenopus* oocytes and examined the activity of these NBC1s. We could find the NBC1 activity in kNBC1 cRNA injected oocyte but could not find that in pNBC1 cRNA injected oocyte, although pNBC1 protein expressed well as kNBC1 protein did. We further injected the IP_3R interacting protein cRNA together with pNBC1 or kNBC1 cRNA, and found that co-expression of the IP_3R interacting protein enhances the activity of pNBC1. In other hand, co-expression of the IP_3R interacting protein does not change the activity of kNBC1. These results strongly sug-

gest that the IP_3R interacting protein enhances the activity of pNBC1 through the intracellular interaction with p-type specific 85 amino acid sequence. We are now examining the molecular mechanism through which the IP_3R interacting protein regulates pNBC1 activity.

Structural Analysis of N-linked sugar chain on IP3R

Ichiro Fujimoto, Hidetoshi Miyashita, and Katsuhiko Mikoshiba:

The expression patterns of N-linked sugar chains are believed spatiotemporally regulated in the central nervous system. We constructed a two-dimensional (2D) HPLC map of pyridylaminated sugar chains to separate most of the N-linked sugar chains and to reliably quantify them. However, identification of each spot on the 2D map is requisite. We determined their structures using a combination of MALDI-TOF/MS and exoglycosidase digestion, followed by HPLC 2D mapping. The results show that 2D mapping of pyridylaminated sugar chains is a powerful method for glycomic analysis. We identified the 4 different structures of N-linked sugar chains on IP_3R glycoprotein that purified from mouse cerebellum. Among these four structures, three were High-Mannose type and the rest one was complex type, A2G0F. This complex type sugar chain is modified in protein at Golgi compartment; even this IP_3R is the ER protein. These results suggest this ER protein go through the ER-Golgi system and return to the ER membrane. Sialic acid does not appear to be a major component of this receptor (less than 4%) that the existence was previously reported. These finding suggested the specific structures of N-linked sugar chain would modulate the function of this protein. We are going to analyze the functional change of current activity of this receptor channel after removing sugar chains using lipid bilayer method.

Publications

Suhara, W., Kobayashi, M., Sagara, H., Hamada, K., Goto, T., Fujimoto, I., Torimitsu, K., and Mikoshiba, K. Visualization of inositol 1,4,5-trisphosphate receptor by atomic force microscopy. *Neurosci Lett*. 391(3): 102-107, 2006

Sakuma, K., Fujimoto, I., Hitoshi, S., Tanaka, F., Ikeda, T., Tanabe, K., Toyokuni, S., Wada, H., Mio, T., Mishima, M., and Ikenaka, K. An *N*-glycan structure correlates with pulmonary metastatic ability of cancer cells. *Biochem Biophys Res Commun*. 340: 829-835, 2006.

Tsuchiya, N., Yamanaka, R., Yajima, N., Homma, J., Sano, M., Komata, T., Ikeda, T., Fujimoto, I., Takahashi, H., Tanaka, R., and Ikenaka, K. Isolation and characterization of an N-linked oligosaccharide that is increased in glioblastoma tissue and cell lines. *Int J Oncol*. 27(5): 1231-1239, 2005.

Menon, K.N., Ikeda, T., Fujimoto, I., Narimatsu, H., Nakakita, S., Hase, S., and Ikenaka, K. Changes in N-linked sugar chain patterns induced by moderate-to-high expression of the galactosyltransferase I gene in a brain-derived cell line, CG4. *J Neurosci Res*. 80(1): 29-36, 2005.

Johnson, C.P., Fujimoto, I., Rutishauser, U., and Leckband, D.E. Direct evidence that neural cell adhesion molecule (NCAM) polysialylation increases intermembrane repulsion and abrogates adhesion. *J Biol Chem*. 280(1): 137-145, 2005.

Donation Laboratories and Research Units

Department of Molecular and Developmental Biology
再生基礎医科学研究部門

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 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) Use of dendritic cells for cell therapy. 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 2005 are as follows.

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

Hirokazu Tabe, Astumi Iida, Mariko Inoue, Jing Zhao, Tomonori Izumi[1], Yoko Tabata, Hideto Koso, Shinya Satoh, Sumiko Watanabe: [1]Division of proteomics research

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.

We screened the expression pattern of cell surface proteins in mouse immature retina by flow cytometer using a large number of antibod-

ies 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.

Results obtained with SSEA-1 and c-kit positive populations showed that these molecules marked temporally and spatially distinct retinal progenitor subsets. SSEA-1 positive cells are in the peripheral region of retina of the E17 embryo and then dramatically disappeared along with retinal development. SSEA-1 strong positive cells were Ki-67 antigen positive and had prolonged proliferation activities than that of SSEA-1 negative cells in reaggregation culture. Moreover, differentiation of SSEA-1 cells into late born retinal subtypes took longer period, suggesting that these cells are at more immature stage than SSEA-1 negative cells. Differential expression of Wnt signal-related genes between SSEA-1 positive and negative subpopulations of retinal cells was revealed, and involvement of Wnt signaling pathway for maintenance of SSEA-1 positive cells was suggested. This observation was in accordance with our in vitro results suggesting the role of Wnt as prevention of premature differentiation of retina. c-kit also labeled a subset of progenitor cells, and c-kit/SSEA-1 double positive cells had more immature characters than single positive cells. Taken together, we showed cell surface molecules, c-kit and SSEA-1, as useful tools not only to mark temporally and spatially distinct retinal subpopulations, but also to study the regulation of differentiation of neural retina.

As a marker for differentiated cells, we identified CD73 as a such marker in mice neural retina. CD73 expression was first appeared around birth. And 5 days after birth, CD73 was expressed almost all the retinal cells and continued to be expressed until adult age. CD73 positive cells do not proliferate and expressed rhodopsin, suggesting CD73 positive cells are subpopulation of photoreceptor lineage cells. In fact, CD73 positive cells expressed transcription factors nrl and crx, which are known to be specifically expressed in photoreceptors.

To obtain new cell markers defining retinal stem cells, we took an approach of proteomics. Membrane fractions were purified from embryonic and adult retina, and the profile of the expression pattern of membranous proteins were examined by shotgun analyses on a nanoflow LC-MS/MS system. Several proteins which were expressed specifically in embryo or adult retina were identified, and biological functions of these proteins are currently examined by RNAi.

Molecular mechanisms controlling photoreceptor differentiation by COUP transcription factors

Shinya Satoh, Mariko Inoue, Sumiko Watanabe

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 retinal subtypes. For example, nuclear orphan receptor NR2E3 is expressed exclusively in rod and acts as transcriptional factor to regulate opsin expression. We are now focusing on chicken ovalbumin upstream promoter-transcription factor (COUP-TF), one of the most extensively studied orphan nuclear receptors. COUP-TF family consists of three genes, COUP-TFI, COUP-TFII, and COUP-TFIII. Each of them forms homodimer and heterodimer with each other or other nuclear receptors, and dimers bind to enhancers present in a wide range of genes. In retinal studies, only mRNA expression patterns of COUP-TFI and COUP-TFII were reported. Initially, we used an immunofluorescence to assay for COUP-TFs expression. Each COUP-TF was expressed in specific retinal subtype and expression pattern of each protein was different in dorsal-ventral axis, suggesting that COUP-TFs are involved in specification of some retinal subtypes and dorsoventral-patterning of retina. To clarify the function of COUP-TFs in retinal development, we are currently investigating the effect of overexpression of each gene in retinal explant culture system and obtaining some interesting data. For example, COUP-TFII-overexpressed retinal progenitor cells preferentially differentiated to amacrine cells, in which COUP-TFII is localized at differentiated states. Analysis of COUP-TF null mice is planned, and by combining these data, we would like to calrify the roles of each COUP-TF in retinal development.

β-Catenin signaling regulates maturation of mouse retinal progenitor cells by preventing premature differentiation

Yasuo Ouchi, Mark M. Taketo[2], Sumiko Watanabe: [2]Department of Pharmacology, Graduate School of Medicine, Kyoto University

Wnt signal is a key factor that controls stem cells expansion in many tissues. In the retina, several studies using lower vertebrates show that Wnt signal plays important roles in the maintenance and proliferation of CMZ retinal progenitor cells. Although specific Wnt members are expressed in a peripheral region of

mammalian retina, it remains unknown whether this region is enriched in immature progenitor cells. In addition, roles of Wnt signaling in early retinal development are not clarified in mammals. In this study, we investigate the Wnt-β-catenin signalling pathway regarding proliferation and differentiation of retinal progenitor cells (RPCs).

Using a retrovirus vector, we expressed various mutants of Wnt signal members, such as constitutively active (ca) forms of β-catenin and LEF, and dominant-negative (dn) form of LEF, in E17.5 mouse retinal explant cultures. Proliferation, differentiation and cell morphology of virus-infected cells were analyzed. Molecular mechanisms were analyzed using PC12 cells. For in vivo studies, genetically engineered mice were used to activate or inactivate β-catenin signals in a tissue specific manner.

The results with retinal explants and PC12 cells suggested: 1) Activation of β-catenin-Lef1 inhibits neurite outgrowth of both retinal and PC12 cells. 2) Conversely, neurite outgrowth was enhanced in cells expressing dominant-negative Lef-1. 3) Proliferation and differentiation remained unaffected in these cells. The retina-specific activation of β-catenin in mice suggested: 1) Expansion of the immature progenitor cell region in embryonic mice. 2) Proliferating RPCs remained in retina derived from 7-week-old mice.

Rab11-FIP4 plays essential role for proliferation and differentiation of neural retina

Akihiko Muto, Yutaka Aoki, Sumiko Watanabe

Rab11-family interacting protein 4 (Rab11-FIP 4) was initially identified in human as a Rab11-binding protein, but its biological function has remained unknown. We cloned the zebrafish orthologue of (zRab11-FIP4) and analyzed its function by using antisense morpholino. zRab11-FIP4 was expressed as two alternative transcripts, the longer A-form predominantly expressed in neural tissues including the retina and the shorter B-form expressed ubiquitously, but in situ hybridization revealed that the A-form was dominantly expressed. In the developing retina, zRab11-FIP4 was first expressed in proliferating progenitors and then, along with the differentiation, the expression was gradually restricted into retinal ganglion cells and amacrine cells as well as ciliary marginal zone. We found that zRab11-FIP4 knockdown embryos exhibited eye phenotypes similar to those of the *shh* mutant, such as small eye with impaired proliferation and the retardation of cell cycle exit and differentiation. Ectopic expression of either p57Kip2 or dominant negative PKA could rescued delayed cell cycle exit in the zRab11-FIP4 morphant retina, suggesting functional interaction of zRab11-FIP4 A with the *shh* signaling during development of zebrafish retina.

We further analyzed the function of Rab11-FIP4 in mouse neural retina. Mouse Rab11-FIP4 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 populations of 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. Interestingly, Rab11-FIP4A promoted cell cycle exit but not the progression. Accordingly, reversed phenotype was observed when Rab11-FIP4A was downregulated by using shRNA. Taken together, it is suggested that Rab11-FIP4 plays a role in retinal development also in the mammalian system.

Melk-like kinase plays a role in haematopoiesis in the zebrafish

Rika Saito, Yoko Tabata, Ken-ichi Arai[3] and Sumiko Watanabe: [3]Tokyo Metropolitan Institute of Medical Science

A serine/threonine kinase, *Melk* was initially cloned in mouse oocytes as a maternal gene, but whose function was unknown. In adult mice, *Melk* was strongly expressed in thymus and bone marrow, suggesting a role for *Melk* in haematopoiesis. We cloned a *Melk-like gene* from zebrafish (z). *zMelk-like gene* was expressed in brain and lateral mesoderm at 12 hpf and in several tissues of adult fish including kidney and spleen, both of which are known to be haematopoietic tissues in zebrafish. Abrogation of *zMelk-like gene* function by *zMelk-like gene*-specific MO resulted in abnormal swelling around the tectum region. In addition, the start of blood circulation was severely delayed, but, in contrast, the vessel formation seemed normal. Expression of *scl, gata-1*, and *lmo-2* was down regulated at 12-14 hpf in the *zMelk-like gene* MO-injected embryos and co-expression of gata-1 rescued the anemic phenotype induced by *zMelk -like gene* MO. Expression of zMelk-like gene in embryos enhanced *gata-1* promoter dependent EGFP expression, suggesting zMelk-like gene affects *gata-1* expression in transcriptional level. Taken together, our data suggest that the *zMelk-like gene* may play a role in primitive haema-

topoiesis by affecting the expression of genes critical for haematopoiesis.

Ly49Q is a marker for immature plasmacytoid dendritic cells

Yumiko Kamogawa[4], Makiko Tohma, Naoko Arai[4], Ken-ichi Arai[3], Sumiko Watanabe: [4]Ginkgo Biomedical Research Institute

Plasmacytoid Dendritic cells (pDC) are the unique cells that produce large amount of type I interferon upon viral or bacterial encounter, thus play an important role for eradication of infectious organisms. In order to investigate the biological importance of pDC, we have searched the molecules that regulate pDC by generating monoclonal Ab, 2E6 that recognized murine pDC. We found that Ab, 2E6, recognized type II C type lectin Ly49Q that is a specific marker for peripheral pDCs and that expression of Ly49Q defines two subsets of pDCs in the bone marrow. Recent report suggested that B220+CD11c+CD11b-pDC from bone marrow can differentiate into myeloid DC(mDC) upon viral infection. We hypothesized Ly49Q-pDC might become mDC upon viral infection. Thus, we purified either Ly49Q- or Ly49Q+pDC in bone marrow after polyIC treatment in vivo by sorting, then analyzed a marker for mDC few days after purification. Ly49Q-pDC but not Ly49+pDC could differentiate into mDC upon polyIC treatment. Moreover, those effects diminished by using type I IFN KO mice bone marrow suggesting that those effects depend on type I IFN. In conclusion, Ly49Q-pDC is immature and can differentiate into mDC upon viral infection by IFN dependent manner. The conversion of pDC to mDC may help the viral clearance by more efficient Ag presentation by mDC in vivo.

Publications

Kamogawa-Schifter, Y., Ohkawa, J., Namiki, S., Arai, N., Arai, K., Liu, Y. (2005) Ly49Q defines two pDC subets in mice. Blood, 105, 2787-2792

Kubota, Y., Oike, Y., Satoh, S., Tabata, Y., Niikura, Y., Morisada, T., Akao, M., Urano, T., Ito, Y., Miyamoto, T., Yasunaga, K., Watanabe, S., Suda, T. (2005) Isolation and expression patterns of three angiopoietin-related proteins in zebrafish. Gene Expr Patterns. 5, 679-85.

Saito, R., Tabata, Y., Muto, A., Arai, K., Watanabe, S. (2005) Melk-like-kinase plays a role for haematopoiesis in zebrafish. Mol. Cell. Biol. 25, 6682-6693

Ouchi, Y., Tabata, Y., Arai, K. Watanabe, S., (2005) Negative regulation of retinal-neurite extension by Wnt-Lef-1 signalling pathway. J. Cell Sci. 118, 4473-4483

Ohtsuka Y, Manabe A, Kawasaki H, Hasegawa D, Zaike Y, Watanabe S, Tanizawa T, Nakahata T, Tsuji K. (2005) RAS-blocking bisphosphonate zoledronic acid inhibits the abnormal proliferation and differentiation of juvenile myelomonocytic leukemia cells in vitro. Blood 106, 3134-3141

Kubota, Y., Oike, Y., Satoh, S., Tabata, Y., Niikura, Y., Morisada, T., Akao, M., Urano, T., Ito, Y., Miyamoto, T., Nagai, N., Watanabe, S., Suda, T., (2005) Cooperative interaction of angiopoietin-like proteins, 1 and 2 on the vascular development of zebrafish. Proc. Natl. Acad. Sci. USA 102, 13502-13507

Koso, H., Ouchi, Y., Tabata, Y., Aoki, Y., Satoh, S., Arai, K., Watanabe, S., (2006) SSEA-1 marks regionally restricted immature subpopulations of embryonic retinal progenitor cells that are regulated by the Wnt signaling pathway. Dev. Biol. in press

Muto, A., Aoki, Y., Arai, K., Watanabe, S. (2006) Identification of mouse Rab11-FIP4 as a gene expressed in developing retina in a spatiotemporally regulated manner. Dev. Biol. in press

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)寄附研究部門

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

本研究部門の目的は，先端医療の確立と普及に必要な方法論を研究し，新たな先端医療研究遂行モデルを提示することです．

わが国では新規薬剤の探索的研究は進んでいますが，臨床試験を通じた先端医療開発体制の整備が遅れています．この結果，国内で発見された新規薬剤，治療法の臨床開発は海外で行われることが多いです．この原因としては，財源問題，臨床試験の高コスト，非効率な臨床試験システム，規制の影響などが指摘されています．また，先端医療が確立されても，それが国内に普及する速度が遅く，医療供給のための財源問題，先端医療の担う人材・情報の流動性が低いことも問題です．しかしながらこれらの問題点の実態に関しては不明な点が多いです．

本研究部門では先端医療の確立・普及を阻害するボトルネックを明らかにし，その解決策を探り，具体化していくために以下の諸活動と提案を行っています．

1）プロテオソーム阻害剤であるBortezomibの日本人における有効性・安全
2）国内未承認薬について
3）外来患者向けフリーペーパー“lohasmedical”による患者啓蒙活動

The mission of this division is to create a new model of translational research (TR). Dramatic progress has been madeon several fronts to improve the lives of patients afflicted with cancer, and both new technologies and the pace of scientific discovery have set the stage for rapid advancement in the years to come, however, the methodology of TR has not been established yet. Division of Exploratory Research aims at breaking the bottle-neck of TR and hastening the translation of basic science into innovations in clinical care.

The focuses of Division of Exploratory Research works are:

1) investigate safety and efficacy of Bortezomib (Velcade®) in Japanese patients with hematological malignancy
2) conduct Japanese survey on the clinical use of anti-cancer drug which is not approved by MHLW (the Japanese Ministry of Health, Labor, and Welfare) and PMDA (the Pharmaceuticals and Medical Devices Agency)
3) provide high medical literacy for outpatients by publishing hospital freesheet “lohasmedical”

Publications

Miyakoshi S, Kami M, Yuji K, Matsumura T, Takatoku M, Sasaki M, Narimatsu H, Fujii T, Kawabata M, Taniguchi S, Ozawa K, and Oshimi K. Severe pulmonary complications in Japanese patients after bortezomib treatment for refractory multiple myeloma. Blood, in press, 2006.

Kami M, Murashige N, Tanaka Y and Narimatsu H. Antifungal prophylaxis following reduced intensity stem-cell transplantation. Transplant Infect Dis, in press, 2006.

上　昌広．移植領域における感染症の変遷．細胞．上　昌広編集．ニューサイエンス社．38(2)：2–3，2006.