Donation Laboratories

Division of Cell Processing (CERES Consortium)

細胞プロセッシング(CERES)寄附研究部門

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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 processed about 20,000 cord blood units, registered about 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 more than 450 units to transplant facilities by the end of 2007. This facility obtained the certification of ISO 9002: 1994 in March 2000 as the first cell-processing center in Japan and Asia, and ISO 9001: 2000 in May 2003. The quality control system developed in this laboratory has been accepted by other cell therapy related facilities in Japan and Asia.

This department has supported the clinical departments through cord blood transplantation for child and adult patients with haematological malignancies. We have also studied 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 stem and progenitor cells and those could differentiate into osteoblasts, chondrocytes, adipocytes and neural type cells. The study has been extended to the mesenchymal stem and progenitor cells in cord blood, and those placenta-derived and cord blood mesencyhmal stem cells (MSCs) should be considered as one of the possible allogeneic cell sources for cell therapies and tissue engineering.

1. Cord Blood Banking in IMUST

Masako Hirai[1], Tokiko Nagamura-Inoue[1], 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 and Donated Blood Distribution Foundation has worked as the carrier of cord

blood and administrative office, and the cord blood processing facility of 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 15 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 has been considered as one of the leading cord blood banks in Japan and Asia, and the effort will continue to develop new technology to improve clinical outcome of CBT.

2. Isolation of mesenchymal stem cells from cord blood

Xiaohong Zhang, Tsuneo A. Takahashi

The mesenchymal 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 mesenchymal stem cells but the number of these cells is estimated to be less than 1% of those in the marrow. We have started isolating mesenchymal 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. Characteristics of mesenchymal stem cells from human umbilical cord blood were compared with those derived from bone marrow, adipose tissue and chorionic villi of placenta. Though surface antigens of these MSCs did not show significant differences among the cell sources, different differentiation abilities existed and cord blood derived MSCs showed high differentiation ability to chondrocyte as well as those from bone marrow, but not for mesenchymal stem cells isolated from adipose tissue.The in vivo transplantation of cord blood derived MSCs with the scaffold showed that these cells could form chondrocyte like tissue in vivo as well as bone generation. The critical factors for successful isolation of MSCs from cord blood were the volume of umbilical cord blood at collection and time between delivery and isolation of cells. If both conditions were achieved (more than 60 ml, less than 5 hours), MSCs could be isolated from cord blood with the frequency of more than 70%. We also examined the suppressive effect of CB-MSCs to immunological functions. Various cells number of MSCs were cultured with PHA stimulated T cells or cells in mixed lymphocyte culture. Suppressive effect by the MSCs was calculated by measuring T cells or lymphocyte proliferation. The suppressive effect of CB-MSCs inhibited T cell proliferation were similar to BM-MSCs and AT- MSCs.

Publications

Parolini, O., Alviano, F., Bagnara, GP., Bilic, G., Bühring, HJ., Evangelista, M., Hennerbichler, S., Liu, B., Magatti, M., Mao, N., Miki, T., Marongiu, F., Nakajima, H., Nikaido, T., Portmann-Lanz, CB., Sankar, V., Soncini, M., Stadler, G., Surbek, D., Takahashi, TA., Redl, H., Sakuragawa, N., Wolbamk, S., Zeisberger S., Zisch, A. and Strom SC. Isolation and characterization of cell from human term placenta outcome of the first international workshop on placenta derived stem cell. Stem Cell. 26 (300-311), 2007.

Donation Laboratories

Division of Cellular Proteomics (BML)
細胞ゲノム動態解析(ビー・エム・エル)寄附研究部門

Visiting Professor Seisuke Hattori, Ph.D.
Assistant Professor Hidetaka Kosako, Ph.D.

客員教授 理学博士 服部成介
助教 理学博士 小迫英尊

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.

1. A Phosphoproteomic Approach for the Discovery of Protein Kinase Substrates: Identification of Cytoplasmic Dynein and the Nuclear Pore Complex as Novel ERK Targets.

Hidetaka Kosako[1,★], Nozomi Yamaguchi[1,2], Masato Ushiyama[1,3], Eisuke Nishida[4], and Seisuke Hattori[1,5]: [1]Division of Cellular Proteomics (BML), [2]BML, Inc., [3]GE Healthcare Bio-Sciences KK, [4]Department of Cell and Developmental Biology, Graduate School of Biostudies, Kyoto University, [5]Division of Biochemistry, School of Pharmaceutical Sciences, Kitasato University.

Many ERK MAP kinase substrates have been identified, but the diversity of ERK-mediated processes suggests the existence of additional targets. Here we present a phosphoproteomic approach to identify putative ERK substrates by combining the steroid receptor fusion system, immobilized metal affinity chromatography (IMAC), fluorescent two-dimensional difference gel electrophoresis (2 D-DIGE), and phosphomotif-specific antibodies. Purification of phosphoproteins from whole cell lysates by IMAC enabled sensitive detection of minor phosphorylated signaling components that would otherwise be obscured by abundant cellular proteins. Changes in phosphoprotein profiles between selective activation and inhibition of the Raf-MEK-ERK pathway were globally analyzed by 2D-DIGE. Quantitative analysis detected 37 reproducibly changed protein spots, several of which were recognized by the ERK consensus motif-specific antibodies. Mass spectrometric analysis identified 38 proteins as ERK pathway-associated proteins. These included MEK1/2 and ERK1/2 as well as previously known ERK substrates such as RSK2, cPLA2, hnRNP K, caldesmon, cortactin, and vinexin, demonstrating the feasibility of our approach. The remaining 24 proteins were considered candidates for novel ERK targets, which suggest as yet undefined roles for this signaling pathway in cytoskeletal regulation, mRNA processing, vesicle transport, proteolysis, and protein folding. We purified 14 proteins fused to GST, 13 of which were phosphorylated by ERK in vitro. Among them, cytoplasmic dynein intermediate chain 2 and the nucleoporin Nup50/Npap60 were shown to be phosphorylated by ERK in intact cells. Moreover, ERK phosphorylation of the FG repeat region of Nup50 was found to reduce its affinity for importin-b. This approach is ap-

plicable to other protein kinases and may be useful for large-scale identification of cellular substrates.

2. ERK MAP Kinase Regulates Actin Organization and Cell Motility by Phosphorylating the Actin Cross-Linking Protein EPLIN.

Mei-Ying Han[1,2], Hidetaka Kosako[1,*], Toshiki Watanabe[2] and Seisuke Hattori[1,3]: [1]Division of Cellular Proteomics (BML), [2]Graduate School of Frontier Sciences, The University of Tokyo, [3]Division of Biochemistry, School of Pharmaceutical Sciences, Kitasato University.

Extracellular signal-regulated kinase (ERK) is important for various cellular processes including cell migration. However, the detailed molecular mechanism by which ERK promotes cell motility remains elusive. Here we characterize epithelial protein lost in neoplasm (EPLIN), an F-actin cross-linking protein, as a novel substrate for ERK. ERK phosphorylates Ser360, Ser 602, and Ser692 on EPLIN in vitro and in intact cells. Phosphorylation of the C-terminal region of EPLIN reduces its affinity for actin filaments. EPLIN colocalizes with actin stress fibers in quiescent cells, and stimulation with PDGF induces stress fiber disassembly and relocalization of EPLIN to peripheral and dorsal ruffles, wherein phosphorylation of Ser360 and Ser602 is observed. Phosphorylation of these two residues is also evident during wound healing at the leading edge of migrating cells. Moreover, expression of an ERK nonphosphorylatable mutant, but not wild type, EPLIN prevents PDGF-induced stress fiber disassembly and membrane ruffling, and also inhibits wound healing and PDGF-induced cell migration. We propose that ERK-mediated phosphorylation of EPLIN contributes to actin filament reorganization and enhanced cell motility.

3. Purification of phosphoproteins by immobilized metal affinity chromatography and its application to phosphoproteome analysis.

Mitsuyo Machida[1], Hidetaka Kosako[1], Kyoko Shirakabe[1], Michimoto Kobayashi[1], Masato Ushiyama[2], Junichi Inagawa[2], Joe Hirano[2], Tomoyo Nakano[3], Yasuhiko Bando[3], Eisuke Nishida[4], and Seisuke Hattori[1]: [1]Division of Cellular Proteomics (BML), [2]GE Healthcare Bio-Sciences Corp KK, [3]AMR Inc., [4]Graduate School of Biostudies, Kyoto University.

Prefractionation procedures facilitate the identification of lower abundance proteins in proteome analyses. Here we have optimized conditions for immobilized metal ion affinity chromatography (IMAC) to enrich for phosphoproteins. Metal ions, Ga(III), Fe(III), Zn(II) and Al(III), were compared for their abilities to trap phosphoproteins, among which Ga(III) showed the best performance. Detailed analyses of the pH and ionic strength for IMAC enabled us to fix the optimal conditions (pH 5.5 and 0.5 M NaCl). When total cell lysates were fractionated in this way, about one tenth of total protein was recovered in the eluate, and the recovery of phosphorylated ERK (extracellular signal-regulated kinase) was over 90%. Phosphorylated forms of RSK (ribosomal S6 kinase) and Akt were also enriched efficiently under the same conditions. Our Ga(III)-IMAC and a commercially available purification kit for phosphoproteins gave similar performances with a slight difference in the spectrum of phosphoproteins. When phosphoproteins enriched from NIH3T3 cells in which ERK was either activated or suppressed were analyzed by two-dimensional fluorescence difference gel electrophoresis (2-D DIGE), phosphorylated ERK was detected as discrete spots unique to ERK-activated cells, which overlapped with surrounding spots in the absence of prefractionation. We also applied the same strategy to search Akt substrates and identified Abi-1 (Abelson interactor-1) as a novel potential target. These results demonstrate the efficacy of phosphoprotein enrichment by IMAC and suggest that this procedure will be of general use in phosphoproteome research.

4. Global analysis of dynamic changes in lipid raft proteins during T-cell activation.

Michimoto Kobayashi[1], Takuya Katagiri[1], Hidetaka Kosako[1], Naoyuki Iida[1], Seisuke Hattori[1,2]: [1]Division of Cellular Proteomics (BML), [2]Division of Biochemistry, School of Pharmaceutical Sciences, Kitasato University.

Lipid rafts are considered as specialized micro domains within the plasma membrane with unique lipid compositions different from surrounding membranes. Following T-cell receptor (TCR) stimulation, lipid rafts assemble in T-cell/antigen-presenting cell (APC) contact site known as the immunological synapse, inner leaflets of which serve as activation or docking sites for downstream signaling components. To understand the signaling events occurring in lipid rafts, we globally analyzed dynamic changes in lipid raft proteins during TCR/CD28 costimulation using two-dimensional fluorescence difference gel electrophoresis (2D-DIGE). We detected multiple spots intensities of which were

enhanced after co-stimulation, and identified proteins in these spots by peptide mass fingerprinting. Identified proteins include Src family tyrosine kinases, tyrosine phosphatase, phosphatidylinositol 3-kinase (PI3-kinase), actin binding proteins, and regulators for small GTPases. Of particular interest, a number of pleckstrin homology (PH) domain-containing proteins were identified. Biochemical and histochemical analyses confirmed the translocation of these proteins from cytosol to lipid rafts. We also demonstrated that these proteins assembled at the T-cell/APC interface. These results indicate the efficacy of our system to systematically analyze dynamics of lipid raft proteins during extracellular stimulation.

Publications

Han, M.-Y., Kosako, H., Watanabe, T. and Hattori, S. Extracellular signal-regulated kinase/mitogen-activated protein kinase regulates actin organization and cell motility by phosphorylating the actin cross-linking protein EPLIN. Mol. Cell. Biol. 27: 8190-8204, 2007.

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Machida, M., Kosako, H., Shirakabe, K., Kobayashi, M., Ushiyama, M., Inagawa, J., Hirano, J., Nakano, T., Bando, Y., Nishida, E. and Hattori, S. Purification of phosphoproteins by immobilized metal affinity chromatography and its application to phosphoproteome analysis. FEBS J. 274: 1576-1587, 2007.

Donation Laboratories

Division of Stem Cell Engineering (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)寄附研究部門

Visiting Professor	Minoru Ueda, D.D.S., Ph.D.		客員教授(併)	医学博士	上田 実
Visiting Associate Professor	Hiedaki Kagami, D.D.S., Ph.D.		客員准教授	医学博士	各務秀明
Assistant Professor	Masaki Honda, D.D.S., Ph.D.		助 教	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 cells in epithelial or mesenchymal tissue from tooth germ, 2) search for molecules to affect the differentiation of the stem cells, 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 biodegradable scaffold as an each element.

1. Clinical study

Bone marrow stromal cells (BMSCs) have been widely used for bone tissue engineering. Although bone tissue engineering using BMSCs is considered feasible, little is known about the clinical efficacy of this novel treatment. We have developed a clinical trial to test if BMSCs will be clinically effective in regenerating bone in patients with severe maxillary and/or mandibular bone absorption who undergo dental implant surgery. 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 pre-

treatment 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. Side population cells expressing ABCG2 in human dental pulp tissue

Pluripotential side populations (SP) of hematopoietic stem cells, which are mediated by the ATP-binding cassette transporter ABCG2 have been defined. However, the stem cells in dental pulp tissue have been less well characterized. In this study, we examined the presence of SP cells by the Hoechst exclusion method in human adult dental pulp. *Methodology*: Human dental papilla cells were generated from third molars. The cells were stained with Hoechst 33342 and sorted into SP cells or non-SP (Main Population cells; MP) cells. We compared both cell types with cell growth and RT-PCR analyses. *Results and Conclusion*: SP cells that co-express ABCG2, Nestin, Notch-1 and α-smooth muscle actin were found at frequencies ranging from 0.67% to 1.02%. This SP profile disappeared in the presence of verapamil. These SP cells expressed dentin sialophosphoprotein when cultured in osteogenic medium. This study demonstrated that dental papilla contain SP cells that differentiate into odontoblast-like cells.

3-2. Subcultured odontogenic epithelial cells in combination with dental mesenchymal cells produce enamel-dentin-like complexes structures

We demonstrated in a previous study that odontogenic epithelial cells can be selectively cultured from the enamel organ in serum-free medium and expanded using feeder layers of 3T 3-J2 cells. The subcultured epithelial cells retain the capacity for ameloblast- related gene expression, as shown by RT-PCR. The purpose of this study was to evaluate the potential of subcultured odontogenic epithelial cells to generate tooth structures in cell-polymer constructs maintained in vivo. Enamel organs from 6-month-old porcine third molars were dissociated into single odontogenic epithelial cells and subcultured under 3T3-J2 cells. Amelogenin expression was detected in the subcultured odontogenic epithelial cells by immunostaining and Western blotting. The subcultured odontogenic epithelial cells were seeded onto collagen sponge scaffolds in combination with fresh dental mesenchymal cells, and transplanted into athymic rats. After 4 weeks, enamel-dentin-like complex structures were present in the implanted constructs. These results show that our culture system produced differentiating ameloblast-like cells that were able to secrete amelogenin proteins and form enamel-like tissues in vivo. This application of the subculturing technique provides a foundation for further tooth-tissue engineering and for improving our understanding of ameloblast biology.

3-3. The sequential seeding of epithelial and mesenchymal cells for tissue-engineered tooth regeneration.

Progress is being made toward generating teeth by seeding dissociated postnatal tooth cells onto scaffolds and implanting them in vivo, but tooth morphology remains difficult to control. In this study, we aimed to facilitate tooth regeneration using a novel technique to sequentially seed epithelial cells and mesenchymal cells so that they generated appropriate interactions in the scaffold. Dental epithelium and mesenchyme from porcine third molar teeth were enzymatically separated and dissociated into single cells. Mesenchymal cells were seeded onto the surface of the scaffold and epithelial cells were then plated on top so that the two cell types were in direct contact. The cell-scaffold constructs were evaluated in vitro and also implanted into immunocompromised rats for in vivo analysis. Control groups included constructs where direct contact between the two cell types was prevented. In scaffolds seed using the seeding technique, alkaline phosphatase activity was significantly greater than controls, the tooth morphology in vivo was developed in similar to that of natural tooth, and only one tooth structure formed in each scaffold. These results suggest that the sequential seeding technique could be useful for regulating the morphology of regenerated teeth.

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Donation Laboratories

Division of Molecular and Developmental Biology (Oriental, Tomy, SBI Holdings)

再生基礎医科学(オリエンタル・トミー・SBIホールディングス)寄附研究部門

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客員教授 医学博士 渡 辺 すみ子
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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 models ranging from culture cells, zebrafish, mouse, to monkey. Based on our research background on the area of haematology and cytokine signals, we now focuses on analysis of development of neural retina from stem cells.

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. Various signaling molecules and transcriptional factors are under investing for their roles on retinal development. For developmental biological analyses, we use zebrafish system in addition to mouse system as model animals. We 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 2007 are as follows.

Role of nuclear receptors in retinal development: Regulation of retinal cone cell differentiation by BMP signaling and nuclear receptor COUP-TFII

Shinya Satoh, Mariko Inoue, Atsumi Iida, Yasuhide Furuta[1], Tatsuhiko Kodama[2], Sumiko Watanabe: [1]M.D. Anderson Cancer Center, Univ. of Texas, USA, [2]RCAST, Univ. of Tokyo.

A panel of antibodies against all known nuclear receptors had been produced by Prof. Kodama's group in RCAST, Univ. of Tokyo. We conducted screening of the expression patterns of antigens of the antibodies in mouse retina at different developmental stages. Among them, we found that several antigens have interesting and specific expression pattern in certain stages of retina and focused on the analysis of their biological roles for development and function of

retina.

Cone cell is a subtype of photoreceptors in retina, and in mouse eye, each cone cell expresses one of two types of color photopigment, which are sensitive for short wavelength (s-opsin) or middle wavelength (m-opsin) of lights. The opsin has unique dorsoventral expression pattern in the retina, i.e., s-opsin is expressed in ventral half of retina, and in contrast, m-opsin expresses mainly in dorsal half of the retina. However, the precise mechanism of spatial patterning of opsin expression has not been understood. Using retinal explant culture system, we found that retinas from mouse at embryo 12-day (E12) stage already possess dorsoventral axis information of retina. Dorsal BMP signaling activated by BMP4 at early embryonic periods is important to determine the dorsoventral axis of retina and its disruption resulted in abnormal retinotectal projection of retinal ganglion cells. In the BMP receptor mutant retina, s-opsin expression was de-repressed at dorsal side of retina and the gradient of m-opsin expression was absent even though the number of cone cells was comparable to that of control retina. These data indicate that dorsoventral patterning of mouse cone opsins is regulated by BMP signaling. We found that COUP-TFII, one of nuclear receptors, is also expressed at dorsal side of retina. In addition, we found that overexpression of COUP-TFII in retinal explant organ culture system leads to the induction of cone cell differentiation but the suppression of rod photoreceptor cell differentiation. Using retinoblastoma cell line, Y79 cells, we conducted experiments of gain and loss of functions of COUP-TFII and obtained evidence of suppression of s-opsin expression by COUP-TFII. In addition, the expression of COUP-TFII was absent at dorsal side of BMP receptor mutant retina, suggesting that BMP signaling plays essential roles for COUP-TFII expression. From these results, we hypothesize that spatial patterning of opsin expression is regulated by BMP signaling and this activity is mediated by the expression of COUP-TFII.

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

Although neural stem cells has been extensively studied, the character of the immature retinal cells has not been 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 (CD antigens) 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. Until now, 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. We continue to characterize retinal cell subsets which are labeled by certain antibodies anti CD antigens.

1. CD44 is a novel surface antigen identified retinal Mueller glial progenitor cells

Hirokazu Tabe and Sumiko Watanabe

Subtypes of neurons and a glial cell, Müller glia, are believed to be differentiated from a common progenitor cell. However, molecular mechanisms of fate decision of neuron and glial cells in retina has not been known. We found that CD44 marks retinal progenitor cells which committed to Müller glial cell lineage. The expression of CD44 was dramatically changing during retinal development. First peak of the expression was at around postnatal day 1, and then the expression was disappeared and seen again after completion of retinal differentiation. In the early stage of retinal development, CD44 positive cells were proliferating and a part of c-kit positive cell population. Purified CD44 positive cells exclusively differentiated into Müller glia cells but not neurons in in vitro reaggregation culture, suggesting that the fate of CD44 positive progenitor cells were specified into Müller glial cells. Although gain- or loss- of function analysis of CD44 did not alter retinal differentiation, CD44 inhibited extension of processes of Müller glial cells. In previous report, it was reported that gliogenesis was regulated by Notch signalling. Activation of Notch signalling dramatically reduced number of CD 44 positive cells and mature Müller glia cells in retinal explant culture. Furthermore, structure of retina was severely damaged in the absence of Müller glia cell, suggesting that Müller glia plays important role for retinal tertiary structure formation. Taken these results together, by the identification of CD44 as an earliest marker of the Müller glia lineage allowed to clarify the role of Müller glia cells for formation of retinal structure. Furthermore, CD44 is first molecule which is suggested to regulate process formation of Müller glia cells.

2. Screening of CD antigen expression patterns of adult peripheral eye structures

Yukihiro Baba and Sumiko Watanabe

For regenerative medicine of retina, characterization of retinal stem cells is essential issue to be clarified. Many works suggested that multipotent retinal stem cells to locate in ciliary margin or iris of mammalian retina, however, their nature had not been revealed. To identify surface antigens expressed in retinal stem cells, we screened the expression of a panel of CD antigens in ciliary margin and iris of adult mouse retina. Antigens showing positive signals were further examined for their expression pattern in developing and mature retina by immunohistochemistry. Immunohistochemical analysis revealed that the antigens expressed specifically either in non-pigmented ciliary epithelium, pigmented ciliary epithelium or RPE. Transition of distinct populations in ciliary margin of mouse retina were defined by examining expression of different CD antigens. Commitment to the non-pigmented cells was suggested to occur in an early stage of development. Although pigmented cells maintained proliferation ability until maturation, non-pigmented cells of mouse retina lost it, suggesting a difference from previous observations in avian retina.

Role of Sox transcription factors in retinal development:

1. Group E Sox genes, Sox8 and Sox9, are regulated by Notch signaling and required for the Müller glia development in mouse retina

Akihiko Muto and Sumiko Watanabe

Müller glia plays pivotal roles in vertebrate adult retina, whereas the mechanism regulating the development of Müller glia is poorly understood. Previous studies have revealed that Notch signaling is involved in the multiple steps of retinal development in a stage-specific manner and required for the Müller glia development in postnatal stage. Hes5 was reported to contribute this process, however, presence of Müller glia genesis in Hes5 deficient mice suggested involvement of other molecules in this process. We found that 2 members of group E Sox genes, Sox8 and Sox9, were expressed in proliferating progenitors in embryonic retina and then exclusively in Müller glia at later stages. The expression of shRNA specific to each significantly decreased the population of Müller glia hence increased rod photoreceptors, but not other retinal neurons, suggesting that these Sox proteins play roles in the specification of Müller glia. We also found that Notch signaling regulated the expression of these Sox genes transcriptionally by using an activated form of Notch and a γ-secretase inhibitor DAPT. This is the first evidence for the role of group E Sox genes in developing vertebrate retina.

2. The HMG-transcription factor Sox2 is sufficient for the induction of amacrine cells in mouse retina

Ya-ping Lin, Yasuo Ouchi, Shinya Satoh and Sumiko Watanabe

The transcription factor Sox2 plays important roles in both human and mouse retinal development. Although loss-of-function mutations in Sox2 have been studied in mice, gain-of-function experiments in the neural retina have not been investigated. Immunohistochemistry showed that Sox2 was expressed throughout the neuroblastic layer in the embryonic retina, but only in the inner nuclear layer in the mature retina. Double immunostaining revealed that Sox2 was expressed in Müller glial cells and in some amacrine cells. Forced expression of Sox2 in a mouse retinal explant culture resulted in the dramatic accumulation of amacrine cells in the inner nuclear layer; in addition, cells expressing amacrine cell markers were also found on the innermost side of the outer nuclear layer. The expression of Pax6, which plays an important role in amacrine cell differentiation, was observed in the Sox2-expressing cells, and Sox2 activated the Pax6 promoter to drive luciferase expression in Y79 cells. Retinal progenitor cell proliferation was also decreased. The suppression of Sox2 expression by shRNA resulted in a decreased number of cells in the inner nuclear layer. Therefore, Sox2 expression is sufficient to induce amacrine cells in the mouse retina from stage E17 onward, possibly by facilitating cell cycle exit.

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Donation Laboratories

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

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

The aim of our division is to establish and popularize state-of-the-art medicine and to promote translational research (TR). We develop clinical research infrastructures by unveiling bottlenecks for such establishment and popularization and exploring solutions.

1. Clinical Research

Tomoko Matsumura, Masahiro Kami

After various multiple-center clinical researches, we have reported on adverse events, effectiveness, and safety of state-of-the-art medicine (1-10, 23-31, 43, 44) and case studies (11-13, 19-22).

2. Patient Recruitment

Masahiro Kami

Since medical practice is rooted in a regional community, investigating patient migration is one of the key factors to promote state-of-the-art practice and clinical researches.

We surveyed hematology practice in Tokushima Prefecture as a model region to investigate optimal coordination among regional medical associations, public administration and regional media, and patient migration.

3. Structure for Medical Provision

Tomoko Matsumura, Masahiro Kami

We studied the structure for medical provision which can respond to the people's demand with increasing diversity. Our collaborative study with home medical care staff demonstrated that home medical care of an elderly patient with malignant lymphoma is possible (14). In our collaborative study with clinics, we identified significant medical needs of young adults with growing health concerns and demands for medical care located on pathways of their daily living such as railway stations.

4. Information Provision for the People

Tomoko Matsumura

We investigate the media effects on the people to improve infrastructures which provide medical information to the people.

In our study on NHK Special released in 2005, we reported the possibility of harmful media effects on patients which increased physicians' prescription and led to significant number of deaths due to adverse drug effects (15).

We investigated websites containing cancer information. The website of the National Cancer Center Hospital was easily accessible by Internet search, which covered large amount of general cancer information and was linked to websites of many hospitals. Yet, user friendliness can be improved (16).

5. Drug Approval and Safety Management

Masahiro Kami

A proteasome inhibitor for multiple myeloma (Velcade) was used before approval in Japan, and some patients died of adverse drug effects. We reported the lack of safety management of unapproved drugs. We continue collaborative studies with Pharmaceuticals and Medical Devices Agency to investigate the structures of clinical trials and drug approval, including distribution systems (17).

6. Medicolegal Issues

Tomoko Matsumura, Masahiro Kami

After an obstetrician was arrested at Fukushima Prefectural Ohno Hospital, Article 21 of Physician Law and introduction of criminal penalty to medical practice induced shrinkage of medical practice and became a nationwide concern. Research articles reporting adverse events, complications, safety and effectiveness dramatically decreased in 2007, suggesting researchers' fear of legal reference. We collaborate with law schools to propose drafts for creation of constructive relationship between legal issues and medical practice/researches.

7. Patient Literacy

Yuji Tanaka

a) Patient Associations

We continue researches on patient feelings with patient associations. We collaborate with the Liaison Council of Inhospital Patient Association Representatives which coordinates 20 inhospital patient associations all over Japan and the Research Division led by Professor Ohsawa at Faculty of Engineering, the University of Tokyo.

b) Pancreatic Islet Transplantation

In our collaborative research with Baylor University in Texas, USA, we study for improvement in quality of life of patients with type I diabetes before and after pancreatic islet transplantation. We aim to develop an informed consent format for pancreatic islet transplantation which improves patient understanding and better convince them, doing patient surveys to evaluate the current version.

8. Collaborative Studies

Masahiro Kami

a) Regenerative Medicine

In the field of regenerative medicine, we develop clinical trial protocols for regeneration of corneal endothelium in collaboration with Department of Ophthalmology, the University of Tohoku. After accumulation of clinical cases in concordance with guidelines at each institute, we plan to collect information on system development at each institute and to standardize necessary steps for clinical application into a database. We also review the safety of each protocol by investigating individual cases.

b) Fungal Infections

We investigate the dynamics of infections introduced from abroad in a research project granted by the Ministry of Health, Labour and Welfare. At present, we conduct nationwide surveys on pathways of communication on fungal infections and on the people's awareness.

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Collaborative Research Unit

Division of Systems Biomedical Technology (Konica Minolta Technology Center)

システム生命医科学技術開発共同研究ユニット (コニカミノルタテクノロジーセンター)

Project Associate Professor Noriko Gotoh, M.D., Ph.D. | 特任准教授 医学博士 後 藤 典 子

Our major research interest is to elucidate the molecular mechanisms regulating cancer cells, stem cells and development, aiming at applying the basic biology to translational medicine. In particular, we are focusing on growth factor signaling. This year we launched as a new department, challenging to a new field, cancer systems biology, by close collaboration with members of Laboratory of DNA Information Analysis, Human Genome Center.

1. An FGF4-induced Cdx2-mediated BMP4 Signaling Pathway Regulates a Stem Cell Niche in Mammalian Embryos

Michiko Murohashi, Takahisa Nakamura[1], Satoshi Tanaka[2], Taeko Ichise[3], Nobuaki Yoshida[3], Tadashi Yamamoto[1], Masabumi Shibuya[4], Joseph Schlessinger[5], and Noriko Gotoh: Division of [1]Oncology, [3]Gene Expression and Regulation, and [4]Genetics, Institute of Medical Science, University of Tokyo, [2]Laboratory of Cellular Biochemistry, Animal Resource Science/Veterinary Medical Science, University of Tokyo, [5]Department of Pharmacology, School of Medicine, Yale University

A variety of stem cells are controlled by the actions of multiple growth factors *in vitro*. However, it remains largely unclear how growth factors control the proliferation and differentiation of stem cells *in vivo*. Here, we describe a novel paracrine mechanism for regulating a stem cell niche in early mammalian embryos, which involves communication between the inner cell mass (ICM) and the trophectoderm, from which embryonic stem (ES) cells and trophoblast stem (TS) cells can be derived, respectively. It is known that ES cells produce fibroblast growth factor (FGF) 4 and that TS cells produce Bmp4. We provide evidence that FGF4 derived from the ICM activates an FRS2α-ERK pathway resulting in enhanced expression of transcription factor Cdx2 in TS cells. Cdx2 in turn binds to an FGF4-responsive enhancer element of the promoter region of *Bmp4*, leading to production and secretion of Bmp4. Moreover, exogenous Bmp4 is able to rescue the defective growth of *Frs2*α-null ICM. These findings highlight an important role of Cdx2 for production of Bmp4 in TS cells to regulate the stem cell niche.

2. *FRS2*$\alpha^{2F/2F}$ mice lack carotid body and exhibit abnormalities of the superior cervical sympathetic ganglion and carotid sinus nerve.

Yoko Kameda[6], Masataka Ito[7], Toshiyuki Nishimaki[6], and Noriko Gotoh: [6]Department of Anatomy, Kitasato University School of Medicine, [7]Department of Anatomy, National De-

fence Medical College

The docking protein FRS2α is an important mediator of FGF-induced signal transduction, and functions by linking FGF receptors to a variety of intracellular signaling pathways. We show that the carotid body is absent in *FRS2*$\alpha^{2F/2F}$ mice, in which the Shp2-binding sites of FRS2α are disrupted. We also show that the carotid body rudiment is not formed in the wall of the third arch artery in mutant embryos. In wild-type mice, the superior cervical ganglion of the sympathetic trunk connects to the carotid body in the carotid bifurcation region, and extends thick nerve bundles into the carotid body. In *FRS2*$\alpha^{2F/2F}$ mice, the superior cervical ganglion was present in the lower cervical region as an elongated feature, but failed to undergo craniocaudal migration. In addition, few neuronal processes extended from the ganglion into the carotid bifurcation region. The number of carotid sinus nerve fibers that reached the carotid bifurcation region was markedly decreased, and baroreceptor fibers belonging to the glossopharyngeal nerve were absent from the basal part of the internal carotid artery in *FRS2*$\alpha^{2F/2F}$ mutant mice. In some of the mutant mice, baroreceptors and some glomus cells were distributed in the wall of the common carotid artery, onto which the sympathetic ganglion abutted. We propose that the sympathetic ganglion provides glomus cell precursors into the third arch artery derivative in the presence of sensory fibers of the glossopharyngeal nerve.

3. The Docking Protein FRS2α Mediates Fibroblast Growth Factor signaling for the Proliferation and Self-renewal of Neural Stem/Progenitor Cells

Takuya Sato, Makoto Watanabe, Takako Horii, Noriko Gotoh

It is important to manipulate neural stem/progenitor cells (NSPCs) *in vitro* or *in vivo* in order to use them for cell replacement therapy. FGF2 is a mitogen commonly used to propagate NSPCs *in vitro*, however, it is still unclear how FGF2 controls NSPCs. Here, we provide evidence that FGF2 controls not only proliferation but also self-renewal of NSPCs through the docking protein FRS2α. We expressed wild-type FRS2α or its mutant forms in neurospheres, spherical cell clusters of NSPCs, using a retroviral system. Expression of the wild-type or the active form FRS2α-8V promoted the proliferation of NSPCs in the presence of FGF2 but not epidermal growth factor (EGF). Interestingly, the number of FGF2-induced neurospheres, an indicator of self-renewing activity, was also increased by the expression of either protein. In contrast, expression of FRS2α-6F, a mutant in which phenylalanine replaced tyrosine at all six phosphorylation sites, did not have these effects on NSPCs. As expected, the activation of both Erk and Akt was enhanced in neurosphere cells expressing wild-type FRS2α or FRS2α-8V but not FRS2α-6F. Furthermore, knockdown of *Frs2*α by short hairpin RNA (shRNA) resulted in a reduction in both the proliferation and self-renewing activity of NSPCs. Thus we demonstrate that FGF2-induced tyrosine phosphorylation of FRS2α is important not only for the proliferation but also for the self-renewal of NSPCs and raise the possibility of manipulating FRS2α in NSPCs for cell replacement therapy.

4. Membrane linked-FRS2/SNT family docking proteins

Noriko Gotoh, and Tsuchida Nobuo[8]: [8]Department of Molecular Cellular Oncology and Microbiology, Tokyo Medical and Dental University

FRS2/SNT family consists of 2 members: FRS2α/SNT-1 and FRS2β/SNT-2/FRS3. Each protein consists of a membrane-anchoring N-myristylation signal, a PTB domain and a C-terminal side region containing tyrosine residues which serve as the binding sites, when phosphorylated, for the SH2 domains of Grb2 adaptor protein and SHP2 tyrosine phosphatase. Although both FRS2 family members are highly homologous, their different expression patterns point to the presence of specific roles for each member. *Frs2*α is ubiquitously expressed at every developmental stage of the mouse, whereas high levels of expression of *Frs2*β are confined to several tissues of neuronal or epithelial origin.

Tyrosine phosphorylated FRS2 proteins serve as a platform for multiprotein complexes induced by activation of several receptor tyrosine kinases with their corresponding ligands, such as FGF, nerve growth factor (NGF), brain-derived growth factor (BDNF) and glial cell-derived growth factor (GDNF). The PTB domain of FRS2 binds to phosphorylated tyrosine residues on TrkA, TrkB and RET, although in the case of FGF, it binds to both phosphorylated and unphosphorylated forms of FGF receptor. The SHP2 binding sites on FRS2α play a primary role for Ras-ERK activation and the Grb2 binding sites on FRS2α recruit Gab1 and ubiquitin ligase Cbl; tyrosine phosphorylated Gab1 recruits and activates PI3-kinase, whereas Cbl activates the ubiqutination, leading to deg-

radation pathway.

RET gene rearrangements lead to generation of chimeric oncoprotein, RET-PTC, in papillary thyroid carcinomas. Point mutations on *RET* gene result in expression of constitutively active RET in inherited multiple endocrine neoplasia types 2A and 2B (MEN 2A and MEN 2B) and familial medullary thyroid carcinoma. FRS2α couples these oncogenic forms of RET with Ras-ERK activation and is implicated in oncogenesis. FRS2 proteins is also implicated in oncogenesis of another chimeric oncoprotein, nucleophosmin (NPM)-anaplastic lymphoma kinase (ALK), which was identified in anaplastic large-cell lymphoma with the t(2:5) chromosomal translocation.

Contrary to the case of FRS2α, FRS2β is not tyrosine-phosphorylated significantly in response to epidermal growth factor (EGF) (one of the epidermal growth factor receptor ligands) but that it inhibits EGF signaling and cell transformation via forming a complex with ERK2. FRS2β thus acts as an adaptor protein for negative regulation of EGF receptor tyrosine kinase signaling pathways.

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