Donation Laboratories and Research Units

Division of Stem Cell Regulation (AMGEN)
幹細胞シグナル分子制御(アムジェン)研究部門

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Department of Stem Cell Regulation is a donation laboratory supported by Amgen. Our research interest is to elucidate molecular mechanisms in organogenesis, especially kidney development. We also aim at derivation of kidney progenitors from stem cells, by utilizing knowledge obtained from molecular genetics.

Organogenesis -Molecular mechanisms of kidney development

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

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

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

1. Sall1, a causative gene for Townes-Brocks syndrome, enhances the canonical Wnt signaling by localizing to heterochromatin

Akira Sato, Ryuichi Nishinakamura

The *Spalt (sal)* gene family plays an important role in regulating developmental processes of many organisms. Mutations of human *SALL1* cause an autosomal dominant disorder, Townes-Brocks Syndrome, and result in ear, limb, anal, renal and heart anomalies. Targeted deletion of mouse *Sall1* results in kidney agenesis or severe dysgenesis. Molecular mechanisms of *Sall*, however, have remained largely unknown. Here we report that Sall1 synergistically activates the canonical Wnt signaling. The transcriptional activity of Sall1 is related to its nuclear localization to punctate nuclear foci (pericentromeric heterochromatin), but not to the association with b-catenin, the nuclear component of Wnt signaling. Thus we propose a new mechanism of Wnt signaling activation by the heterochromatin localization of Sall1.

2. Identification of kidney mesenchymal genes by a combination of microarray analysis and *Sall1-GFP* knockin mice

Minoru Takasato, Yuko Matsumoto, Nobuaki Yoshida,[1] Hiroyuki Aburatani,[2] Ryuichi Nishinakamura: [1]Laboratory of Gene Expression and Regulation, IMSUT [2]Genome Science Division, Research Center for Advanced Science and Technology, The University of Tokyo

SALL1, a causative gene for Townes-Brocks syndrome, encodes a zinc finger protein, and its mouse kidney (*Sall1*) is essential for metanephros development, as seen on gene targeting. In the embryonic kidney, *Sall1* is expressed abundantly in mesenchyme-derived structures from condensed mesenchyme, S-, comma-shaped bodies, to renal tubules and podocytes. We generated mice in which a green fluorescent protein (*GFP*) gene was inserted into the *Sall1* locus and we isolated the *GFP*-positive population from embryonic kidneys of these mice by fluorescein-activated cell sorting (FACS). The *GFP*-positive population indeed expressed mesenchymal genes, while the negative population expressed genes in the ureteric bud. To systematically search for genes expressed in the mesenchyme-derived cells, we compared gene expression profiles in the *GFP*-positive and -negative population using microarray analysis, followed by in situ hybridization. We detected many genes known to be important for metanephros development including *Sall1, GDNF, Raldh2, Pax8* and *FoxD1*, and genes expressed abundantly in the metanephric mesenchyme such as *Unc4.1, Six2, Osr-2* and *PDGFc*. We also found groups of genes including *SSB-4, Smarcd3*, μ-*Crystallin, TRB-2*, which are not known to be expressed in the metanephric mesenchyme. Therefore a combination of microarray technology and *Sall1-GFP* mice is useful for systematic identification of genes expressed in the developing kidney.

3. Multipotent Progenitors in the Embryonic Mouse Kidney Epithelialize through Wnt/PCP pathway

Kenji Osafune, Ryuichi Nishinakamura

Renal stem or progenitor cells with a multilineage differentiation potential remain to be isolated, and the differentiation mechanism of these cell types in kidney development or regeneration process has been unknown. To overcome this issue, we set up an *in vitro* culture system using NIH3T3 cells stably expressing Wnt4 (3T3 Wnt4) as a feeder layer, in which a single renal progenitor in the metanephric mesenchyme forms colonies consisting of several types of epithelial cells that exist in glomeruli and renal tubules. We found that only cells strongly expressing Sall1 (Sall1$^{+/high}$), a zinc finger nuclear factor essential for kidney development, form colonies, and that they differentiate into renal epithelia *in vitro*. We further showed that planar cell polarity (PCP)/Rac/c-Jun N-terminal kinase (JNK) pathway downstream of Wnt4 signal is essential both for proliferation and differentiation of renal progenitors. Thus our colony-forming assay identifies multipotent progenitors in the embryonic mouse kidney, and can be used for dissecting mechanisms of renal progenitor differentiation.

4. Murine homologue of *SALL4*, a causative gene for Okihiro syndrome, is essential for early embryogenesis and proliferation of embryonic stem cells.

Masayo Sakaki-Yumoto, Akira Sato, Chiyoko Kobayashi,[3] Yuko Matsumoto, Minoru Takasato, Hiroyuki Aburatani,[2] Nobuaki Yoshida,[1] and Ryuichi Nishinakamura.: [3]Division of Integrative Cell Biology, Institute of Molecular Embryology and Genetics, Kumamoto University

Mutations of *SALL4*, a human homologue of the *Drosophila* homeotic gene *spalt (sal)*, cause an autosomal dominant disorder Okihiro syndrome, characterized by limb deformity, eye movement, anorectal and kidney anomalies. We here show that a targeted null mutation of mouse *Sall4* leads to lethality at peri-implantation and that epiblast formation is severely impaired. Growth of inner cell masses from the knockout blastocysts was reduced, and *Sall4*-null embryonic stem (ES) cells proliferated poorly, with altered epigenetic status of selected

imprinted genes. Some heterozygous mice showed anorectal anomalies and exencephaly, and compound heterozygotes of *Sall4* and *Sall1* exhibited increased incidence of these phenotypes, as well as kidney agenesis. We propose that some symptoms of Townes-Brocks syndrome caused by *SALL1* truncations result from inhibition of *SALL4* functions by heterodimer formation. Our data clearly demonstrate the unexpected importance of *Sall4* for early embryogenesis and proliferation of ES cells as well as organogenesis in later development.

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Donation Loboratories and Research Units.

Division of Cell Processing (CERES: Cell Research and Supply)

細胞プロセッシング(CERES)研究部門

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Visiting Research Associate	Tokiko Nagamura-Inoue, M.D. PhD.	助 手	医学博士	長 村(井上)登紀子
Visiting Research Associate	Nobukazu Watanabe, M.D. PhD.	助 手	医学博士	渡 辺 信 和

Division of Cell Processing was established in IMSUT on September 1995 to support the clinic through cell therapy. This division established Tokyo Cord Blood Bank on September 1997 and has registered 3,500 units in Japan Cord Blood Bank Network, International organization NETCORD and AsiaCORD and Bone Marrow Donor Worldwide and delivered more than 320 CB units by the end of 2004. Our facility obtained the certification of ISO 9002:1994 in March 2000 and ISO 9001:2000 in May 2003. We support the clinical departments through dendritic cell therapy for patients with malignancies. We study the expansion of hematopoietic stem cells including $CD34^+$ cells, NK/T cell progenitors in cord blood. Since 2001 we have started the research on regeneration medicine using placenta -derived mesenchymal progenitor cells (PDMPCs), which could differentiate into osteoblasts, chondrocytes, adipocytes and neural cells, which may be considered as one of the possible for cell therapies and tissue engineering.

1. Quality management and internationalization of Tokyo Cord Blood Bank:

Tokiko Nagamura-Inoue, Michiko Sugo, Yan Cui, Mika Shioya, Atsuko Takahashi, Masako Hirai, Kei Takada, Atsushi Taguchi, Kenji Takahashi, Michiko Takahashi, Tsuneo A. Takahashi

The 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, our Tokyo Cord Blood Bank 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, we shipped 12 CB units for 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 for the future, 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. Tokyo Cord Blood Bank will keep the effort to grow with high quality valid to the world.

2. Immune reconstitution of post-cord blood transplantation and T cell phenotypic alternation and appearance of Cytotoxic T cell analysis for CMV after cord blood transplantation (CBT) Cord Blood Transplantation

T. Nagamura-Inoue, N. Watanabe, Satoshi Takahashi,[2] N. Watanabe, M. Shioya, Y. Mori, A. Mituru, S. Asano,[2] TA. Takahashi.: [2]Department of Hematology/Oncology, The Institute of Medical Science, The University of Tokyo,

Cord blood (CB) has the characteristics to have amount of progenitors, immature naïve T cells and suppressed NK cell activity. After transplantation with the CB cells, however, immune reconstitution after CBT for adult patients remained to be clarified. We investigated the immunological cell reconstitution after CBT in adult patients by the FACS analysis. T cell, NK cell, monocyte and B cells were monitored by the intra-beads technique to put out the absolute number of the cells. Monocytes rapidly recovered and overshoot in one month, NK cells also were induced in one month with no remarkable change after then, T cells showed gradually increasing in number after CBT up to 3 months, while B cells showed zero and suddenly recovered around 2 to 3 months. We are now analyzing the data with clinical symptoms.

We (main investigator, Watanabe N) analyzed Th1/Th2 balance, Tc1/Tc2 balance, and perforin expression among T cells post transplantation. We found that $CD8^+$ T cells showed large amount of IFN-γ production and high expression level of perforin molecules around day 30 to 60 post cord blood transplantation. These finding suggested that cytotoxic $CD8^+$ T cell activity is very strong during early stage of CBT. However, these $CD8^+$ T cells had immature surface phenotype, $CD45RA^-CD62L^+$ (central memory) and $CD45RA^-CD62L^-$ (immature effecter). This discrepancy between high expression level of killing-related molecules and immature surface markers of $CD8^+$ T cells post CBT might be one of the reasons why equal levels of GVL reaction occur despite of lower incidence of acute graft versus host disease post CBT compared with post BMT.

Several researchers reported that cytomegalovirus (CMV) infection frequently occurred in recipients post umbilical cord blood transplantation (CBT). We analyzed the CMV-specific T cells using intracellular IFN-γ staining post CMV antigen stimulation in recipients post CBT and bone marrow transplantation (BMT). Every CBT recipients developed CMV antigenemia and were treated with anti-viral drug (DHPG). However, they did not have any clinical symptoms caused by CMV infections. CMV-specific $CD4^+$ T cells were detected around day 35 in most recipients. However, CMV-specific $CD8^+$ T cells were not detected in CBT recipients around day 30. The major population of these CMV-specific $CD8^+$ T cells belonged to $CD45RA^-CD62L^-$ phenotype (immature effector). Taken together, the quick establishment of CMV-specific $CD4^+$ T cells might play an important role in controlling CMV infection and blocked the progression to CMV diseases in CBT recipients.

3. Functional analysis of cryopreserved tumor lysate loaded-dendritic cell cultured in serum free medium

Eiji Akagawa, Kaori Sato,[1] Tsuneo A. Takahashi and Naohide Yamashita[1]

In the past decade there has been increasing evidence that tumor antigen-loaded dendritic cells (DCs) are able to elicit anti-tumor responses. Initial clinical data shows tumor regressions were observed in some patients. However, production of DC for clinical vaccination protocol is a time- and cost-intensive procedure. Cryopreservation of DC in aliquots ready for clinical use would significantly facilitate DC-based vaccination in the clinic. Therefore, we asked whether freezing and thawing alters the phenotype or functional properties of DC. DCs from healthy volunteers were analyzed after freezing and thawing for their viability, morphology, immunophenotype, T-cell stimulatory capacity and mobility. Our results demonstrated that cryopreservation did not cause significant changes in the phenotype or function of DC. Immunophenotype and mobility of DC cultured in the serum free medium were superior to the DC cultured in human serum. These data indicated that cryopreserved aliquots of DC are suitable for clinical application in DC-based immunotherapy protocols.

4. Neural differentiation of human placenta-derived mesenchymal progenitor cells

Koichi Igura, Tadashi Yamashita, Kenji Takahashi, Xiaohong Zhang, Ayako Mitsuru, and T.A. Takahashi.

We previously described that human placenta -derived mesenchymal progenitor cells (PDMPC) displayed neuron-like structures under chemical induction medium including dimethyl sulfoxide (DMSO) and butylated hydroxyanisol (BHA). These cells formed a network with numerous cell-cell contacts similar to neuronal

cells. However, recent reports have raised the question that neuron-like cells, differentiated from bone marrow stromal cells, may be the results of artificial effects by DMSO and BHA. To progress in understanding the mechanism for cellular differentiation and the pluriopotency of PDMPC, we induced the neurite formation *in vitro* and transplant PDMPC into brain. We found that PDMPC displayed neuron-like morphology induced by Rho-associated kinase (ROK) inhibitor and cAMP analog. These results provided evidence that inactivation of Rho pathway and elevation of intracellular cAMP level is closely related with neurite formation in PDMPC. Moreover, pre-treatment of PDMPC with bFGF effected for increasing the number of these neuron -like cells. To determine whether the PDMPCs could differentiate and actual neuronal functions *in vivo*, we are now investigating the characterization of engraftment, migration, differentiation, phenotypic expression, and long-term survival after transplantation of PDMPC into neonatal mouse brain.

5. 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

We have reported that mesenchymal progenitor cells (PDMPC) from chorionic villi in human placenta could differentiate into osteoblast, chondrocyte, adipocyte and neural cell under appropriate condition *in vitro*. To further clarify the repair process of a full-thickness osteochondral defect using by PDMPCs, we describe an *in vitro* system for the induction of chondrocyte and *in vivo* evaluation system of bone synthesis. PDMPCs loaded in 3D-collagen sponge were cultured *in vitro* for 2 weeks with chondrogenic induction medium. After *in vitro* induction, these sponges were implanted into nude mice subcutaneously or into knee joints of nude rat. PDMPCs represent one of useful cell source candidate for cartilage tissue engineer.

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Donation Laboratories and Research Units

Division of Hematopoietic Factors (Chugai)

造血因子探索(中外製薬)研究部門

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Our major projects are (1) identification and characterization of novel cytokines and cytokine receptors (2) analysis on the molecular mechanism of leukemogenesis, particularly in leukemias resulted from chromosomal translocations, and development of therapeutic small molecules against the leukemic cells, (3) investigation of signal transduction through cytokine receptors, and (4) study on the stem cell biology.

Division of Hematopoietic Factors was established in September 1996. In the lab, we apply a retrovirus-mediated expression cloning system and gene targeting strategy to study hematopoiesis, signal transduction, leukemogenesis, embryogenesis, and so on. Our goals are (1) to clone novel cytokines and cytokine receptors using retrovirus-mediated expression screening strategies, (2) to identify and characterize transforming genes and to relate these to *in vivo* leukemogenesis, (3) to develop various retrovirus vectors and packaging cell lines, and (4) to clarify the molecular mechanism of cell proliferation, differentiation, self-renewal, and transformation.

1. Isolation and characterization of new genes by a novel signal sequence trap method SST-REX.

Yuichi Ikeda, Wing-Chun Bao,[1] Toshihiko Oki,[1] Tetsuo Kojima, Hidetoshi Kumagai, Yoshihiro Morikawa,[2] Tetsuya Nosaka, and Toshio Kitamura[1]: [1]Division of Cellular Therapy, Advanced Clinical Research Center, Institute of Medical Science, The University of Tokyo, [2]Department of Anatomy and Neurobiology, Wakayama Medical University.

Secreted and cell-surface proteins play essential roles in cell-cell interaction. We established a novel and efficient signal sequence trap method (SST-REX), in which cDNA fragments fused to an extracellular deletion mutant of the constitutively active MPL were transduced into IL-3-dependent cells via retrovirus infection followed by the selection of factor-independent clones. Our method is quick and more accurate than the previously published methods. In addition, type II membrane proteins, which had never been isolated by the previous SST methods, were also obtained by our SST-REX.

Several interesting genes have been isolated by this method from various tissues including hemopoietic stem or progenitor cells, AGM (aorta-gonad-mesonephros) cells, mast cells, and

cardiocytes, and their functions are currently being investigated. One of the most interesting novel molecules we have isolated is *vasorin*. We have found that Vasorin is a cell-surface modulator of TGF-β signaling in vascular smooth muscle cells.

2. Functional characterization of the *septin* family genes that are fused to *MLL* in infant leukemias with chromosomal translocations, and molecular analysis on the mechanism of leukemogenesis mediated by MLL-SEPTIN6.

Ryoichi Ono, Yasuhide Hayashi,[3] Hideaki Nakajima,[1] Katsutoshi Ozaki, Hidetoshi Kumagai, Tomohiko Taki,[4] Yuki Kataoka,[5] Nobuaki Yoshida,[5] Toshio Kitamura,[1] and Tetsuya Nosaka: [3]Gunma Children's Medical Center, [4]Department of Molecular Laboratory Medicine, Kytoto Prefectural University of Medicine Graduate School of Medical Science. [5]Laboratory of Gene Expression and Regulation, Center for Experimental Medicine, Institute of Medical Science, The University of Tokyo.

We identified a human homolog to mouse *Septin6* as a fusion partner of *MLL (ALL-1, HRX)* in three cases of *de novo* infant acute myeloid leukemia with complex chromosomal abnormalities involving 11q23 and Xq22-24 (Ono, R. et al., Cancer Res. 62, 333-337, 2002). Septins comprise a eukaryotic GTPase subfamily and are known to be involved in cytokinesis. Among *septin* family genes, nearly complete ORFs of *septin6, CDCREL1*, and *septin9 (MSF /AF17q25)* are fused to 5' half of *MLL* in leukemic patients, resulting in formation of chimeric proteins. We are investigating the oncogenic potentials of these chimeric proteins in various assay systems *in vitro* and *in vivo*, and trying to identify the transformation-responsible genes downstream of the *MLL-Septin*. Furthermore, *septin6*-deficient mice have been generated by gene targeting experiments to define the biological roles of this gene *in vivo*.

3. Functional characterization of the *AF5q31* gene that is fused to *MLL* in infant acute lymphoblastic leukemia by chromosomal translocation.

Atsushi Urano,[1] Masaki Endoh,[6] Tadashi Wada,[6] Yoshihiro Morikawa,[2] Miyuki Itoh,[1] Yuki Kataoka,[5] Tomohiko Taki,[4] Hideaki Nakajima,[1] Nobuaki Yoshida,[5] Yasuhide Hayashi,[3] Hiroshi Handa,[6] Toshio Kitamura,[1] and Tetsuya Nosaka: [6]Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology.

We identified a gene *AF5q31* from an infant early pre-B acute lymphoblastic leukemia (ALL) with ins (5; 11) (q31; q13q23) as a fusion partner of *MLL*. The *AF5q31* gene, which encoded a protein of 1163 amino acids, is homologous to *AF4*-related genes, including *AF4*, *LAF4*, and *FMR2* (Taki T et al., PNAS 96, 14535, 1999). The AF4 is directly involved in infant ALL with t (4; 11) (q21; q23) of poor prognosis, and *AF4* knockout mice display altered lymphoid development. The LAF4, a lymphoid-restricted nuclear protein, is expressed at the highest level in pre-B cells and is suggested to play a regulatory role in early lymphoid development. On the other hand, *FRM2* was identified as a gene associated with FRAXE mental retardation. Although *MLL* is known to play an essential role in leukemogenesis by regulating *HOX* family gene expression, the fusion partner of *MLL* also appears to be important to modify the MLL function, thereby determining the phenotype of the leukemia. To clarify the role in leukemogenesis and the biological function of *AF5q31*, we have generated the *AF5q31* knockout mice. While most AF5q31$^{-/-}$ mice showed embryonic and neonatal lethality, 13% of the AF5q31$^{-/-}$ mice thrived, but male mice displayed infertility. AF5q31 was found to be preferentially expressed in Sertoli cells, and AF5q31$^{-/-}$ mice had severely impaired expression of the genes for protamines that are essential for remodeling of the haploid germ cells. In contrast, AF5q31 did not affect phosphorylation of RNA polymerase II *in vitro* and *in vivo*. These results may have clinical implications for the understanding of human male infertility.

4. Development of retrovirus vectors and packaging cell lines.

Yuko Koshino,[1] Sumiyo Morita, Fumi Shibata,[1] Toshihiko Oki,[1] Tetsuo Kojima, Hideaki Nakajima,[1] Tetsuya Nosaka, Hidetoshi Kumagai, and Toshio Kitamura[1]

We previously developed an MuLV-derived efficient retrovirual vector pMX which is suitable for library construction. Combination of transient retrovirus packaging cell lines such as Bosc23 and the pMX vector produced high titer (10^6-10^7/ml) retroviruses which gave 100% infection efficiency in NIH3T3 cells, 10-100% infection efficiency in various hemopoietic cell lines, and 1-20% in primary culture cells including T cells, monocytes, and mast cells. However, pMX did not work well in immature cells such as EC cells and ES cells. We have now developed pMY

and pMZ vectors that utilize PCMV's LTR and primer binding site, and can express GFP in EC cells and ES cells.

Recently, usefulness of transient packaging cells has been recognized, however the titers of retroviruses are rather unstable during culture. In order to establish more stable packaging cell lines, we used the IRES sequence which allows simultaneous expression of both *gag-pol* or *env* gene and drug resistance gene from one transcript. We used the strongest promoter EF-1α in making packaging constructs. In addition, to avoid inclusion of retrovirus sequences as much as possible, we used only coding sequence of *gag-pol* and *env* genes for the packaging constructs, which will not allow the formation of replication-competent retroviruses by recombination in packaging cell lines. We established high-titer ecotropic (PLAT-E) and amphotropic (PLAT-A) packaging cell lines where the EF-1α-*gag-pol*-IRES-puror together with the corresponding EF-1 α -*env*-IRES-bs^{r} were introduced into 293T cells. We have also established another new packaging cell line (PLAT-F) for efficient infection to human hematopoietic stem cells by using *env* gene of feline endogenous retrovirus RD114.

5. A GTPase-activating protein binds STAT3 and is required for IL-6-induced STAT3 activation and for differentiation of a leukemic cell line.

Yukio Tonozuka,[1] Yukinori Minoshima,[1] Ying Chun Bao,[1] Yuseok Moon,[1] Yohei Tsubono,[1] Tomonori Hatori,[1] Hideaki Nakajima,[1] Tetsuya Nosaka, Toshiyuki Kawashima,[1] and Toshio Kitamura[1]: [1]Division of Cellular Therapy, Institute of Medical Science, The Univertsity of Tokyo.

We previously identified a male germ cell Rac GAP (MgcRacGAP) that enhanced IL-6-induced macrophage differentiation of murine M1 leukemia cells. Later, MgcRacGAP was found to play crucial roles in cell division. However, how MgcRacGAP enhanced IL-6-induced differentiation remained elusive. Here we show that MgcRacGAP enhances IL-6-induced differentiation through enhancement of STAT3 activation. MgcRacGAP, Rac, and STAT3 formed a complex in IL-6-stimulated M1 cells, where MgcRacGAP interacted with Rac1 and STAT3 through its cysteine-rich domain and GAP domain. In reporter assays, the wild-type MgcRacGAP enhanced transcriptional activation of STAT3 while a GAP-domain deletion mutant did not significantly enhance it, suggesting that the GAP domain was required for enhancement of STAT3-dependent transcription. Intriguingly, forced expression of MgcRacGAP rendered M1 cells hyperresponsive to the IL-6-induced differentiation. Moreover, knockdown of MgcRacGAP by RNA interference profoundly suppressed STAT3 activation, implicating MgcRacGAP in the STAT3-dependent transcription. All together, our data not only reveal an important role for MgcRacGAP in STAT3 activation, but also demonstrate that MgcRacGAP regulates IL-6-induced cellular differentiation in which STAT3 plays a pivotal role.

6. Overexpression of IL-21 induces extramedullary hematopoiesis.

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The IL-21 receptor is expressed on T,B, and NK cells, and IL-21 is critical for regulating immunoglobulin production in vivo in cooperation with IL-4. However, so far, little is known about a role for IL-21 outside the immune system. Previously, we reported that overexpression of IL-21 increases myeloid cell numbers in the spleen. Here we report that it increases the number of c-Kit^{+}, Sca-1^{+}, and lineage$^{-/low}$ cells (KSL cells) and CFU-GM in the spleen, indicating extramedullary hematopoiesis. Even in RAG2$^{-/-}$ mice, which lack mature T and B cells, IL-21 induced an increase in KSL cells and CFU-GM in the spleen. IL-21 augmented the proliferation of KSL cells in vitro and freshly isolated KSL cells express IL-21 receptor. These results demonstrate that IL-21 induces extramedullary hematopoiesis in vivo even in the absence of mature T and B cells.

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Donation Laboratories and Research Units

Division of Genetic Diagnosis (Otsuka) (1)
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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. Polymorphisms in the prostaglandin E_2 receptor subtype 2 gene confer susceptibility to aspirin-intolerant asthma: A Candidate Gene Approach

Nobuyoshi Jinnai, Takuro Sakagami, Takashi Sekigawa, Miho Kakihara, Toshiaki Nakajima, Kenichi Yoshida, Shin Goto, and Ituro Inoue

Aspirin-intolerant asthma (AIA) is a subtype of bronchial asthma characterized by development of bronchoconstriction evoked by nonsteroidal anti-inflammatory drugs (NSAIDs). NSAIDs inhibit the cyclooxygenase pathway, leading to enhancement of the lipoxygenase pathway. We evaluated allelic association of 370 single nucleotide polymorphisms (SNPs) of 63 candidate genes, mostly from the arachidonic acid metabolic cascade, with AIA. After two rounds of screening with 198 AIA patients, multiple SNPs in the prostaglandin E_2 receptor subtype 2 (EP2) gene were associated with AIA ($P<0.05$). Among the 77 SNPs identified in the EP2 gene, we selected 17 SNPs based on linkage disequilibrium and allelic frequencies (minor allele frequency$>$0.1) for further association study. SNPs in the promoter region of the EP2 gene, uS5, uS5b, and uS7, were significantly associated with AIA (permutation $P=0.039$-0.001). Analysis of haplotypes constructed according to the LD pattern showed a significant association with AIA (permutation $P=0.001$). The most significantly associated SNP, uS5, located in the regulatory region of the EP2 gene, was in a STATs-binding consensus sequence (AIA 31.1% vs. control 22.1% (permutation $P=0.0016$) or vs. ATA 22.2% (permutation $P=0.0017$)). Although STAT1 binding was not observed in gel mobility shift assay with HeLa nuclear extract, an unidentified protein was specifically bound to the allelic sequence. In *in vitro* reporter assay in HCT116 cells, the site containing the uS5 allele showed reduced transcription activity. Taken together, these results suggest that uS5 allele serves as a target of a transcription repressor protein. A functional SNP of the EP2 gene associated with risk of AIA should decrease the transcription level, resulting in reduction of the PGE_2 braking mechanism of inflammation and involvement in the molecular mechanism underlying AIA.

2. Population genetics for mapping of disease gene

Takuro Sakagami, Nobuyoshi Jinnai, Toshiaki Nakajima, and Ituro Inoue

A 25.6 kb region at chromosomal location 5q 31, covering the entire human interleukin 13 (*IL-13*) and interleukin 4 (*IL-4*) genes has been reported to be associated with bronchial asthma. We have examined nucleotide variation at this locus in African, European American, and Japanese populations, using 120 diallelic variants and a microsatellite marker. A block of strong linkage disequilibrium ($|D'|>0.7$) spans a 10 kb region containing *IL-4* in European American and Japanese populations, and is present but less clear in African samples. Two major haplotypes at *IL-4*, account for $>80\%$ of haplotypes in Europeans Americans and Japanese. These haplotypes are quite diverged from each other and the ancestral haplotype, and tests of neutrality show that these haplotypes appear to have been maintained by balancing selection. *FST* statistics show that European American and Japanese populations are unusually distinct at the *IL-4* locus. The most common haplotype in the European American population is much less common in the Japanese population, and vice versa. The evidence of balancing selection at *IL-4* suggests that *IL-4* may account for some genetic variance underlying susceptibility to asthma and other allergic diseases, while the strong LD observed in the *IL-4* region may allow more efficient disease-association studies using this locus.

3. Ossification of the posterior longitudinal ligament of the spine (OPLL): *COL6A1*, the candidate gene for OPLL, is associated with diffuse idiopathic skeletal hyperostosis

So Tsukahara, Hiroyuki Akagawa, Toshihiro Tanaka, Atsushi Tajima, 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. In multipoint linkage analysis using GENEHUNTER-PLUS, evidence of linkage to OPLL was detected on chromosomes 1p, 6p, 11q, 14q, 16q, and 21q. 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. One hundred-fifty positional candidate genes lie in the region and 600 gene-based SNPs were genotyped. There were positive allelic associations with 7 genes ($P<0.01$) in 280 patients and 210 controls and 4 of the 7 genes were clustered within a region of 750 kb, about 1.2 Mb telomeric from *D21S1903*. Extensive linkage disequilibrium and association studies of the 4 genes indicated that SNPs in the collagen 6A1 gene (*COL6A1*) were strongly associated with OPLL ($P=0.000003$ for SNP in intron32 (-29)). Haplotype analysis with 3 SNPs in *COL6A1* gave a single point P value of 0.0000007. 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.

Genetic screening of *COL6A1* in patients with diffuse idiopathic skeletal hyperostosis (DISH) recruited in Japan and the Czech Republic. A possibility that *COL6A1* constitutes the genetic susceptibility to DISH was investigated. DISH is a skeletal hyperostotic disease characterized by ligamentous ossification of the anterolateral side of the spine. OPLL is a related disorder with DISH, and *COL6A1* was identified as a susceptibility gene to OPLL. Accordingly, *COL6A1* was examined for susceptibility in DISH patients from Japan and the Czech Republic. Seven single nucleotide polymorphisms (SNPs) of *COL6A 1* were genotyped by direct sequencing. The allele frequencies were compared between 97 Japanese DISH patients and 298 Japanese controls, and between 96 Czech DISH patients and 96 Czech controls by the chi-square test. The intron 32 (-29) SNP of *COL6A1* was significantly associated with Japanese DISH patients ($x^2=9.33$; $P=0.0022$), but not with Czech patients. Because *COL6A1* could be associated with the occurrence of DISH and OPLL in Japanese population, we consider that the SNP of *COL6A 1* would have functional impact on the hyperostotic state leading to ectopic bone formation in the spinal ligament.

4. PLZF promotes osteoblastic differentiation of human mesenchymal stem cells as an upstream regulator of CBFA1

Ryuji Ikeda, Kenichi Yoshida, So Tsukahara, Yoshiko Sakamoto, Ituro Inoue

Ossification of the posterior longitudinal ligament of the spine (OPLL) is the leading cause of

myelopathy in Japan, and is diagnosed by ectopic bone formation in the paravertebral ligament. OPLL is a systemic high bone mass disease with a strong genetic background. To detect genes relevant to the pathogenesis of OPLL, we performed a cDNA microarray analysis of systematic gene expression profiles during osteoblastic differentiation of ligament cells from OPLL patients (OPLL cells), ossification of yellow ligament patients, and non-OPLL controls, and human mesenchymal stem cells (hMSCs) after stimulating them with osteogenic differentiation medium (OS). Twenty-nine genes were upregulated during osteoblastic differentiation in OPLL cells. Zinc finger protein 145 (PLZF) was one of the highly expressed genes during osteoblastic differentiation in all the cells examined. 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 in the expression of osteoblast-specific genes such as the alkaline phosphatase (ALP), collagen 1A1 (COL1A1), Runx2/cbfa1 (CBFA1), and osteocalcin (OCN) genes, even in the presence of OS in hMSCs. The expression of PLZF was unaffected by addition of bone morphogenetic protein 2 (BMP-2) and the expression of BMP-2 was not affected by PLZF in hMSCs. In C2C12 cells, overexpression of PLZF increased the expression of Cbfa1 and Col1a1, on the other hand, overexpression of CBFA1 did not affect the expression of Plzf. These findings indicate that PLZF plays important roles in early osteoblastic differentiation as an upstream regulator of CBFA1 and thereby might participate in promoting the ossification of spinal ligament cells in OPLL patients.

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Division of Genetic Diagnosis (Otsuka) (2)
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Our main interest is to study molecular mechanisms to maintain genomic integrity, what is called "caretaker" function, in hematopoietic stem cells (HSCs). This function is critical for HSCs to fulfill their capacity for long term repopulation and regulated proliferation and differentiation. Dysfunction of caretakers in HSCs leads to development of myeloid aplasia and neoplasms. As a model disease, we study "Fanconi anemia (FA)", a genetic disease characterized by chromosome instability and hematopoietic disorders. The disease is genetically heterogeneous, and 9 FA genes (FANCA, FANCB, FANCC, FANCD1/BRCA2, FANCD2, FANCE, FANCF, FANCG/XRCC9, FANCL/PHF9) have been identified. These products function in a common pathway, termed the FA pathway, wherein nuclear multiprotein complex FANCA/B/C/E/F/G/L-dependent monoubiquitination of FANCD2 plays a critical role in DNA damage response.

1. Hsc70/Hsp90/CHIP chaperone-ubiquitin ligase system regulates the Fanconi anemia pathway.

T. Oda, T. Yamashita

Wild-type (wt) FANCA protein is predominantly localized in the nucleus, whereas multiple patient-derived FANCA mutants fails to enter the nucleus, suggesting that nuclear localization of FANCA is critical for downstream activation of the FA pathway. Wt-FANCA is present in the nucleus in most of cells, whereas, in a small fraction of cells, it is predominantly cytoplasmic, suggesting that subcellular localization of FANCA is regulated in physiological situations. However, the underlying molecular mechanisms are largely unknown. To better understand the mechanisms, we tried to identify FANCA-regulatory proteins in the cytoplasm. For this purpose, we stably expressed Flag-tagged FANCA in HeLa cells and, from cytoplasmic extracts, we purified FANCA-containing protein complex using sequential immunoaffinity chromatography, and analyzed eluted proteins on SDS gel electrophoresis. We identified Hsc70 and Hsp90, constituting a molecular chaperone machinery, as FANCA-binding proteins. A specific inhibitor of Hsp90, 17-AAG, blocked FANCD2 mono-ubiquitination, thus suggesting this chaperone machinery regulates the FA pathway. When we examined effects of 17-AAG on FANCA, we found that this agent induced a rapid reduction in FANCA protein levels and nuclear localization of FANCA. whereas proteasome inhibitors completely blocked this reduction. 17-AAG induced polyubiquitination of FANCA. Taken together, these results suggest that Hsp90 plays an important role in protein folding of FANCA and that unfolded FANCA is degraded via a ubiquitin/proteasome pathway. We reasoned that CHIP (carboxyl terminus of Hsc70-interacting protein), a co-chaperone molecule containing a U-box ubiquitin-ligase motif, may be involved in the degradative pathway of FANCA. In support for

this notion, CHIP co-immunoprecipitated with FANCA and exogenous expression of CHIP decreased expression levels of FANCA in a dose-dependent manner. In conclusions, we propose that the Hsc70/Hsp90/CHIP system plays an important role in the regulation of the FA pathway.

2. FANCG negatively regulates nuclear import of FANCA through masking of a nuclear localizing signal.

T. Oda, T, D Adachi, T. Yamashita

We found that FANCG negatively regulates nuclear localization of FANCA. First, FANCA (Leu25Pro) mutant, which disrupts interaction with FANCG, localized in the nuclei than wt-FANCA. Furthermore, enforced expression of FANCG induced cytoplasmic redistribution of wt-FANCA. However, this effect was not observed on FANCA (Leu25Pro) mutant. Since FANCG binds to NLS of FANCA, we reasoned that FANCG prevents interactions of NLS with importins. To test this notion, we examined the interaction of FANCA with importins. Overexpresssion of FANCA revealed its co-immunoprecipitation with importins α and β, in an NLS-dependent manner. Co-expression of FANCG increased FANCA protein levels but decreased co-immuopreciptated importin α and importin-β. Taken together, these results suggest that FANCG negatively regulates nuclear import of FANCA through masking NLS and blocking access of importins.

2. Myeloid-lineage selective growth of revertan cells in Fanconi anemia

S. Hamanoue, T. Yamashita

An increasing number of reports suggest that reverse mosaicism noted in peripheral blood lymphocytes (PBL) is associated with mild hematological symptoms in FA. We now report a patient with inherited heterozygous mutations of FANCA, 2546delC and 3720-3724del, in whom mild pancytopenia was prolonged yet no apparent reversion was detected in PBL. On the other hand, lymphoblastoid cells from the patient had 2546C>T, instead of 2546delC, resulting in expression of a functional missense protein. Since the identical reversion was detected in polymorphonuclear granulocytes, sustained hematopoiesis in the patient is attributed to selective growth advantage of revertant myeloid cells. Hematological consequences of reverse mosaicism depend on in which lineage of cells the reversion occurs and revertant cells proliferate. Mild pancytopenia in FA mosaic patients is considered to result from occurrence of reversion in lymphohematopoietic progenitors and expansion of the revertant progenitors along myeloid as well as lymphoid lineages. On the other hand, the somatic reversion was detected in myeloid cells and B-LCL but not in PBL in the present case, suggesting that revertant progenitors failed to expand in post-thymic T cells. Lineage-selective growth advantage of revertant cells was repeatedly noted in inherited lymphohematopoietic disorders such as X-linked severe combined immunodeficiency , adenosine deaminase deficiency and Wiskott-Aldrich syndrome. Since FA proteins seem to have a major role in myeloid cells rather than in lymphoid cells based on clinical phenotypes of the disease, myeloid-lineage selective reverse mosaicism may be relatively common but overlooked in routine cytogenetic tests of PHA-stimulated PBL. Recognition of this status will provide new insights into the pathophysiological role of reverse mosaicism in FA.

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Division of Functional Proteomics
機能プロテオミクス共同研究ユニット

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Our laboratory aims to understand functional signaling networks of proteins in cells. The major strategy is functional proteomics; large-scale analysis of protein expression and comprehensive analysis of protein-protein interactions to reveal their functional significance.

One of the major ways to elucidate cell function at the molecular level is a large-scale analysis of the expression and interactions of proteins. Current methods being applied to these problems include the use of microarrays for analyzing expression profiles of genes, and yeast 2-hybrid screens for systematic protein interaction analysis. Proteomics probes protein expression and interaction by direct and large-scale analysis of proteins from cells or tissues. Proteomics analyses are exemplified by determination of quantitative changes in protein concentrations, often comparing two or more cell populations, to assess the effects of a wide variety of perturbations to cells. They are further exemplified by comprehensive analyses of protein-protein interactions through mass identification of components in functional protein complexes, membrane domains and cellular organelles. Besides conventional methods for proteomics based on two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and mass spectrometry (MS), our laboratory is equipped with advanced liquid chromatography (LC)-based technologies to combine proteomics with functional cellular analyses. Using these technologies as analytical platforms, we are dissecting protein signaling networks and their spatiotemporal regulation during fundamental cellular processes such as differentiation, growth and apoptosis. We have chosen embryonic stem (ES) cells as a paradigm of this functional proteomic approach and now describe some of our work in this area.

1. Comparative analysis of protein catalogs obtained from ES cells, their derivatives and NIH3T3 cells

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We have performed large-scale analyses of proteins expressed in mouse ES cells and characterized a major subset of the ES cell proteome. Here we describe the comparative analysis of protein expression between ES cells and different examples of their differentiated derivatives. We used three sets of catalogs comprising 1,370 proteins from undifferentiated ES cells (udES), 1,277 proteins from ES cells differentiated by the

withdrawal of leukemia inhibitory factor (dES), and 1,014 proteins from NIH3T3 cells (NIH3T3), respectively. Comparison of protein catalogs revealed a trend in which relatively multipotent cells were richer (udES>dES>NIH3T3) in proteins having nuclear localization (32%, 29%, 22%) or cellular roles in transcription (9%, 8%, 7%) and RNA metabolism (9%, 8%, 6%). udES shared 68% of proteins with dES but only 54% with NIH3T3 in contrast to a typical value (~75%) obtained from duplicate analyses of cellular proteins. Among 2041 proteins in a combined catalog, 549 proteins were commonly identified from all three cell types, whereas 353 proteins, including Oct-3/4, were detected only in udES, 286 proteins in dES, and 331 proteins in NIH3T3. To identify protein molecules closely associated with functions of udES cells, udES-specific proteins detected multiple times were selected from the catalog and further investigated. Western blotting analyses revealed a group of regulatory molecules (most of which lacked known functions in ES cells) were highly expressed in udES, but not in NIH3T3. Furthermore, expression of members of this group was down-regulated during the course of retinoic acid-induced differentiation of udES. These results were consistent with important roles for these proteins in the maintenance and renewal of udES cells. We therefore conclude that comparative analysis of protein catalogs is a powerful tool with which to investigate stochastic protein expression for a given specific cell type.

2. Proteomic analysis of cell surface proteins reveals diversity of potential signaling pathways in undifferentiated mouse ES cells

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Controlled differentiation of ES cells has been attempted extensively using different extracellular stimuli, including combinations of growth factors and/or feeder cells. However, little is known about properties of the surface of undifferentiated ES cells that might mediate these signals to differentiate. We report here a protein subset expressed on the cell surface of mouse D3 ES cell line, which has been identified by a combination of cell surface labeling with sulfo-NHS-LC-biotin, subcellular fractionation of plasma membranes, and mass spectrometry-based proteomics. The subset consisted of 324 proteins assigned from 965 peptides carrying biotin labels, and included 235 proteins that have potential signal sequences and/or transmembrane segments. The identified peptide sequences were restricted to the extracellular domain of well-characterized or suggested plasma membrane proteins having single transmembrane segments (n=122), demonstrating the cell surface selectivity of this approach. Receptor (19%), transporter (15%), cell adhesion (13%) and proteolysis (7%) were major functional categories attributed to the identified proteins; 10% were classified as uncharacterized. Besides known cell surface markers of ES cells such as alkaline phosphatase, the analysis identified 50 cluster of differentiation (CD) molecules and approximately 80 components of multiple cell-signaling pathways. Thus, we identified LIF receptor and IL6-signal transducer (known to play an important role in the maintenance of undifferentiated mouse ES cells in vitro), receptors for BMP, EGF, FGF, IGF, PDGF, VEGF, ephrin, delta, hedgehog and Wnt (each of which transduces signals for cell differentiation and embryonic development), 8 integrins and 10 cell adhesion molecules (defining cell-matrix and cell-cell interactions), and 6 matrix metalloproteases (with the potential to modify the extracellular matrix). By comparing the number of peptide assignments, we estimated the relative abundance of these proteins and identified major signaling molecules expressed in D3 cells. These analyses identified several signaling pathways known to function in ES cells, particular types of cells or most cell types. From the results presented here, we propose that the sheer diversity of expressed proteins is one characteristic of pluripotent ES cells.

3. Abl-catalyzed tyrosine phosphorylation of Rb is necessary for survival of certain types of human tumor cells

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The retinoblastoma gene product (Rb) is a tumor suppressor that is mutated or inactivated in many types of human cancers. Although Rb is known to be an upstream negative regulator of Abl protein tyrosine kinase, we propose here that Rb also functions as a downstream effector of Abl that plays a role in survival of some human tumor cell types including Bcr/Abl-positive

chronic myelogenous leukemia (CML). We show that Rb is constitutively phosphorylated at tyrosine in certain tumor cells and that Abl phosphorylates Rb specifically at Y805 within the C-terminal domain of the molecule. We also show that ectopic expression of Rb induces apoptosis in tumor cells in which Rb is constitutively phosphorylated at tyrosine, and that Rb-induced apoptosis is compromised by Abl-catalyzed phosphorylation of Rb at Y805. Our findings suggest that Abl-dependent tumor cells might escape apoptosis by site-specific phosphorylation of Rb at Y805, and raise the possibility that this phosphorylated Rb can be a molecular target for cancer therapy aimed at inducing apoptosis of Abl-dependent tumor cells, such as Bcr/Abl-positive CML.

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Donation Laboratories and Research Units

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

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Our aim is to reveal intracellular signaling mechanism by using proteomic approaches. Although 2-D gel electrophoresis is a powerful tool to analyze proteome, it is still difficult to analyze the components of signal transduction due to their low abundance. To overcome this difficulty, we established the protocols to prefractionate proteins of low abundance. This protocol is applied to identify ERK and ERK-family kinase substrates, as well as raft proteins.

Proteomic investigation of signal transduction pathways

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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 using 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 O'Farrell in 1975, since then over millions of 2-D gels were performed to compare the protein profile 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 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 column using Fe^{3+} has been shown to be useful to purify phos-

phopeptides. We applied this simple method to isolate phosphoproteins and got successful results. Phosphorylated ERK (extracellular signal-regulated kinase) or proteins phosphorylated by Akt kinase are recovered with relatively good yield. By this affinity column procedure, nearly six-fold purification of these phosphoproteins was achieved. Commercially available phosphoprotein purification column was also found to be useful.

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 idetified 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. Akt substrates are also being identified by a similar approach. For this approach NIH3T3 cells stimulated with PDGF in the presence or absence of wortmannin, a specific inhibitor for PI3-kinase (an upstream activator of Akt kinase) are used.

Proteomic identification of Bcl2-associated athanogene 2 as a novel MAP kinase-activated protein kinase 2 substrate

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The p38 MAP kinase cascade is activated by various stresses or cytokines. Downstream of p 38 MAP kinases there are diversification and extensive branching of signaling pathways. Fluorescent two-dimensional difference gel electrophoresis of phosphoprotein-enriched samples from HeLa cells in which p38 MAP kinase activity was either suppressed or activated enabled us to detect approximately 90 candidate spots for factors involved in p38-dependent pathways. Among these candidates, here we identified four proteins including Bcl-2 associated athanogene 2 (BAG2) by peptide mass fingerprintings. BAG family proteins are highly conserved throughout eukaryotes and regulate Hsc/Hsp70-mediated molecular chaperone activities and apoptosis. The results of two-dimensional immunoblots suggested that the phosphorylation of BAG2 was specifically controlled by a p38 MAPK-dependent manner. Furthermore, BAG2 was directly phosphorylated at serine 20 *in vitro* by MAPKAP kinase 2, which is known as a primary substrate of p38 MAP kinase and mediates several p38 MAPK-dependent processes. We confirmed that MAPKAP kinase 2 is also required for phosphorylation of BAG2 in vivo. Thus, p38 MAPK- MAPKAP kinase 2- BAG2 phosphorylation cascade may be a novel signaling pathway for response to extracellular stresses.

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

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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.

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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

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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 epitherial 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.

1. Bone regeneration

Autogenous Injectable Bone for Regeneration with Mesenchymal Stem Cells and Platelet-Rich Plasma

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We have attempted to regenerate bone in a significant osseous defect with minimal invasiveness and good plasticity, and to provide a clinical alternative to autogenous bone grafts. Platelet-rich plasma (PRP) may enhance the formation of new bone and is nontoxic, nonimmunoreactive, and accelerates existing wound-healing pathways. We have used a combination of PRP as an autologous scaffold with in vitro-expanded mesenchymal stem cells (MSCs) to increase osteogenesis, compared with using the scaffold alone or autogenous particulate cancellous bone and marrow (PCBM). The newly formed bones were evaluated by radiography,

histology, and histomorphometric analysis in the defects at 2, 4, and 8 weeks. According to the histological observations, the dog MSCs (dMSCs)/PRP group had well-formed mature bone and neovascularization compared with the control (defect only), PRP, and PCBM groups at 2 and 4 weeks. Histometrically, at 8 weeks newly formed bone areas were 18.3+/−4.84% (control), 29.2+/−5.47% (PRP), 61.4+/−3.38% (PCBM), and 67.3+/−2.06% (dMSCs/PRP). There were significant differences between the PCBM, dMSCs/PRP, and control groups. These results demonstrate that the dMSCs/PRP mixture is useful as a osteogenic bone substitute.

2. Mucosa regeneration

A double-layered mucosa membrane cultured on collagen sheet

Imaizumi F, Asahina I*, Moriyama T, Ishii M, Omura K: Oral Surgery, Tokyo Medical and Dental University Graduate School, *Division of Stem Cell Engineering IMSUT

We developed a novel cultured mucosal membrane, that was facile to prepare, easy to handle, and that could be applied to mucosal defects in the oral cavity. The human oral keratinocytes and fibroblasts were prepared from the oral mucosa. We made the following two types of cultured mucosal cell sheets: a monolayer sheet of keratinocytes cultured on a collagen membrane (K-S), and a double-layered sheet of keratinocytes and fibroblasts on a collagen membrane (KF-S). Each type of the sheet was transplanted onto a dorsum dorsal skin defect of a nude -mouse. Wound contraction of KF -S was minimal in all types of grafts. Although the histologic examination showed normal differentiation of the epithelium in all types of graft, the involucrin expression pattern of KF-S was most similar to that of the normal epithelium. These results indicate that the double-layered sheet of keratinocytes and fibroblasts cultured on the a collagen membrane may facilitate epithelial healing and prevent the wound contraction.

3. Cartilage regeneration

Cartilage formation by serial passaged cultured chondrocytes in a new scaffold: hybrid 75:25 poly(L-lactide-epsilon-caprolactone) sponge.

Honda MJ* Yada T, Ueda M* Kimata K.: *Division of Stem Cell Engineering IMSUT, Institute for Molecular Science of Medicine, Aichi Medical University

This study was designed to determine whether multipled chondrocytes immersed in a new scaffold, 75:25 poly(L-lactide-epsilon-caprolactone) sponge coated with type I collagen (75-PLC scaffold), could be used to generate cartilage tissue in vivo and to evaluate the correlation between cartilage generation and the phenotype of the proliferated chondrocytes. Rat chondrocytes were suspended in 75-PLC scaffold at a density of 1×10^7cells/mL after proliferation in a monolayer for 1 (P1) to 4 passages (P4) and implanted in nude mice for 4 weeks. Cells were characterized by the expression of genes encoding type II collagen, aggrecan, and type I collagen by Northern hybridization, and consequently, the newly formed tissue was evaluated histologically. The expression of aggrecan messenger RNA gradually decreased with the passaged cultures; however, the expression of type I collagen messenger RNA increased with time. The cartilage formations in all specimens were found not only in P1 chondrocytes but also in P2 chondrocytes, although when P3 chondrocytes were grafted, approximately 50% of cartilage formation was still observed up to but not beyond P4. It is suggested that cartilage tissue is generated with cultured chondrocytes up to P2 but not beyond P4. Northern blot analysis is useful for the assessment of whether the cells are capable of regeneration.

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Donation Laboratories and Research Units

Laboratory of Biostatistics (Biostatistics Training Unit)

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The main projects of our laboratory are to reveal new biological meanings at molecular level by various statistical approaches, and to train the researchers for the appropriate use of statistical techniques. The subjects under investigation cover a wide range of fields in computational biology: network inference from gene expression profiles, genome comparison in view of gene configuration and DNA sequence, fold recognition and structure modeling in protein structure analysis, and kinetic parameter optimization in systems biology.

1. Network Inference

a. Statistical analysis of gene expression in repair mechanism under UV irradiation.

Sachiyo Aburatani, Katsuhisa Horimoto

E. coli elicit the so-called "SOS response" in the condition of UV irradiation. To reveal the complicated mechanism of SOS response, the comprehensive analysis of gene expressions by DNA microarray has been reported. In this study, we analyze the DNA microarray data under UV irradiation by statistical approach, especially focusing on the relationships between LexA regulated genes. We analyzed the 15 DNA microarray data, in which the gene expressions were monitored during the first hour under UV irradiation in the wild-type strain, MG1655, and in an isogenic *lexA1* mutant on *E.coli*]. In particular, among the expression data of 4,101 genes, 47 LexA-related genes were focused.

We performed two types of statistical analyses. One is the calculation of correlation coefficients between the expression data of the two genes to investigate the two-gene relationship, and another is the calculation of partial correlation coefficients by graphical Gaussian modeling (GGM) to investigate the multi-gene relationship.

From our analysis, 34 LexA regulated genes were detected as the co-expressed with mutual and the other genes at 10^{-7}, and 45 genes were detected at 5×10^{-5}. Interestingly, the Lex-A regulated genes show a trend in terms of the repair function; the fraction of the genes related with the repair function to all co-expressed genes is proportional to the fraction of the LexA -regulated genes to all co-expressed genes. In other words, the genes that co-expressed within Lex-A regulated genes are associated with repair function, and the genes that co-expressed between Lex-A regulated genes and the other genes are not.

To further investigate the multi-gene relationship, we performed the graphical Gaussian modeling by ASIAN that has been recently developed. The clustering results of 34 and 45 genes in the two cases, which are a preprocessing to apply the GGM, are well consistent with

the two-gene relationships described in the preceding section. Furthermore, the inferred network between the clusters by GGM describes the relationship between the functional steps in the repair system. The present statistical analyses of gene expression profiles of LexA regulated genes provide new insights to the regulation of LexA regulated genes.

b. Genetic network reconstruction in tryptophan metabolism from gene expression profiles.

Akira Imaizumi[1], Sachiyo Aburatani, Katsuhisa Horimoto: [1]AJINOMOTO CO. LTD.

The expression profiles of genes that were related with tryptophan metabolism in a previous study were subjected to the network reconstruction. The expression profile data analyzed were cited from Khodursky et al. (2000) (*Proc. Natl. Acad. Sci*., 97: 12170-12175), and according to the previous study, we analyzed the 169 gene profiles to infer a network between them. The expression profiles were analyzed by the tools on the recently constructed ASIAN web site (http://eureka.ims.u-tokyo.ac.jp/asian). The tool in the web site performs simultaneously the different types of hierarchical clusterings, the estimation of cluster number, and the network inference. To progress the profile analyses beyond the clustering, we inferred a network between clusters by the graphical Gaussian modeling (GGM).

The network revealed more concrete and quantitative relationship between *trp* related operons and the influencing genes on them, in comparison with the previous analyses by the statistical classification of up-and down regulated genes and the clustering for gathering profiles with similar patterns.

Furthermore, the inferred network suggests some new insights into the genetic relationship in tryptophan metabolism, especially between tryptophan and aromatic amino acids metabolisms, leading to propose the candidates of binding sites for transcription factors by the sequence analyses of upstream regions.

c. ASIAN on personal computer.

Shigeru Saito[1], Kousuke Goto[1], Sachiyo Aburatani, Hiroyuki Toh[2], Katsuhisa Horimoto: [1]INFOCOM LTD., [2]Kyoto University

Recently, we have developed a system, named ASIAN (Automatic System for Inferring A Network), to infer a network from a large amount of data. The system is also provided as a website (http://eureka.ims.u-tokyo.ac.jp/asian) for enhanced utilization of the ASIAN system. Here, we introduce the ASIAN that is operated on the personal computer. The PC ASIAN runs on WINDOWS and LINUX operation systems. In addition to the various options in the ASIAN website, furthermore, the PC ASIAN is equipped with the newly developed functions.

One of the remarkable improvements in the PC ASIAN is that the PC version can handle an enormous size of data that requires large physical memory for dissimilarity matrix to perform hierarchical clustering. For this purpose, we add the Ward's method using the reciprocal nearest neighbor data structure to the set of hierarchical clustering procedures. Even in other parts, we further improve algorithms and data structures to operate stably the processing of data at high speed. Another high-light of improvement is the visual aspect of operation. A user can use various graphical tools such as a correlation viewer, a dendrogram viewer, and a network viewer for interactive analyses.

2. Genome Comparison

a. Relationship between chromosome rearrangements and repeat sequences in human chromosome 7.

Hiroo Murakami, Sachiyo Aburatani, Katsuhisa Horimoto

We analyze the relationships between the large-scale segmental duplications of chromosome and the periodic sequences. For this purpose, the periodic sequences are detected by three tools, *Tandem Repeat Finder (TRF), mreps*, and *STEPSTONE*, to detect repeats of genomic sequence, and the distribution of detected periodic sequences is corresponded with the locations on the chromosome where the segmental duplications are observed. We analyze human chromosome 7 genomic sequence. The distribution of detected repeat patterns is compared with the locations of segmental duplications of self-or other-chromosomes.

Many periodic patterns with large periodicity appeared around the telomere regions and near in the region of segmental duplication. In the 'un-masked' sequence, *STEPSTONE* detects periodic patterns with the large periods, *Mreps* detects periodic patterns with short periods, and *TRF* detects patterns with the middle range of periods. Interestingly, all tools commonly detect the periodic sequences around the location of 60,000,000 bp where a huge segmental duplication is observed. In the 'repeat-masked' sequence, both *TRF* and *STEPSTONE* detect peri-

odic patterns with large periodicity around the segmental duplication region. This suggests that unknown periodic sequences with large periodicity are related with the segmental duplication.

b. Gene-transpositions on functional categories in prokaryotic genomes.

Nobuyoshi Sugaya, Hiroo Murakami, Sachiyo Aburatani, Kunio Shimizu[1], Katsuhisa Horimoto: [1]Keio University

We estimate the transposition degrees of genes categorized by their functions, by taking into account not only the numbers of genes but also the gene locations. In this study, we focus on the variation of gene number and gene-transposition degrees estimated by the two measures in the functional categories, in comparison with the prokaryotic genomes in different genome sizes. We analyzed 10 genomes from the organisms in distinct genome sizes. We estimate the gene transposition degree by two statistical measures: one is a measure of similarity (S*) based on the Von Mises distributions, and another is the gene-location distance (GLD) based on the correlation coefficient for circular data. The former measures the similarity of the distribution form of all gene locations on two circular genomes, irrespective of orthologous relationship between genes, and the latter measures the similarity of the configuration of orthologous genes.

The gene number and the two gene-transposition degrees between the 10 genomes were compared in 14 functional categories. As a result, the gene numbers were partly consistent with the trend in previous studies. In some metabolism-related categories, CVs are large, being consistent with the previous trend, but CVs are small. The similarity of gene configuration shows a similar trend of that of gene content. Although the correspondence between the gene content and GLD in each genome is further required, the change of gene configuration is closely related with that of gene content, as a first approximation.

c. Systematic survey of sequence similarity between sense and anti-sense strands in *Escherichia coli* genome.

Makihoko Sato[1], Sachiyo Aburatani, Katsuhisa Horimoto: [1]Maebashi Institute of Technology

We performed comprehensive similarity search between the coding sequences on sense strand and the corresponding sequences on anti-sense strand in *E. coli* K12 MG1655 from GenBank of NCBI. Three different frame shifts of anti-sense DNA sequences were assumed from the data, and then decoded to respective amino acid sequences. The genes in which the coding regions mutually overlap and those including insertion sequences (IS) on the anti-sense strand were removed from the decoding. We searched similarities for each gene against the decoded sequences using "ssearch" program with default parameters; the significance threshold of the similarity was set at 0.001 of E-value.

We found 14 hit pairs of the gene and the corresponding region on the anti-sense strand. Some hit pairs are symmetrically located against the axis of replication sites, and the partners in hit pairs are frequently found in terminus site. The wide range of similar regions between the sense and anti-sense strands are concentrated in the regions of frame shift by 0. In the functional classification, five of 14 pairs are related with the function of transport.

3. Protein Structure Analysis

a. Performance comparison between profiles from hidden Markov models and from pseudo-count method.

Makihiko Sato[1], Sachiyo Aburatani, Katsuhisa Horimoto: [1]Maebashi Institute of Technology

We have proposed a novel profile, match-node profile, constructed via profile HMM. To evaluate the performance of match-node profile, we construct the profile by two popular HMM packages, HMMER and SAM, and compared the performance of match-node profile for the remote homolog detection with that of a representative profile-profile method, COMPASS, in which the profile is constructed by a psedocount method.

The fractions of proteins with the highest score that belongs to the same category of a query sequence are shown in each SCOP level. The three methods show equivalent performance; in detail, the recognition performance of match-node profile methods is better in the family level, while that of COMPASS is slightly better in superfamily and fold levels. The profile alignment and the structural alignment by CE are compared, and the mean length is estimated as the alignment accuracy from the number of residue pairs that are correctly aligned in both alignments. In this case, the match-node profile methods show the superiority to COMPASS in the accurate alignment length. Overall, two match-node profile methods and COMPASS are equal in the performance of remote homolog de-

tection.

b. Computational method for determining global protein topology from distance restraints by chemical cross-linking

Katsuhisa Horimoto, Tatsuya Akutsu[1], Akira Kinjyo[2], Ken Nishikawa[2]: [1]Kyoto University, [2]National Institute of Genetics

The atomic model is constructed by the improved distance-geometry method from the information on the distance restraints simulated by two kinds of cross-linkers, in addition to the contact residue number and the secondary structure. The performance is demonstrated by five proteins with distinct folds.

We assume two kinds of cross-linkers used in previous studies for protein structures; a homogeneous cross-linking between amines of two lysine residues, and a proto-chemical cross-linker between amines of lysine and arbitrary residue. We constructed the atomic models in six cases: all, N, $N/2$, $N/4$, $N/8$, and $N/10$ numbers of simulated restraints by cross-linking, when N is a number of amino acid residues in the tested protein. We evaluated the performance of our procedure in five proteins; 1mba (myoglobin, α, 146a.a.,), 1bla (fibroblast growth factor, β, 155 a.a.), 1gbs (lysozyme, α+β, 185a.a.), 5nll (flavodoxin, α/β, 138a.a.), and 1tph (triosephosphate isomerase, TIM barrel, 247 a.a.) in PDB database. The present procedure produces one hundred of atomic models for each protein, and the accuracy of all models was evaluated by the program, MATRAS, against the SCOP database.

The evaluation of model accuracy reveals that the global topology can be identified with $N/8$ number of simulated restraints in 5nll and 1tph, $N/10$ in 1mba, 1bla and 1gbs, by the native contact number and secondary structure, and $N/2$ in 1bla, $N/4$ in 1mba and 1tph, and $N/10$ in 1 gbs and 5nll, by the predicted number and structure. In summary, the performance of our procedure promises to detect the global topology of protein structure with a small number of distance restraints, and to serve a high-throughput determination of protein structure for the structural genomics.

4. Systems Biology

a. Symbolic-numeric optimization for biological kinetics by quantifier elimination

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We introduce a new approach to optimization for biological kinetics that deals with numerical data by symbolic quantifier elimination (QE). In this study, we illustrate the feasibility of the symbolic-numeric method in comparison with previous numerical methods.

The symbolic-numeric approach is applied to an optimization problem estimating five reaction parameters to fit a simulated signal with the five parameters to observed one, in the model described by ODE for the mechanism of irreversible inhibition of HIV protainase.

The reaction parameters $k''(k''_{22}, k''_{3}, k''_{42}, k''_{52}, k''_{6})$ with minimum SSq is the same magnitude as those by the numerical optimization methods in previous studies. Furthermore, the present method has the following merits: 1) The model parameters $\underline{k'}$ $(i, j)$and $\underline{k''}$ are estimated with a few points (e.g. two points) of the observed signal. 2) Feasible ranges of $\underline{k'}$ (i, j) and $\underline{k''}$ (i, J) are selected because unfeasible region can be confirmed exactly by the result "false" obtained by QE. 3) Our method enables us to estimate exactly how much the uncertainties of numerical simulation and observation should be so that the constraints become feasible. 4) The symbolic-numeric approach provides feasible ranges of reaction parameters.

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Donation of 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.
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特任助教授 理学博士 藤本一朗
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Our research interest is to characterize the functional and structural changing molecules of neural signaling pathway. We are studying the following subjects by introducing molecular imaging, atomic force microscope (AFM), molecular and cellular biology, and polymer hydrogel electrodes. We are normalizing the AFM microscope probe in the solution environment to observe the membrane protein structure changing without fixation. The other hand, we are focusing on Na^+/HCO_3^- co-transporter protein that functioning in brain.

Atomic force microscopy (AFM) observation of calcium-induced conformational change of inositol 1,4,5-trisphosphate receptor (IP_3R) tetramer structure

Wakako Suhara, Touichiro Goto[1], Mime Kobayashi[1], 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) receptor (IP_3 R) 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 co-agonists IP_3 and Ca^{2+} induces the IP_3 R 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. The type 1 IP_3R (IP_3R1) is a predominant type in cerebellar ER and spine apparatus and plays an integral role in Ca^{2+} signaling and neural plasticity.

In previous studies, IP_3R1 protein purified from mouse cerebella has been used to reveal its tetrameric structure. By analyzing the negative-stained IP_3R1 by electron microscopy, three-dimensional structure was reconstituted. It was demonstrated that its tetramer structure strikingly different depending on Ca^{2+} concentration in the cell. IP_3R1 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_3R1 actually change its structure, tapping mode AFM is expected to be a suitable method.

In attempt to observe a single molecule of IP_3 R1 stuck into membrane in aquatic environment, we prepared recombinant mouse IP_3R1 protein over expressed in insect Sf9 cell line. Compared to crude microsome, samples from the second gradient preparation was highly enriched with IP_3R1 and ~95% of the protein appeared to be IP_3R1 on a gel. AFM experiments were performed on a SPI3000 / SPA300-HV (SII NanoTechnology Inc., Chiba, Japan) with a 20 µm scanner using a liquid chamber. Oxide sharpened Si_3N_4 probes with a resonance frequency of 34kHz and a nominal spring constant of 0.08N/m (Olympus Corporation, Tokyo, Japan) were used for tapping-mode imaging of the membrane proteins. In liquid condition, resonant frequency of the tips were tuned to ~ 12kHz with amplitude of around 0.5V. Scans in liquid were recorded in imaging buffer with a rate of 0.5-1.0Hz.

On mica, flat membrane surface with many protrusions in the center were evident. The height of the membrane from mica surface was about 5nm that corresponds to a typical thickness of lipid bilayer. Heights of each central protrusion were about 5nm from lipid surface and were 30nm in diameter. To prove that the protrusions on the membrane are IP_3R1 molecules, we tried an immuno staining with gold particles. Both height and diameter were increased compared to the one that was not treated with IP_3R1 specific antibody. These results support that most of protrusions observed in the membranes are IP_3R1.

Next, we performed AFM imaging of IP_3R1 that was purified from mouse brain with detergent. 300ng of IP_3R1 was adsorbed to mica surface and observed in imaging buffer without detergent. IP_3R1 was singly distributed and the high magnification image shows the partial substructure details of IP_3R1. Section analysis shows a height of ~6nm and a width of 25nm. While the globular structure was observed in the Ca^{2+} free buffer, the IP_3R1 with a dent in the center was observed in the presence of Ca^{2+}. Although the resolution of our imaging was not the best, the shape observed was similar to windmill-like structures that were observed with electron microscope.

Identify the binding sites between sodium/bicarbonate co-transporter and its interacting protein

Giuseppina Priori[2], Kyoko Shirakabe, and Katsuhiko Mikoshiba: [2]JSPS Postdoctoral Fellowship Program for Foreign Researchers, Japan Society for the Promotion of Science (JSPS).

The electrogenic sodium/bicarbonate cotransporter (NBC1) exists in 3 different isoforms (kNBC1, pNBC1, bNBC1), which are the result of different splicing of the same gene. The NBC1 proteins were first described in kidney proximal tubule and then they were shown to be expressed in many different tissues including pancreas, brain, liver, colon, cornea, heart and lung. All the proteins are involved in the maintaining of intracellular pH homeostasis and playing an important role in trans-epithelial absorption and secretion of HCO_3^-. This is due to the fact that NBC1 proteins can transport Na^+ and HCO_3^- with stoichiometric ratio of $1Na^+$: $2HCO_3^-$ or $1Na^+$: $3HCO_3^-$ leading to efflux or influx of the ions.

Recently it has been proposed that the phosphorylation status of NBC1 is responsible for its stoichiometry changing, and that the interaction with carbonic anhydrase II and IV is essential for full pH recovery activity.

Nevertheless not many data about NBC1 binding proteins are present in literature. We have identified an interaction between NBC1 and other protein that probably involved in regulation of NBC1 function. All three isoforms are highly conserved: the only differences are in the N-terminal sequence of kNBC1 and in the C-terminal sequence of bNBC1. We showed by in vitro experiments that our protein specifically binds to the first 88 amino acids of pancreatic sodium-bicarbonate cotransporter (pNBC1). The binding occurs through the N-terminal region of our protein and is phosphorylation-dependent.

Functional study of sodium/bicarbonate co-transporter 1

Hideomi Yamada[3], Kyoko Shirakabe Giuseppina Priori, George Seki[3], and Ichiro Fujimoto: [3]Department of Internal Medicine, Faculty of Medicine, The University of Tokyo.

The NBC1 proteins can be expressed in *Xenopus* oocytes for functional studies by injecting each specific cRNA into the cells and for investigating the transport activities of Na^+/HCO_3^-. In order to investigate the interaction between NBC1 isoforms and their binding proteins elucidating its physiological significance

and its functional role, we are co-expressing the pNBC1 or kNBC1 cRNAs in *Xenopus* oocytes in presence or absence of the partner binding protein. Co-expression studies with pNBC1 (pancreas type) or kNBC1 (kidney type) in *Xenopus* oocyte confirmed that interaction between NBC1 and bindind protein is specific to pNBC1 and demonstrated that increased pNBC1 transport activity in the presence of binding protein results from an increased amount of pNBC1 protein in the plasma membrane. These findings suggest that our protein plays a role in pNBC1 trafficking to the plasma membrane.

Polymer Hydrogel Electrodes for the Study of Neural Network Formation

Tobias Nyberg[2], Keiichi Torimitsu[1], and Ichiro Fujimoto: [1]Materials Science Laboratory, NTT Basic Research Laboratories, Nippon Telegraph and Telephone Corporation, [2]JSPS Postdoctoral Fellowship Program for Foreign Researchers, Japan Society for the Promotion of Science (JSPS).

We have evaluated conducting polymer hydrogel electrodes (PHEs) for neural interfacing. Neural interfacing puts high demands on the biocompatibility and the electrical parameters of the materials to create desired neural networks as well as good electrical coupling to neurons. An interesting category of materials for neural interfacing is the conducting polymer hydrogel family (1,2). These materials allow for the fabrication of 3-dimensional biomimetic scaffoldings for cell attachment in addition to having parameters suited for electrical interfacing.

Neuronal cells were cultivated on PHE substrates and PHE microelectrode arrays fabricated using electropolymerization from a solution of the monomer ethylenedioxythiophene and a dispersion of poly (3,4-ethylenedioxythiophene)-poly (styrenesulfonate).

Neurons grown on PHE substrates exhibited axonal sprouting and interconnections between cells. The versatile characteristics of the PHE make it an interesting novel tool for the study of neuronal networks.

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Donation of Laboratories and Research Units

Core Facility for Therapeutic Vectors
研究拠点形成　治療ベクター開発室

Professor	Hideaki Tahara, M.D., D.M.Sc.		教授(室長)	医学博士	田　原　秀　晃(併)
Associate Professor	Katsunori Sasaki, Ph.D.		特任助教授	医学博士	佐々木　勝　則
Research Associate	Hisako Katano, D.D.S., Ph.D.		特任教員	歯学博士	片　野　尚　子

The primary function of the Core Facility for Therapeutic Vectors (CFTV) is to support clinical trials that require the genetic modification and/or ex vivo manipulation of patients' tissue under current Good Manufacturing Practice (cGMP) conditions.

1. Preparation of Standard Operating Procedures (SOPs)

Marimo Sato, Asuka Asami, Takako Shibayama, Hiroaki Uchida, Hideaki Tahara

The cGMP compliance is maintained using written SOPs which codify all aspects of laboratory activities including facility design and operations of the personnel.

2. Establishment of Master and Working Cell Banks (MCB and WCB)

Marimo Sato, Asuka Asami, Takako Shibayama, Toshihiro Suzuki, Ichiro Watanabe, Yukiko Haraguchi, Setsuko Nakayama, Hideaki Tahara

We created the cGMP compliant MCB and characterized and extensively tested for not containing contaminations such as bacteria, fungi, mycoplasma and viruses. The aliquot of the MCB were expanded to form the WCB to be used for preparing individual lots of vectors.

3. Review of CFTV by GMP consultants

Marimo Sato, Shouko Nasu, Asuka Asami, Katsunori Sasaki, Hideaki Tahara

The GMP consultants has reviewed and evaluated the facility, management, and individual protocols of CFTV. It was reported by the GMP consultants that this facility has been properly organized and functional in accordance with the GMP level set by FDA.

4. Adoption of ISO

Marimo Sato, Shouko Nasu, Asuka Asami, Naoya Ichikawa, Hideaki Tahara

Quality management system of the CFTV has been assessed and found to be in accordance with the requirements of the quality standards detailed ISO9001:2000; in the scope of development and manufacture of cell and gene therapy products.

5. Validation of CFTV

Hisako Katano, Marimo Sato, Asuka Asami, Katsunori Sasaki, Hideaki Tahara

The CFTV is organized with two distinct units; 1)vector unit, the primary adenovirus production suite which may also function as ex-vivo transduction suite; 2)cell unit, processing capabilities for patients in an environment which allows for the induction of dendritic cells for adoptive immunotherapy and gene therapy. The Class 100,000 area which has storage areas and, receipt, and storage of raw materials and final production. The Class 10,000 GMP production area has two self-contained vector production suites in the vector unit and two self-contained tissue culture suites in the cell unit. There are many features incorporated into the design of this CFTV to minimize the risk of cross-contamination between products; i.e., unidirectional traffic flow, individual airlocks to each production suite, single-pass HEPA filtered supply air, 100 percent exhaust from the biological safety cabinets through dedicate ducts, among others.

6. Acceptance of project

Takuya Takayama, Hideaki Tahara

Cancer gene therapy using IL-12 transduced dendritic cells.

7. Support

This CFTV is partially supported by 21 century COE program from Japan Society for the Promotion of Science.

Donation Laboratories and Research Units

Promotion of Genome-Based Medicine Project

研究拠点形成　ゲノム医療プロジェクト推進

Professor　Yoichi Furukawa, M.D., Ph.D.

特任教授　医学博士　　古　川　洋　一

Our research group started in March 2004, and we are working on the application of expression profiles of a wide range of human tumors to clinics in collaboration with Laboratory of Molecular Medicine and Laboratory of Genome Technology in Human Genome Center, and Department of Infectious Diseases and Applied Immunology and Department of Applied Genomics in Research Hospital. Following two major projects are ongoing; one is prediction of sensitivity of anticancer drugs to human tumors, and the other is development of novel diagnostic and therapeutic strategies of human cancers.

1. Prediction of sensitivity of Imatinib to CML, and that of Gefitinib to lung cancer.

Yoichi Furukawa, Yusuke Nakamura[1,2], Toyomasa Katagiri[1], Yataro Daigo[2], Noriharu Sato[3], Naoyuki Takahashi[3], Tsuyoshi Fujii[4]: [1] Laboratory of Genome Technology, Human Genome Center, IMSUT, [2]Laboratory of Molecular Medicine, Human Genome Center, IMSUT, [3] Department of Applied Genomics, Research Hospital, IMSUT, [4]Department of Infectious Diseases and Applied Immunology, Research Hospital, IMSUT

In an earlier study, expression profiles of chromic myeloid leukemia (CML) with high-sensitivity and those with low-sensitivity to Imatinib (Glivec) were investigated, and a total of 79 genes differently expressed between high-sensitivity group and low-sensitivity group were identified. In addition, a prediction system of the sensitivity to Imatinib was developed using expression of 15 genes that were statistically selected from the 79 genes. To apply the findings to clinics, we launched a prospective study to evaluate the system and analyze expression profiles of additional CML samples in collaboration with Department of Applied Genomics, and Department of Infectouo Diseases and Applied Immunology in Research Hospital, IMSUT. An outpatient clinic for consultation of applicants was opened in June, 2004, and three patients with CML visited the clinic. All three patients passed the criteria of our prospective study, and were given detailed information of the study and analyses. After written informed consent was obtained, blood samples were taken from the three patients. We carried out FISH and expression profile analyses, and calculated predictive scores of their sensitivity to Imatinib. The results were informed to the patients in the clinic.

Expression profiles of lung adenocarcinomas with high-sensitivity, and those with low-sensitivity to Gefitinib (Iressa) were analyzed. A system to predict sensitivity to Gefitinib was developed using expression of 12 genes. Two other groups reported that genetic alteration of *EGFR*, the target of Gefitinib, was associated with high-sensitivity to Gefitinib. However, since these studies and ours analyzed a limited number of

clinical samples, sensitivity and reliability of the two prediction methods have not been evaluated. Therefore, we started to apply our prediction system and the mutation analysis of *EGFR* for patients who are going to be treated with Gefitinib, as a prospective study in collaboration with Department of Applied Genomics, and Department of Infectious Diseases and Applied Immunology, in Research Hospital. An outpatient clinic for consultation of applicants was opened in September 2004, and a total of twelve patients with lung cancer visited the clinic in 2004. Among the twelve, nine passed the criteria of our prospective study, and were given detailed information of the study and analyses. Tumor specimens were obtained from six patients who agreed the analyses and gave a written informed consent, by TBLB, excision of lymph node, or needle aspiration biopsies using CT. Finally we could analyze expression profiles and genetic alterations of *EGFR* in three of the six patients, and the results were reported to the patients.

2. Identification of genes differently expressed between human diffuse-type and intestinal-type gastric cancers

Yoichi Furukawa, Yusuke Nakamura[1]: [1]Laboratory of Molecular Medicine, Human Genome Center, IMSUT

Carcinogenesis involves not only genetic alterations but also deregulated expression of multiple genes. We have been analyzing global gene expression profiles of human cancers using cDNA microarray in order to clarify human tumorigenesis and identify novel molecular targets for their diagnosis and treatment. We previously analyzed 20 intestinal-type gastric cancers, and identified genes that showed altered expression between the tumors and non-cancerous mucosal epithelia of the stomach. In this study, we analyzed expression profiles of 20 diffuse-type gastric cancers, and compared their expression profiles with those of 20 intestinal-type tumors. The analysis identified a total of 153 genes that were commonly up-regulated and more than 1,500 genes that were commonly down-regulated in the diffuse-type tumors compared to the corresponding non-cancerous mucosa. We also identified a number of genes related to tumor progression. Furthermore, comparison of the expression profiles of diffuse-type with those of intestinal-type gastric cancers identified 46 genes that may represent distinct molecular signatures of each histological type. The putative signature of diffuse-type cancer exhibited altered expression of genes related to cell-matrix interaction and extracellular-matrix (ECM) components, whereas that of intestinal-type cancer represented enhancement of cell growth. These data provide insight into different mechanisms underlying gastric carcinogenesis, and may also serve as a starting point for identifying novel diagnostic markers and/or therapeutic targets for diffuse-type gastric cancer.

3. Identification of novel molecular targets for the treatment of human cancers

Yoichi Furukawa, Yusuke Nakamura[1], Ryuji Hamamoto[1]: [1]Laboratory of Genome Technology, Human Genome Center, IMSUT

Using the data of expression profiles in colorectal and hepatocellular carcinomas, we identified a gene that was frequently over-expressed in the great majority of colorectal and hepatic tumors. The gene encoded a putative 428-amino acid protein containing a MYND-zinc finger domain and a SET domain, and was termed *SMYD3*. Introduction of *SMYD3* into NIH3T3 cells enhanced their growth, and its reduced expression by siRNAs resulted in significant growth suppression of colorectal and hepatic tumor cells. Immunohistochemical staining showed that the subcellular localization of SMYD3 changed from cytoplasm to nucleus in a cell cycle-dependent manner. Yeast two-hybrid screening identified HSP90alpha and HELZ, as SMYD3-interacting proteins. Subsequent immunoprecipitation assay corroborated that SMYD3 interacts with HSP90alpha, or HELZ together with RNA polymerase II, suggesting that SMYD3 might function as a transcription factor. We additionally revealed that SMYD3 binds to a DNA element containing "5'-CCCTCC-3'", and that the SET domain of SMYD3 contained a histone H3-lysine 4 (H3-K4)-specific methyltransferase activity, which was enhanced in the presence of HSP90alpha. We searched for genes regulated by SMYD3 and identified a set of genes including oncogenes, homeobox genes, and genes associated with cell cycle regulation including *Nkx2.8*. We found two SMYD3-binding elements in the 5' flanking region of *Nkx2.8*, and showed that the elements were responsible for the association with SMYD3 by ChIP and gel-shift assays. A reporter assay revealed that the promoter of *Nkx2.8* containing the elements was activated by SMYD3. Our findings suggest that SMYD3 plays an important role in transcriptional regulation as a component of RNA polymerase II complex, and that its activation is one of the key factors in human carcinogenesis. These data will be useful for the development of novel anticancer drugs targeting

SMYD3.

We additionally identified three novel human genes that were frequently up-regulated in colorectal cancers (CRCs). One of the three genes, termed *CLUAP1* (Clusterin Associated Protein 1), encodes a nuclear protein of 413 amino acids containing a coiled-coil domain. To investigate its function, we searched for CLUAP1-interacting proteins using yeast two-hybrid system and identified nuclear Clusterin. Expression of CLUAP1 was gradually increased in the late S to G2/M phases of cell cycle and it returned to its basal level in G0/G1 phases. Suppression of *CLUAP1* by siRNAs resulted in growth retardation in the transfected cells. These data suggest that inactivation of CLUAP1 may serve in the future as a novel therapeutic intervention for treatment of colon cancer.

The second novel gene was termed *RNF43* (RING finger protein 43), because it contains a RING finger domain. Multiple-tissue northern blot analysis revealed undetectable expression of *RNF43* in normal adult tissues examined, and low levels of expression in fetal kidney and lung. Its exogenous expression conferred growth -promoting effect in COS7 and NIH3T3 cells, and suppression of its expression by specific short interfering RNAs retarded the growth of colon cancer cells. Interestingly, RNF43 protein was shown to be a secreted protein, and addition of the conditioned media of the RNF43-transfected cells into culture media of NIH3T3 cells revealed a significant enhancement of cell growth. These data suggest that RNF43 may exert its growth promoting effect in an autocrine manner, and that it may be a novel diagnostic marker for colorectal cancer.

The third novel gene was termed *LEMD1* (*LEM domain-containing1*). Northern blotting revealed that *LEMD1* was expressed only in testis among the 16 normal adult tissues examined. Subsequent analysis identified six alternatively spliced forms of *LEMD1* transcripts in normal testis, but only one of the six was expressed in CRCs. Since the LEMD1 protein appears to fall in the category of cancer-testis antigens (CTAs), it may represent a promising target antigen for immunotherapy of CRCs.

We also identified a novel human gene that was up-regulated in diffuse-type gastric cancer compared to the corresponding non-cancerous mucosa. The gene encodes a putative 150-kDa protein with an RNA-recognition motif (RRM) domain in its amino-acid terminal region, and was termed as *NOL8*. Comparison of expression profiles between diffuse-type and intestinal-type gastric cancers showed that *NOL8* was specifically up-regulated in diffuse-type cancers. Immunocytochemical staining of NOL8 showed its specific localization in the nucleolus. Subsequent protein phosphatase analysis coupled with western analysis revealed a presence of its phosphorylated form. Furthermore, transfection of short-interfering RNA (siRNA) specific to *NOL8* into three diffuse-type gastric cancer cell lines, St-4, MKN45 and TMK-1, effectively reduced expression of this gene and induced apoptosis in these cells. These findings provide a new insight into diffuse-type gastric carcinogenesis and may contribute to development of new therapeutic strategies for diffuse-type gastric cancer.

4. Genetic diagnosis of HNPCC

Yoichi Furukawa

Hereditary non-polyposis colorectal cancer (HNPCC) is an autosomal dominant hereditary disease accompanied by tumors arising mainly in colon, rectum, and other associated organs, such as stomach, renal pelvis, and endometrium. The frequency of HNPCC in colorectal cancers was reported between two and five percent. However the frequency in Japanese colorectal cancers has not been undetermined. We have been analyzing mutations in *MSH2*, *MLH1*, and *MSH6*, the responsible genes for HNPCC in the patients who are diagnosed as HNPCC and registered in the HNPCC registration project of Japanese Study Group for Colorectal Cancer. We have investigated genetic alteration in *MSH2*, *MLH1*, and *MSH6*, in a total of 77 patients using direct sequencing and Multiplex Ligation-dependent Probe Amplification. The results will be used for genetic diagnosis of the affected family members of the probands. Furthermore, these data may provide useful information for further understanding of the frequency, penetrance and phenotypes of HNPCC in Japanese population.

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Donation Laboratories and Research Units

Project of Mesenchymal Stem Cells
研究拠点形成　間葉系幹細胞プロジェクト

Associate Professor　Hideaki Nakajima, M.D., Ph. D.　特任助教授　医学博士　　中　島　秀　明

Our major research interests are focused on the dissection of molecular networks of hematopiesis, especially the molecular control of hematopoietic stem cell function and transcriptional control of progenitor differentiation. We are taking a variety of approaches including cDNA cloning by signal sequence trap and gene-deficient or transgenic mice model. The final goal is to apply the findings to the manipulation of hematopoietic stem cells and progenitors ex vivo, and contribute to develop new treatment strategies for catastrophic diseases such as hematological malignancies.

1. Immune suppressor factor confers bone marrow stromal cells with enhanced supporting potential for hematopoietic stem cells

Hideaki Nakajima, Fumi Shibata[1], Yumi Fukuchi[1], Yuko Goto-Koshino[1], Miyuki Ito[1], Hiroyuki Aburatani[2], Toshio Kitamura[1]: [1]Division of Cellular Therapy, Advanced Clinical Research Center, The Institute of Medical Science, The University of Tokyo; [2]Department of Cancer Systems Biology, Research Center for Advanced Science and Technology, The University of Tokyo

Immune suppressor factor (ISF) is a subunit of the vacuolar ATPase proton pump. We earlier identified a short form of ISF (ShIF) as a stroma-derived factor that supports IL-3-independent growth of a mutant subline of Ba/F3 cells. Co-culture of murine bone marrow cells with a stromal cell line overexpressing ISF or ShIF (MS 10/ISF or MS10/ShIF) significantly enhanced their colony-forming activity and the numbers of long-term culture initiating cells (LTC-IC). Moreover, competitive repopulating activity of c -Kit$^+$Sca-1$^+$Lin$^-$HSC was significantly maintained by co-cultivation with MS10/ISF or MS10 /ShIF. These stem cell supporting activities were abolished in the proton pump mutant of ISF/ ShIF, indicating that proton transfer across cellular or endosomal membrane was critical. Gene expression analysis of ISF/ShIF-transfected cell lines revealed downregulation of secreted frizzled related protein (SFRP)-1 and tissue inhibitor of metalloproteinase-3 (TIMP-3), and the restoration of SFRP-1 and TIMP-3 expressions in MS10/ISF cells partially reversed its enhanced LTC-IC supporting activity to a normal level. These results suggest that ISF/ShIF supports HSC by modulating Wnt-activity and the extracellular matrix, and provide new insights in HSC physiology *in vivo*.

2. Molecular mechanism of stem cell self renewal on bone marrow stroma

Hideaki Nakajima, Yuko Koshino[1], Fumi Shibata[1], Yumi Fukuchi[1] and Toshio Kitamura[1]

Hematopoietic stem cells (HSC) keep self-renewing in the bone marrow in order to support continuous blood cell production. These processes are thought to occur in the bone marrow niche, a special microenvironment created by stromal cells. HSC-stromal cell interaction is

thought to provide unknown signals to keep HSC in immature state and makes them undergo extensive self-renewal. However, molecular mechanism of these processes is poorly understood. We are trying to address this question by following approaches. 1) Identify cell surface molecules that are expressed on stromal cells and important for HSC self-renewal by utilizing a variety of technologies (i.e. signal sequence trap, mRNA subtraction) and analyze their function in vitro and in vivo. 2) Identify genes that are induced in HSC by contacting with stromal cells. These genes are strong candidates that are involved in the self-renew processes evoked by stromal cell contact. We are now characterizing two novel molecules that are speculated to be important for these processes.

3. The role of CCAAT/enhancer-binding protein ε and α in normal hematopoiesis and leukemogenesis

Yumi Fukuchi[1], Naohide Watanabe[3], Makoto Handa[3], Yasuo Ikeda[4], James N Ihle[5], Scott Kogan[6], Grant McArthur[7], Toshio Kitamura[1] and Hideaki Nakajima: [3]Deparment of Transfusion and Cellular Therapy, Keio University School of Medicine; [4] Division of Hematology, Department of Internal Medicine, Keio University School of Medicine; [5]Department of Biochemistry, St Jude Children's Research Hospital; [6]Comprehensive Cancer Center, University of California, San Francisco; [7]Division of Hematology/Medical Oncology, Peter MacCallum Cancer Institute

Granulocyte colony-stimulating factor (G-CSF) is a major cytokine that regulates proliferation and differentiation of myeloid cells, although the underlying mechanisms by which G-CSF controls myeloid differentiation are largely unknown. Differentiation of hematopoietic cells is regulated by lineage-specific transcription factors, and gene-targeting studies previously revealed the critical roles of CCAAT/enhancer-binding protein C/EBPα and C/EBPε, respectively, in the early and mid-late stages of granulocyte differentiation. The expression of C/EBPε in 32Dcl3 cells and FDCP1 cells expressing mutant G-CSF receptors was examined and it was found that G-CSF up-regulates C/EBPε. The signal for this expression required the region containing the first tyrosine residue of G-CSF receptor. Dominant-negative STAT3 blocked G-CSF-induced granulocytic differentiation in 32D cells but did not block induction of C/EBPε, indicating that these proteins work in different pathways. It was also found that overexpression of C/EBPε greatly facilitated granulocytic differentiation by G-CSF and, surprisingly, that expression of C/EBPε alone was sufficient to make cells differentiate into morphologically and functionally mature granulocytes. Overexpression of c-myc inhibits differentiation of hematopoietic cells, but the molecular mechanisms of this inhibition are not fully understood. In 32Dcl3 cells overexpressing c-myc that do not differentiate by means of G-CSF, induction of C/EBPε is completely abrogated. Ectopic expression of C/EBPε in these cells induced features of differentiation, including changes in nuclear morphologic characteristics and the appearance of granules. The data show that C/EBPε constitutes a rate-limiting step in G-CSF-regulated granulocyte differentiation and that c-myc antagonizes G-CSF-induced myeloid differentiation, at least partly by suppressing induction of C/EBPε.

Acute promyelocytic leukemia is characterized by the balanced translocation t(15;17), which generates PML-RARα fusion protein. This fusion protein is thought to affect key differentiation pathway of normal myeloid development, one of which is C/EBPε. We employed PML-RARα transgenic mouse model to show that restoration of C/EBPε expression can revert leukemic phenotype of these mice. These observations reveal that C/EBPε is a critical target of PML-RARα and suggest that targeted modulation of C/EBP activities could provide a new approach to therapy of AML

4. Role of C/EBPα in lineage specification, transdifferentiation and stem cell function in hematopoiesis.

Yumi Fukuchi[1], Fumi Shibata[1], Miyuki Ito[1], Yuko Goto-Koshino[1], Yusuke Sotomaru[8], Mamoru Ito[8], Toshio Kitamura[1], Hideaki Nakajima: [8]Central Institute for Experimental Animals

CCAAT/enhancer binding protein α (C/EBPα) is a member of the bZIP family of transcription factors that plays a critical role for early myeloid differentiation. C/EBPα knockout mice showed a complete differentiation block at myeloblast stage in hematopoietic system, and mature neutrophils and eosinophils are absent in the peripheral blood. Lineage specification in developmental tree of hematopoiesis is generally determined by lineage specific transcription factors such as C/EBPα and GATA-1 that allow commitment to the specific lineage with simultaneous extinction of their capacity to differentiate into the other ones. However, recent evidences revealed by the ectopic expression of above transcription factors unveiled the unexpected

developmental plasticity of various progenitors such as MEP (erythroid/megakaryocyte progenitor) and CLP (common lymphoid progenitor). GATA-1 is reported to convert CLP and CMP (common myeloid progenitor) into erythroid/megakaryocyte lineage, however, the effect of C/EBPα on MEP and CLP is still unclear. In order to investigate the role of C/EBPα in the various aspect of hematopoietic differentiation, especially its effect on the lineage specification at different stages of differentiation in vivo, we generated transgenic mice expressing inducible form of C/EBPα (C/EBPα-ER) under H-2K promoter (C/EBPα-ER Tg). In these mice, C/EBPα activity can be induced conditionally by 4-hydroxy tamoxifen (4-HT) in all hematopoietic cells. As expected, C/EBPα-ER was expressed in almost all hematopoietic tissues including bone marrow, spleen and thymus in these mice. Gel shift analysis revealed that C/EBPα-ER was activated by 4-HT, and showed specific binding to C/EBP-specific oligonucleotide in these tissues. Next we tested differentiation plasticity of erythroid and lymphoid progenitors by ectopically inducing C/EBPα-ER activity in these cells. We sorted MEP and CLP by FACS from C/EBPα-ER Tg and examined their clonogenic activities in the presence or absence of 4-HT. In the absence of 4-HT, MEP and CLP exclusively formed erythroid/ megakaryocyte and lymphoid colonies, respectively, as previously reported. Surprisingly however, these cells dramatically changed their fate of differentiation and formed significant numbers of granulocyte/macrophage (GM) colonies in the presence of 4-HT, indicating that ectopic activation of C/EBPα-ER activity skewed their differentiation pathways to myeloid lineage. Cytospin preparation of the colonies and RT-PCR analysis revealed that these were accompanied by the morphological differentiation to granulocytes/macrophages, and upregulation of myeloid-specific genes at mRNA level. These results indicate that MEP and CLP are not fully committed to either erythroid/megakaryocyte or lymphoid lineage, and possess differentiation plasticity that can be redirected to myeloid lineage.

5. Isolation and characterization of placental mesenchymal stem cells (MSCs)

Yumi Fukuchi[1], Fumi Shibata[1], Miyuki Ito[1], Yuko Goto-Koshino[1], Toshio Kitamura[1], Hideaki Nakajima

MSC is a cell that has a capacity to differentiate to muscle cells, bone, cartilage, adipocytes or cardiomyocytes. MSCs are widely distributed in a variety of tissues in the adult human body as well as in the fetal environment. Since the methods for isolation, culture and differentiation induction of MSC are still not fully established for clinical application, and the more feasible, easily-obtainable source for MSC are wanted, we tried to identify cells with MSC-like potency in human placenta. We isolated adherent cells from trypsin-digested term placentas and established two clones by limiting dilution. We examined these cells for morphology, surface markers, gene expression patterns, and differentiation potential and found that they expressed several stem cell markers, hematopoietic/endothelial cell-related genes, and organ-specific genes, as determined by RT-PCR and FACS. They also showed osteogenic and adipogenic differentiation potentials under appropriate conditions. We suggest that placenta-derived cells have multilineage differentiation potential similar to MSCs in terms of morphology, cell-surface antigen expression, and gene expression patterns. These new methods of MSC isolation from human placenta will pave way to their application in the clinical settings.

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Laboratory of Developmental Stem Cells

文部科学省　再生医療の実現化プロジェクト
幹細胞探索研究領域

Associate Professor　Hideo Ema M.D., Ph.D.

特任助教授　医学博士　　依　馬　秀　夫

The mission of this project is to understand basic principles in stem cell biology and to explore tools applicable to stem cell-based regenerative medicine. To this end, we have been determined to work on hematopoietic stem cells (HSCs) because HSCs have already been used in bone marrow transplantation as a prototype of stem cell therapy, but also been extensively studied for the last decades, leading in the field of stem cell biology.

1. Quantification of self-renewal of hematopoietic stem cells

Jun Seita,[1] Kazuhiro Sudo,[1] Azusa Maeda,[1] Yohei Morita,[1] Mitsujiro Osawa,[1] Hiromitsu Nakauchi[1] and Hideo Ema: [1]Laboratory of Stem Cell Therapy, Center for Experimental Medicine, IMSUT.

Stem cells have been heralded as limitless sources for tissue or organ regeneration because of their self-renewal capacity. However, self-renewal capacity has never been quantified for any type of stem cells including hematopoietic stem cells (HSCs). Long-term multilineage repopulating activity detectable by transplantation experiments is the most reliable HSC marker. Based on competitive repopulation, repopulating units (RUs) and competitive repopulating units (CRUs) have been used to express stem cell activity quantitatively. RUs indicate the amount of repopulating activity and CRUs the number of stem cells; these two units thus complement one another. Given both RU and CRU values, the mean activity of stem cells (MAS) can be calculated (MAS＝RU/CRU). We have proposed use of MAS in comparison of stem cell qualities.

Evidence for self-renewal of HSCs has been provided by retroviral marking studies in which HSC clones marked with proviral integration sites could be transplanted into secondary recipients. A high degree of HSC purification enabled successful long-term reconstitution with single HSCs. After transplantation of single CD $34^{-/low}$c-$Kit^{+}Sca\text{-}1^{+}$lineage $marker^{-}$ ($CD34^{-}KSL$) cells, we observed emergence of donor-derived $CD34^{-}KSL$ cells in the recipients' bone marrow (BM), indicating self-renewal and expansion of the originally transplanted single $CD34^{-}KSL$ cells. However, when these cells were sorted and transplanted into secondary recipients, the reconstitution capacity of the $CD34^{-}KSL$ cells appeared significantly diminished. These data imply that while HSCs do self-renew in BM of primary recipients, their capacity to self-renew declines. To examine this possibility, we needed to develop a clonal and quantitative assay for self-renewal of HSCs. First, single-cell-reconstituted mice were created. BM cells of these mice then were analyzed for RU and CRU produced by single donor cells. This let us know the number of HSCs regenerated from single HSCs and let us use the MAS of these regener-

ated HSCs to evaluate their quality.

Establishment of this clonal and quantitative assay for HSC function permitted demonstration that adult mouse HSCs are significantly heterogeneous in the degrees of multilineage repopulation and that the greater the self-renewal activity, the higher the repopulating potential. An HSC with high repopulating potential could regenerate approximately 1,000 HSCs whereas the repopulating activity of regenerated HSCs on average was significantly reduced, indicating extensive but limited self-renewal capacity in adult HSCs.

2. Differentiation model of hematopoietic stem cells

Makoto Kaneda[1], Azusa Maeda[1], Hina Takano[2], Hiromitsu Nakauchi[1] and Hideo Ema: [1]Laboratory of Stem Cell Therapy, Center for Experimental Medicine, IMSUT. [2]Department of Hematology, Musashino Red Cross.

Little is known of how hematopoietic stem cells (HSCs) differentiate. Our paired daughter (PD) cell study of $CD34^-$KSL cells, highly enriched for HSCs, previously showed that particular myeloid lineages, such as a neutrophil/macrophage (nm) lineage, arise asymmetrically through their first division. In this study, we asked whether lymphoid lineage restriction similarly takes place at the level of HSCs.

Single $CD34^-$KSL cells were allowed to divide once in the presence of SCF+TPO or SCF+IL-3 under serum-free culture conditions. Subsequently, resultant daughter cells were separated by micromanipulation and individually transplanted into lethally irradiated mice along with Rag-2 deficient bone marrow cells. Recipient mice were analyzed 7 weeks after transplantation to detect single test cell-derived repopulation in myelolymphoid lineages. The differentiation potential of CD34+KSL cells was similarly examined with limiting dilution type assay.

While most PD cells were previously shown to have a variety of myeloid colony forming activity in vitro, in vivo myeloid lineage (My) repopulation was detectable in about 60% of the PD cells. B and T, B, or T-lymphoid lineage differentiation potential was also detected in 8%, 8%, or 40% of these PD cells with My repopulating activity when generated in the presence of SCF+TPO and in 2%, 5%, or 2% of the cells when generated in the presence of SCF+IL-3. As compared with freshly isolated $CD34^-$KSL cells, T-lymphoid, but not B-lymphoid differentiation potential was maintained in PD cells by SCF+TPO whereas neither potential was maintained by SCF+IL-3. These data indicate the instructive role of cytokines in the maintenance of lymphoid potential in HSCs. A significant number of MyT repopulating cells were detected in $CD34^-$KSL cells. The similar type of cells appeared to be directly generated from MyBT stem cells via their asymmetric division. Because a large number of MyB repopulating cells were estimated to be present in $CD34^+$KSL cells, these data together suggest that T cell commitment takes place prior to B cell commitment at the early stages of HSC differentiation, raising issues concerning the Common lymphoid progenitor and Common lymphoid progenitor P based differentiation model proposed by Weissman and his colleagues.

3. First blood forming activity in the mouse embryo

Chie Furuta[1], Hiromitsu Nakauchi[1] and Hideo Ema: [1]Laboratory of Stem Cell Therapy, Center for Experimental Medicine, IMSUT.

We have shown that adult HSCs have limits in self-renewal capacity, supporting the generation-age hypothesis that the replication capacity of stem cells decreases along with increase of their cell divisions. If this is the case, HSCs in developing embryos should have greater self-renewal capacity than do adult HSCs. Supporting this idea, HSCs in the fetal liver have been shown to have greater repopulating capacity than that in adult bone marrow HSCs. However, the self-renewal capacity in fetal liver HSCs was not high enough to regenerate HSCs with reasonably high RU in secondary transplantation. Thus, we have decided to seek HSCs with more unlimited self-renewal capacity in the mouse embryos.

In the context of the origin of HSCs, the relationship between primitive and definitive hematopoiesis remains unclear. We have postulated that both types of hematopoiesis are derived from the same ancestors in the early stages of development. To begin with, we attempted to quantitatively measure the first wave of hematopoiesis before yolk sac hematopoiesis occurs in the mouse embryos.

We used both methylcellulose colony assay and coculture with OP-9 cells for the detection of hematopoietic precursor cells. Precursor cells detected by the coculture system have been shown to be able to give rise to colony forming cells in the colony assay. Hematopoietic precursor cells in erythroid and macrophage lineages were detected as early as day 6 of embryos before the blood island formation in the yolk sac. Interestingly, endothelial precursor cells were detected at least 24 hours earlier. If heman-

gioblasts are responsible for generation of both types of precursor cells, the appearance of endothelial precursor cells may mark the very first process of blood formation in embryos. Clonal analysis is now planned to verify this assumption. Nonetheless, we are now purifying hematopoietic and/or endothelial precursor cells from E6.5 embryos by FACS. Cells purified to the reasonable extent will be used for the attempts to generate definitive hematopoiesis.

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Award

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Laboratory of Stem Cell Regulation

文部科学省　再生医療の実現化プロジェクト
幹細胞制御研究領域

Associate Professor　Koichi Hattori, M.D, Ph.D.
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特任助手　医学博士　ハイズィーッヒ・ベアーテ

Our main project is to use adult stem cells for tissue regeneration. To accomplish this goal, we are focusing on the following subjects: 1) studying the role of chemokines during tissue regeneration or cell differentiation, 2) identification and characterization of stem cells and their microenvironment, 2) studying the potential use of growth factors for tissue regeneration, 3) understanding the mechanism how proteases are involved in stem cell-mediated tissue regeneration.

1. Chemokine-mediated interaction of hematopoietic progenitors with the bone marrow vascular niche is required for thrombopoiesis.

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The molecular pathways involved in the differentiation of hematopoietic progenitors are unknown. Here we report that chemokine-mediated interactions of megakaryocyte progenitors with sinusoidal bone marrow endothelial cells (BMECs) promote thrombopoietin (TPO)-independent platelet production. Megakaryocyte-active cytokines, including interleukin-6 (IL-6) and IL-11, did not induce platelet production in thrombocytopenic, TPO-deficient (Thpo(-/-)) or TPO receptor-deficient (Mpl(-/-)) mice. In contrast, megakaryocyte-active chemokines, including stromal-derived factor-1 (SDF-1) and fibroblast growth factor-4 (FGF-4), restored thrombopoiesis in Thpo(-/-) and Mpl(-/-) mice. FGF-4 and SDF-1 enhanced vascular cell adhesion molecule-1 (VCAM-1)- and very late antigen-4 (VLA-4)-mediated localization of CXCR4(+) megakaryocyte progenitors to the vascular niche, promoting survival, maturation and platelet release. Disruption of the vascular niche or interference with megakaryocyte motility inhibited thrombopoiesis under physiological conditions and after myelosuppression. SDF-1 and FGF-4 diminished thrombocytopenia after myelosuppression. These data suggest that TPO supports progenitor cell expansion, whereas chemokine-mediated interaction of progenitors with the bone marrow vascular niche allows the progenitors to relocate to a microenvironment that is permissive and instructive for megakaryocyte maturation and thrombopoiesis. Progenitor-active chemokines offer a new strategy to restore hematopoiesis in a clinical setting.

2. A role for niches in hematopoietic cell development

Beate Heissig, Yuichi Ohki, Yayoi Sato, Shahin Rafii[1], Zena Werb[2] and Koichi Hattori

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Stem cells reside in a physical niche, a particular microenvironment. The organization of cellular niches has been shown to play a key role in regulating normal stem cell differentiation, maintenance and regeneration.

Hematopoietic stem cells (HSC) emerge at distinct allocation territories during ontogenesis, notably the aorta-gonad-mesonephros region, the fetal liver. They can be mobilized into the blood stream, but the adult HSC expand and differentiate exclusively in the bone marrow (BM). This implies that stem cells are not autonomous units of development; rather, tissue specific niches control their destiny. Interaction of HSCs with their stem cell niches is critical for adult hematopoiesis in the bone marrow (BM). A niche is composed of stromal cells, which either through direct cell-to-cell contact or via release of soluble factors maintain the typical features of stem cells, mainly stem cell quiescence, maintenance or expansion. HSCs are keeping the balance of the quiescence and the self-renewal in the stem cell niche, and are maintaining long-term hematopoiesis.

Therefore, an understanding of cellular and chemical architecture of the stem cell niche is vital in understanding stem cell behavior. This review summarizes the recent developments in our understanding of the stem cell niche with particular focus on the HSC niche.

3. Granulocyte-Colony Stimulating Factor promotes neovascularization by releasing vascular endothelial growth factor from neutrophils

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The granulocyte colony-stimulating factor (G-CSF) promotes angiogenesis. However the exact mechanism is not known. We demonstrate that vascular endothelium growth factor (VEGF) was released by Gr-1^{+}CD11b^{+} neutrophils, but not Gr-1^{-}CD11b^{+} monocytes pre-stimulated with G-CSF *in vitro*. Similarly in vivo, concomitant with an increase in neutrophil numbers in circulation, G-CSF augmented plasma VEGF level *in vivo*. Local G-CSF administration into ischemic tissue increased capillary density and provided a functional vasculature and contributed to neovascularization of ischemic tissue. VEGF, released from G-CSF activated neutrophils, known to express VEGFR1 promotes neutrophil migration through endothelium, which results in further release of VEGF. On the other hand, as we had shown previously VEGF can induce EPC mobilization. Here, we show that G-CSF also augmented the number of circulating VEGF receptor-2 (VEGFR2) endothelial progenitor cells (EPCs) as compared to untreated controls. Blocking the VEGF/VEGFR1, but to a much lesser extend the VEGF/VEGFR2 pathway in G-CSF treated animals delayed tissue revascularization in a hind limb model. These data clearly show that G-CSF modulates angiogenesis by increasing myelo-monocytic cells (VEGFR1＋neutrophils) and their release of VEGF.

Our results indicated that administration of G -CSF into ischemic tissue provides a novel and safe therapeutic strategy to improve neovascularization.

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