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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Division of Stem Cell Regulation is a donation laboratory supported by AMGEN. Our research interest is to elucidate molecular mechanisms in organogenesis, especially in 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 β-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

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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. Zinc-finger protein Sall2 is not essential for embryonic and kidney development.

Akira Sato, Yuko Matsumoto, Yuki Kataoka[1], Nobuaki Yoshida[1], Ryuichi Nishinakamura

We generated mice lacking *Sall2*, another *Sall* family gene. Though *Sall2* is expressed mostly in an overlapping fashion to that of *Sall1, Sall2*-deficient mice show no apparent abnormal phenotypes. Morphology and gene expression patterns of the mutant kidney were not affected. Mice lacking both *Sall1* and *Sall2* show kidney phenotypes comparable to those of *Sall1* knockout, thereby demonstrating the dispensable roles of *Sall2* in embryonic and kidney development.

4. Generation of *Sall4*-deficient mice

Masayo Sakaki, Akihiro Nakane, Yuko Matsumoto, Nobuaki Yoshida[1], Ryuichi Nishinakamura

SALL4 is a causative gene for Okihiro syndrome, characterized by finger and eye movement abnormalities, and less commonly kidney and heart anomalies. We generated *Sall4*-deficient mice and are currently analyzing the phenotypes. We are also in the process of generating mice lacking all *Sall* genes.

Publications

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Donation Laboratories 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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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 3,150 units in Japan Cord Blood Bank Network, International organization NETCORD and AsiaCORD and Bone Marrow Donor Worldwide and delivered 233 CB units by the end of 2003. 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 10 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. Wash-out of DMSO does not improve the speed of engraftment of cord blood transplantation: follow-up of 46 adult patients with units shipped from a single cord blood bank, Tokyo Cord Blood Bank

T. Nagamura-Inoue, M. Shioya, M. Sugo, Y. Cui, A. Takahashi, S. Tomita, Y. Zheng, K. Takada, Hirai, M. Hideki Kodo[1], Shigetaka. Asano[2] and T.A. Takahashi: [1]Blood Transfusion Department, Metropolitan Fuchu Hospital, Tokyo, 2Department of Hematology/Oncology, The Institute of Medical Science, The University of Tokyo Tokyo, Japan

Prolonged period of marrow hypoplasia has been the problem in cord blood transplantation (CBT). The cryoprotectant, DMSO is thought to produce osmotic shock to the progenitors when the thawed cells are infused into the patients. To solve this problem, two-fold dilution method originally developed in New York Blood Center showed earlier myeloid engraftment, although follow-up clinical studies have not performed. To clarify the influence of the removal of DMSO by this method on a speed of engraftment in unrelated CBT, 46 adult patients with CB units processed by Tokyo Cord Blood Bank from September 1998 to March 31, 2002 were studied. Twenty-four patients received nucleated cells (NCs)/kg of $2.6\pm0.71\times10^7$ without washing (non-washed group), while 22 patients were infused $2.7\pm0.52\times10^7$ cells after two-fold dilution washing (washed group). Cumulative incidence of engraftment was not significantly different between the two groups. Median neutrophil recovery ($\geq 5\times10^9$/L) in non-washed and washed group was 26 days and 25 days and median platelet recovery ($\geq 20\times10^9$/L) in patients with myeloid engraftment was 44 days and 40 days, respectively (not significant). On the other hands, the doses of CFC and CD34+ cells showed the influence on the myeloid and platelet recovery. Two-fold dilution after thawing cord blood did not result in the improvement of myeloid engraftment speed.

3. Expansion of NK/NKT/T cells

T. Nagamura-Inoue, Yuka Mori, Zheng Yizhou, Nobukazu Watanabe, T.A. Takahashi

We investigated the effect of Interleukin-15 (IL 15) with Flt3 ligand (Flt3L) on the expansion and activation of NK cells derived from umbilical cord blood mononuclear cells (UCB-MNCs). UCB-MNCs were cultured at 1 to 100ng/ml of IL15 plus Flt3L (10ng/ml) compared with 1 to 500ng/ml of IL2 plus Flt3L (10ng/ml). Cultured cells were assessed for surface marker and calculated absolute number of NK cells and T cells. The cytotoxic activity was analyzed with purified NKcells. After 2 weeks-culture with 5ng/ml of IL15 with Flt3L, the fold inductions of absolute number of NK cells significantly increased to 20.9 ± 9.3 folds to that of the number of NK cells on day0 ($P<0.05$), with 24.4 ± 16.1 folds of T cells. But with 50ng/ml of IL15 with Flt3L, fold induction of NK cells decreased to 5.1 ± 3.9 folds, while T cells showed 34.8 ± 18.7 folds ($n=8$). The proportion of NK vs. T cell number showed to be significantly higher (1.61 ± 0.91) with 5ng/ml of IL15 than that with 50ng/ml of IL15 (0.12 ± 0.03). Such proportional change of NK/T cell number could not be observed with IL2. Immunophenotypes of CD56, CD16, LFA1, CD94, CD8 and perforin of cultured NK cells with 10ng/ml of IL15 and Flt3L showed the same pattern of those with 50ng/ml of IL2 and Flt3L. Cytotoxic activity against K562 of cultured NK cells resulted in the same level as adult peripheral blood (PB)-derived NK cells. Higher induction of NK cells derived from UCB -MNCs was achieved by low dose (5 to 10ng/ml) rather than high dose (>50 ng/ml) of IL15.

4. *Ex vivo* manipulation of cord blood-derived stem cells to enhance the expression levels of homing-related molecules and intra-bone marrow injection of cord blood cells to facilitate the engraftment of cord blood cells

Y. Zheng, Atsushi Taguchi, N. Watanabe, T. Nagamura-Inoue, Hitomi Nagayama[1], Arinobu Tojo[2], S. Asano[2], Yoichi Takaue[3], Shin-ichiro Okamoto[1], and T.A. Takahashi: [1]Division of Hematology, Department of Medicine, School of Medicine, Keio University, Tokyo, Japan, [2]Department of Hematology/Oncology, The Institute of Medical Science, The University of Tokyo, [3]Department of Medical Oncology, Stem Cell Transplantation Unit, National Cancer Center Hospital, Tokyo, Japan.

We compared the expression levels of the homing-related molecules on CB-, mobilized peripheral blood (mPB) and bone marrow (BM)-derived $CD34^+$ cells using four-color FACS analysis. Significantly lower expressions of CD 49e, CD49f, CD54 and CXCR-4 on CB-derived $CD34^+$ cells were observed compared with those of mPB-, and BM-derived $CD34^+$ cells. *Ex vivo* manipulation of cord blood cells with human stem cell factor (SCF) for 48 hours resulted in the enhanced expression levels of these molecules and engraftment of human blood cells in NOD/SCID mouse. To overcome the physical obstacle for engraftment of stem cells, we tried the intra-bone marrow injection of cord blood cells using NOD/SCID mouse. In this model, engraftment of cord blood cells was enhanced even in the different bone mallows from injected site (right tibial bone). These techniques seem

useful for clinical hematopoietic stem cell transplantation to improve the engraftment of hematopoietic stem cells.

5. Immune reconstitution of post-cord blood transplantation

T. Nagamura-Inoue, Satoshi Takahashi[2], N. Watanabe, M. Shioya, Y. Mori, 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.

6. T cell phenotypic alternation and appearance of Cytotoxic T cell analysis for CMV after cord blood transplantation (CBT) Cord Blood Transplantation

N. Watanabe, Satoshi Takahashi, Ayako Mitsuru, T. Nagamura-Inoue, Asano[2], TA. Takahashi.

We 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.

7. Phase I clinical trial of autologous monocyte-derived dendritic cell therapy for STAGE IV thyroid carcinoma patients

Eiji Akagawa, Mariko Morishita[1], Kaoru Uchimaru[1], Kaoru Sato[1], Naohide Yamashita[1] and T.A. Takahashi,

To clarify the feasibility and efficacy of administering autologous monocyte-derived dendritic cell (DCs) for thyroid carcinoma, we used tumor lysate-pulsed DCs as tumor vaccine in five stage IV patients. Autologous monocytes were harvested from 15 liter of pheresis products and culture with GM-CSF (50ng/ml) and IL -4 (50ng/ml) for 7 days to generate immature DCs. Immature DCs were then pulsed with autologous tumor lysate (100μg/ml) and subsequently cultured with TNF-alpha (50ng/ml) for another 4 days for maturation. 1x107 of loaded DCs were injected subcutaneously once a week for four times and subsequently once in two weeks for four times. This clinical trial has been completed feasibility and two of five patients have showed some improvement reactions.

8. 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 regres-

sions 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.

9. Diffferent signaling pathways for the expression of GM-CSF receptor in the maturation of monocyte-derived dendritic cell

E. Akagawa and T.A. Takahashi

Granulocyte-macrophage colony stimulating factor (GM-CSF) plays an important role in myeloid differentiation. Dendritic cells (DC) are derived from monocyte cultured in response to GM-CSF and interleukin-4 (IL-4). Recent studies have been demonstrated that the receptor for GM-CSF (GM-CSFR) is increased in maturation of DC. In order to understand the mechanism of the expression of GM-CSFR of DC, we investigated the signal transduction and the expression of PU. 1, which is the master transcriptional regulator of GM-CSFR alpha (GM-CSFRa) and common beta (bc). In the GM-CSFRa expression during the process of DC maturation, our result demonstrated that TNF-alpha stimulation activated ERK1/2 pathway, whereas LPS stimulation activated p38 pathway. On the other hand, both ERK1/2 and p38 are required for the expression of bc. Furthermore, PU. 1 was upregulated by ERK1/2 stimulated by TNF-alpha and p38 stimulated by LPS. These results suggested that the expression of GM-CSFR is mediated by different pathways during maturation of DC and that a novel pathway involved in PU. 1 is exist in DC maturation.

10. Expression of corticotropin releasing factor receptors on human monocyte-derived dendritic cells

E. Akagawa and Tsuneo A. Takahashi

Dendritic cells (DCs) are most potent antigen presenting cells required for initiation of the immune response toward foreign proteins. During physiological events of DCs, they are thought to undergo mediations by soluble factors, such as cytokines, endocrine hormones and neuropeptides. Among them, corticotropin releasing factor (CRF) is one of neuropeptides that plays an important role in response to stress. CRF has been shown that it not only stimulates the activation of hypothalamic-pituitary-adrenal, but also has immunomodulating properties. Although CRF is known to stimulate monocyte and leukocyte to produce adenocorticotropic hormone (ACTH), there is no evidence about the interaction between CRF and DCs so far. In this study, we have investigated the effect of CRF on human monocyte-derived DCs function *in vitro*. To establish whether the expression of receptor for CRF is present on DCs, we used flowcytemetory analysis, immunohistochemical analysis or reverse transcription-PCR (RT-PCR). In flowcytemetory and immunohistochemical analysis, result showed that CRF receptor type I (CRF-RI) and type II (CRF-RII) were found on immature and mature DCs. Sequence analysis, RT-PCR, confirmed the expression of mRNA for CRF-RI and CRF-RII alpha, whereas CRF-R II beta was not detected. Incubation of immature DCs with CRF (10-7M) for 24 hours resulted in decrease ($>30\%$, $n=6$) of the migratory response to inflammatory chemokine, RANTES. This reduction was recovered dose dependently by using inhibitor for CRF, Astressin. Mature DCs treated with CRF also decreased the migratory response to MIP-3 beta as well as immature DCs. These data are the first evidence for expression of CRF receptors on human DCs and suggested that CRF may inhibit migratory behavior and functions of DCs.

11. Isolation and characterization of multipotent mesenchymal progenitor cells from human placenta

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We established a method of isolating mesenchymal progenitor cells (MPCs) from chorionic villi of the fetal part of the full-term human placenta and the characteristics of these cells. Placenta-derived mesenchymal progenitor cells (PDMPCs) isolated by the explant culture method consisted of a heterogeneous cell popu-

lation including spindle-shaped cells and large flat cells. The PDMPCs expressed CD13, CD44, CD73, CD90, CD105 and HLA-class I as surface epitopes, but did not CD31, CD34, CD45 and HLA-DR. Under specific induction conditions, these cells differentiated into osteoblasts, chondrocytes, adipocytes and neural cells. PDMPCs may thus be considered as one of the possible sources of MPCs that can be used for cell therapies and tissue engineering. We are investigating the ability of bone or cartilage formation of PDMPCs *in vivo* with supporting biomaterials. On the other hand, PDMPCs have a limited replicative life span when serially passaged in culture. To establish immortal PDMPCs, we transduced PDMPCs with lentiviral vector encording Bmi-1, which downregulates the p16 tumor suppressor genes encorded by the *ink*4a locus and/or telomerase catalytic component (TERT), which prevents the progressive shortening of telomeres at cell divisions. We are trying to isolate clones from these cells and analyze the differentiation ability of these clones.

Publications

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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, (4) study on the roles of small GTPases and GAPs, and (5) study on the stem cell biology.

Division of Hematopoietic Factors was established in September 1996. In the lab, we apply a retrovirus-mediated expression screening 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 cloning 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, (4) to clarify the molecular mechanism of intracellular signal transduction in cell proliferation, differentiation, and transformation, and (5) to elucidate the molecular mechanisms of biologically interesting phenomena such as cytokinesis, self renewal, gastrulation, and so on.

1. Characterization of constitutively active forms of signaling molecules

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We combined retrovirus-mediated screening system and PCR-driven random mutagenesis to identify activating mutations in cytokine receptors, kinases, and other signaling molecules. For example, we introduced random mutations into the STAT (signal transducer and activator of transcription) 5A sequence followed by retrovirus-mediated screening for constitutively active forms of STAT5A, and identified the mutant STAT5A that can induce autonomous proliferation of IL-3-dependent cell lines. Two mutations, one (H298R) in the DNA binding domain and the other (S710F) in the effector domain were required for the constitutive activity of STAT5A. The active STAT5A mutant showed constitutive tyrosine phosphorylation, nuclear localization, and transcriptional activation, and were able to induce factor-independency in IL-3-dependent cell lines. Interestingly, the mutant STAT5A also induced differentiation and/or apoptosis in Ba/F3 cells after IL-3 stimulation. We investigated the mechanisms of this pleiotropic functions of the constitutively active STAT 5A by expressing each target gene of STAT5A using a bicistronic retroviral vector having IRES-GFP. Pim-1, $p21^{WAF1/Cip1}$, and SOCS1 (suppressor of cytokine signaling 1) were found to be re-

sponsible for inducing proliferation, differentiation, and apoptosis, respectively. In addition, we have identified another constitutively active STAT5A mutant which harbors a single point mutation in the SH2 domain (N642H) that shows the same phenotype as the STAT5A mutant with the two mutations. Interestingly, the SH2 mutation resulted in restoration of the conserved critical histidine which is involved in the binding of phosphotyrosine in the majority of SH2-containing proteins. Dimerization was required for the activity of the SH2 mutant of STAT5A as was the case for the wild type. These findings demonstrate that different mutations rendered STAT5A constitutively active, through a common mechanism, which is similar to that of physiological activation. The same things were also true for the STAT5B.

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

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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.

3. Functional analysis of the mouse *twisted gastrulation* (TSG) gene

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In the search for soluble factors which are expressed in the mouse AGM region at 10.5 days p.c., by SST-REX, we have identified a mouse homologue of the *Drosophila* TSG protein which is known to regulate dorsal-ventral patterning of the fly development by modulating BMP (bone morphogenetic protein) signaling. Although the Xenopus TSG has recently been reported to bind BMP-4 and its antagonist Chordin, biological function of the TSG protein is still unknown. To elucidate the biological role of TSG in mammals, we have generated TSG-deficient mice. The mice displayed dwarfism with delayed endochondral ossification and lymphoid deficiency with small thymus and spleen. Since BMP-4 promotes skeletogenesis and inhibits thymus development, our findings suggest that TSG acts as both a BMP-4 agonist in skeletogenesis and an antagonist in T-cell development. These findings may also provide a clue for understanding the pathogenesis of human dwarfism with immunodeficiency.

4. Functional Characterization of the mouse *septin* family genes that are fused to *MLL* in infant leukemias with chromosomal translocations

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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*.

5. Functional Characterization of the mouse *ALF4* gene, a homolog of the human *AF5q31* that is fused to *MLL* in infant acute lymphoblastic leukemia by chromosomal translocation

Atsushi Urano[1], Yasuhide Hayashi[6], Tomohiko Taki[6], Yuki Kataoka[5], Nobuaki Yoshida[5], Katsutoshi Ozaki, Toshio Kitamura[1], and Tetsuya Nosaka

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 *ALF4 (AF5q31)* knockout mice. We are also searching for the target genes of *MLL-ENL* and *MLL-AF9* which are generated by t(11;19) and t (9;11), respectively, through the transforming potential in mouse primary bone marrow cells.

6. Development of retrovirus vectors and packaging cell lines

Yuko Koshino, Sumiyo Morita, Fumi Shibata, Toshihiko Oki[1], Tetsuo Kojima, Hideaki Nakajima[1], Tetsuya Nosaka, Hidetoshi Kumagai[2], 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-puro^r together with the corresponding EF-1α-*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.

7. Analysis of the role of MgcRacGAP as a regulator of the small GTPase Rho family in differentiation and cytokinesis

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In the search for key molecules that prevent murine M1 leukemic cells from undergoing IL-6-induced differentiation into macrophages, we isolated an antisense cDNA that encodes full-

length mouse MgcRacGAP through functional cloning. In human HL-60 leukemic cells, overexpression of the human MgcRacGAP induced growth suppression and macrophage differentiation. Analysis using the mutants revealed that the GAP activity was dispensable, but the myosin-like domain and the cysteine-rich domain were indispensable for growth suppression and macrophage differentiation. Interestingly, MgcRacGAP localized to the nucleus in interphase, accumulated to the mitotic spindle in metaphase, and was condensed in the midbody during cytokinesis. Overexpression of an N-terminal deletion mutant resulted in the production of multinucleated cells in HeLa cells. This mutant lost the ability to localize in the mitotic spindle and midbody. MgcRacGAP was also found to bind α-, β-, and γ-tubulins through its N-terminal myosin-like domain. These findings indicate that MgcRacGAP dynamically moves during cell cycle progression probably through binding to tubulins and plays critical roles in cytokinesis. Furthermore, using a GAP-inactive mutant, we have disclosed that the GAP activity of MgcRacGAP is required for cytokinesis, suggesting that inactivation of Rho family GTPases may be required for normal progression of cytokinesis. We have recently found that MgcRacGAP is phosphorylated by Aurora B that is known to work in the midbody, and expression of a kinase defective mutant of Aurora B inhibited the phosphorylation of MgcRacGAP at Ser387 in the midbody during M-phase. MgcRacGAP colocalized with Rac1 on the mitotic spindles in metaphase and with RhoA on the contractile ring in cytokinesis. These findings indicate that MgcRacGAP is functionally converted to a RhoGAP through phosphorylation induced by Aurora B and plays essential roles in the completion of cytokinesis.

8. Identification of a small molecule which inhibits leukemic cell growth caused by the internal tandem duplication mutations of *Flt-3*

Ken Murata, Hidetoshi Kumagai[2], Toshiyuki Kawashima, Kaori Tamitsu[2], Mariko Irie[2], Hideaki Nakajima[1], Tetsuya Nosaka, Shigetaka Asano[10], and Toshio Kitamura[1]: [10]Division of Molecular Therapy, Advanced Clinical Research Center, Institute of Medical Science, The University of Tokyo.

Internal tandem duplications of the juxtamembrane region of the *Flt-3* are found in about 20% of the human acute myeloid leukemia patients. In screening of the small compounds by the ability to selectively inhibit leukemic cell growth caused by such mutations of *Flt-3*, we have identified several small chemical compounds. These molecules show structural similarity to the tyrosine kinase inhibitor. One of the most effective molecules GTP14564 preferentially inhibited the growth of the Ba/F3 cells transformed by the mutant *Flt-3*, thereby suppressing the tyrosine phosphorylation of STAT5, but not very much in Ba/F3 cells driven by the Flt-3 ligand/wild type Flt-3. Forced expression of the dominant negative STAT5A, but not treatment with the MEK inhibitors suppressed the mutant Flt-3-driven cell growth. On the other hand, the proliferative signal through the wild type Flt-3 was dependent on the activation of MAP kinases. We also revealed that the N-terminal two tyrosine residues of the intracellular domain of the mutant Flt-3 were responsible for STAT5 activation and autonomous cell growth, but the corresponding tyrosine residues of the intracellular domain of the wild type Flt-3 were dispensable for cell growth.

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Division of Genetic Diagnosis (Otsuka) (1)
ゲノム情報応用診断(大塚製薬)研究部門基礎分野

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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-parametriv 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, familial prostate cancer, and intracranial aneurysm and analyze the molecular causality.

1. Linkage disequilibrium analysis and mapping of disease gene

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

A 25.6 kb locus on chromosome 5q31 covering the entire human interleukin 13 gene (*IL*-13) and interleukin 4 gene (*IL*-4) has been reported to be in linkage or association with bronchial asthma. We have evaluated this locus for nucleotide variation among African, European American, and Japanese populations based on 120 diallelic variants and an adjacent microsatellite marker. Two linkage disequilibrium (LD) blocks ($|D'|>0.7$), one spanning 0.8 kb in *IL*-13 and the other spanning at least 10 kb in *IL*-4, were identified in European American and Japanese samples but are less obvious in African samples. Unique haplotype structures are observed within each LD block, for example, at *IL*-13, the most common and ancestral haplotype in African samples is rare in European American samples and absent in Japanese. Two major haplotypes at *IL*-4 found in>80% of European American and Japanese samples are not prominent in Africans. Variations in SNP allele frequencies between populations were examined by F_{ST} statistic. The mean F_{ST} value is high between European American and Japanese samples at *IL*-4 (0.239), but at *IL*-13 and the intergenic region, mean F_{ST} values are low (0.023 and 0.007, respectively), indicating natural selection. Tajima's D, Fu and Li's D, and F statistics were tested among the three populations at *IL*-13, the intergenic region, and *IL*-4. In the African samples, all three regions showed values close to zero, while the values were mostly positive in European American and Japanese samples except in the intergenic region. We also performed mismatch distribution test for the *IL*-13 and *IL*-4 haplotypes to analyze pairwise differences among haplotypes. Mismatch distribution of *IL*-13 haplotypes in European American and Japanese samples show a bimodal pattern, while the distribution of *IL*-4 haplotypes show two discrete peaks. These data indicate a history of balancing selection in *IL*-4. The nucleotide diversity patterns in *IL*-13 and *IL*-4 suggest the importance of comparative disease-association studies, especially in

asthma and other allergic diseases.

2. Natural selection and population history in the human angiotensinogen gene (AGT): 736 complete AGT sequences in world-wide chromosomes

Toshiaki Nakajima and Ituro Inoue

Several lines of evidence suggest that patterns of genetic variability in the angiotensinogen gene (*AGT*) contribute to phenotypic variability in human hypertension. The A (-6) promoter variant of *AGT* is associated with higher plasma angiotensinogen level and the increased risk of essential hypertension. Geographical variation in susceptibility to hypertension has introduced the "sodium retention hypothesis", which posits that populations living in tropical Africa and temperate Eurasian environments are adapted to different levels of salt availability. This hypothesis predicts that the A (-6) variant should be found at higher frequencies in African populations than in non-African populations. To test this hypothesis, we investigated the roles of population history and natural selection in shaping patterns of genetic diversity in *AGT*, by sequencing the entire *AGT* (14,400bp) in 736 chromosomes from Africa, Asia, and Europe. We confirmed that the A (-6) variant is present at higher frequency in African populations than in non-African populations. In addition, haplotypes carrying the G (-6) variant showed elevated levels of linkage disequilibrium, suggesting that they have risen to high frequency recently. Several neutrality tests found no evidence for a departure from selective neutrality when whole *AGT* sequences were compared. However, sliding-window analyses showed that patterns of variation in the vicinity of the *AGT* promoter are consistent with the hypothesis of a recent selective sweep. Departures from neutral expectation in some, but not all, regions of *AGT* indicate that patterns of diversity in the gene cannot be accounted for by human population history, which would affect all regions equally. Taken together, patterns of genetic diversity in *AGT* suggest that natural selection has favored the G (-6) variant over the A (-6) variant in some populations.

3. Ossification of the posterior longitudinal ligament of the spine (OPLL)

Toshihiro Tanaka, So, Tsukahara, Ryuji Ikeda, Kenichi Yoshida, and Ituro Inoue

Ossification of the posterior longitudinal ligament (OPLL) of the spine is a subset of "bone forming" diseases, characterized by ectopic ossification in the spinal ligaments. OPLL is a common disorder among elderly populations in East Asia, and is the leading cause of spinal myelopathy in Japan. We performed a genomewide linkage study with 142 affected sib-pairs to identify genetic loci related to OPLL. 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.

4. Intracranial aneurysm (IA) and molecular variants in collagen type 1 alpha-2 gene (COL1A2)

Taku Yoneyama, Hiroyuki Akagawa, Toshiaki Nakajima, and Ituro Inoue

Rupture of IA causes subarachnoid hemorrhage, with high morbidity and mortality, which remains to be one of major public health problems. Although genetic and environmental factors are thought to play equally important roles in the pathogenesis of IA, recent advances in molecular genetics make it possible to dissect the genetic determinants responsible for IA. Affected sib-pairs analysis based on 104 Japanese showed the positive evidence of linkage with IA around chromosome 5q22-31, 7q11, and 14q22.

COL1A2 on chromosome 7q22.1, which constitutes a positional and functional candidate for intracranial aneurysm (IA), was extensively screened for susceptibility in Japanese IA patients. Twenty one single nucleotide polymorphisms (SNPs) of *COL1A2* were genotyped in

genomic DNAs obtained from 260 IA patients and 293 controls. Differences in allelic and genotypic frequencies between patients and controls were evaluated by chi-square statistics. A significant genotypic association in dominant model was observed between an exonic SNP of *COL1A2* and familial IA patients (χ^2=11.08, df=1, P=0.00087, odds ratio=3.19 (95%C.I. =2.22-6.50)). This SNP induces Ala to Pro substitution at amino acid 459, locating on a triple-helix domain. Circular dichroism (CD) spectrometry was monitored with collagen-related peptides that mimic triple-helical models of type I collagen with Ala-459 and Pro-459 to estimate conformation and stability alterations. CD spectra showed that Pro-459 peptide had a higher thermal stability than Ala-459 peptide. The variant of *COL1 A2* could be a genetic risk factor for IA patients with family history. These observations provide some biological insight about the possible mechanism of a genetic predisposition to IA.

5. Genomewide linkage analysis of familial prostate cancer in a Japanese population

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Prostate cancer (PC) is one of the most common causes of cancer mortality in Western countries and familial aggregation of PC is well known. Multiple PC susceptibility loci have been reported in Western countries, but attempts to confirm the loci in independent data sets have proven to be inconsistent. We performed a genomewide linkage analysis with 53 affected sib-pairs to identify genetic loci related to PC in a Japanese population. Two linkage analyses, GENEHUNTER-PLUS and SIBPAL, were applied and detected nominal statistical significance of linkage for PC at chromosome 1p and 8p, which were reported as being loci for PC in Caucasians. The best evidence of linkage was detected near *D8S550* on 8p23 (maximum Z_{lr}=2.25, P=0.037), and the second best evidence of linkage was observed near *D1S2667* on 1p36 (maximum Z_{lr}=2.24, P=0.034). This is the first genetic mapping of PC in Japanese and the results suggest that susceptibilities of PC lie close to *D8S550* on 8p23 and *D1S2667* on 1p36.

6. Association Studies of Single Nucleotide Polymorphisms in Candidate Genes for Bronchial Asthma

Takashi Sekigawa, Miho Kakihara, and Ituro Inoue

Bronchial asthma (BA), one of the most common of all chronic inflammatory diseases in human populations, is resulted from a combination of detrimental factors, both environmental and genetic. One approach to address the genetic factors associated with BA is to undertake extensive surveys of candidate genes to search for variations, and to test allelic association studies. We selected genes as candidates that may relate to inflammation or apoptosis, such as genes encoding proteins related to cell-cell interactions (cytokines and their receptors) and those involved in the arachidonic acid cascade. These products are well known to have various biological activities and some have been shown to induce inflammation. The differences in response are likely to reflect subtle variations among genes encoding the proteins involved in this pathway. One of our interests is to identify susceptibility for aspirin induced asthma (AIA). AIA is a distinct clinical syndrome characterized by adverse respiratory reactions to aspirin and other non-steroid anti-inflammatory drugs. Because of the pharmacological action of aspirin, candidate genes for AIA could be easily listed on arachidonic acid cascade. We screen candidate genes in the pathway to identify the causality of AIA.

7. Dissecting the molecular mechanism of initiation of DNA replication in human cells

Kenichi Yoshida and Ituro Inoue

Toward a better understanding of how DNA replication is initiated or regulated in human cells, we have employed a genetic approach that efficiently provides suitable assay system to approach to biological functions of essential genes. Because commonly applied knocking out technique for an essential gene only resulted in cell mortality, we have developed a domain specific disruption method through homologous recombination in human cell line. By applying this knock down system for a replication initiator gene, Geminin, we can obtain minimal functional domain disrupted mutant cells that restrain DNA replication of chromosome and of viral episomes in cultured cells. We have clearly showed that the destruction box of Geminin is critical for cell proliferation and tumor growth in human cells. We have also employed tet-regulatable expression system for a putative helicase gene, MCM7, and revealed its function

in tumor progression. We are currently studying on several new mammalian proteins implicated in replication initiation and are examining their roles in DNA replication and regulation throughout the cell cycle.

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Donation Laboratories and Research Units

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. We also study a novel molecular complex, ABT1/ABTAP, which is involved in transcriptional regulation.

1. Molecular analyses of the Fanconi anemia/BRCA pathway

T. Oda, D. Adachi, T. Yamashita

FA is a genetically heterogenous disease classified into>10 different groups and 8 FA genes (FANCA, FANCC, FANCD1/BRCA2, FANCD2, FANCE, FANCF, FANCG and FANCL) have been identified to date. These gene products function in a common pathway, called as "the FA/BRCA pathway". In a current model of the pathway, a multiprotein nuclear complex including FANCA/C/E/F/G/L is critical for activation of FANCD2 into a monoubiquitinated form, which regulates DNA damage response in collaboration with BRCA1 and FANCD1/BRCA 2. This model is based on the following findings: (i) FANCA, C, G, E, F and L forms a stable nuclear complex; and (ii) deficiency of either of FANCA, C, E, F, G or L abolishes nuclear complex formation of the FA proteins and FANCD2 activation. Exceptionally, the FANCA/FANCG binding is not affected by the absence of other FA proteins. FANCG directly binds to the N-terminal region of FANCA, in which Leu at 25 was shown to be critical for the binding. The FANCA/FANCG binding was previously considered to be essential for activation of the FA pathway, based on the observation that deletion of the N-terminal region abolished activation of FANCD2. To verify this notion, we characterized FANCA mutants with amino acid substitution in this region, in comparison with patient-derived dysfunctional FANCA mutants such as H1110P. L25P and LL25/26AA failed to bind FANCG, FANCC, FANCF and FANCE but showed normal interaction with FANCL and nuclear localization. Contrary to the current model of the FA pathway, stable complex formation including all the components is not essential for nuclear localization of FANCA or FANCD2 activation. Unstable interaction among FA proteins, which is not detectable in co-immunoprecipitation assays, may be enough to activate ubiquitin-ligase of FANCD2. Our present findings provide novel insights into regulatory mechanisms of the FA/BRCA pathway.

2. Identification and characterization of novel mutations of the major Fanconi anemia gene *FANCA* in the Japanese population

H. Yagasaki, S. Hamanoue, T. Yamashita

Previous studies of *FANCA* mutations revealed high allelic heterogeneity, frequent occurrence of large deletions and inter-population differences. However, systematic mutational analysis, including gene dosage assay to detect large deletions, has not been documented for Asian populations. We newly developed TaqMan™ quantitative PCR-based gene dosage assay, and this analysis combined with sequencing of exons and cDNA fragments allowed for detection of 48 mutant alleles of *FANCA* in 27 (77%) of 35 unrelated Japanese FA families with no detectable mutations in *FANCC* or *FANCG*. We identified 29 different mutations (21 nucleotide substitutions or small deletions/insertions and 8 large deletions), of which at least 20 were novel. The *FANCA* mutational spectrum of the Japanese was different from that of other ethnic groups so far studied. Our study is the largest scale of mutation analysis of *FANCA* in the Japanese population. Characterization of these mutations including large deletions, splicing mutations and missense mutations provided new insights into the mutagenesis mechanisms and structure-function relationship of FANCA.

3. Somatic reversion and natural gene therapy in Fanconi anemia

S. Hamanoue, Y. Dong, T. Yamashita

Increasing interest has focused on effects of somatic reversion on clinical phenotypes of genetic diseases. In some cases, spontaneous clinical remission occurs in association with genetic reversion, which is called as "natural gene therapy". Reverse mosaicism in FA was previously noted by appearance of a subpopulation of peripheral blood (PB)-T cells with normal sensitivity to DNA crosslinkers, and genetic reversion was demonstrated in PB-T cells and EBV-immortalized B cells (B-LCL). However, few reports are available on analysis of genetic reversion in both lymphoid and myeloid cells in individual patients. In a single reported case with genetic reversion in hematopoietic cells as well as T lymphocytes and B-LCL, pancytopenia was progressive. Thus, little data is available on clinical consequences of genetic reversion in FA. We identified a 22-year-old female patient with long-term persistent hematopoiesis and genetic reversion in myeloid cells and B-LCL but not PB -T cells, unlike previous cases of FA mosaicism. The patient developed mild pancytopenia at the age of 4, which has been basically stable last 19 years. Diagnosis of FA was made, based on her clinical features including microcephaly and a chromosome breakage test. We identified biallelic mutations, 2546delC (maternal) and 3720-3724del (paternal), of FANCA in DNA from cultured PB-T and buccal cells. Consistently, PB-T cells showed MMC hypersensitivity and defective expression of FANCA protein. On the other hand, the patient's B-LCL showed normal MMC sensitivity and normal expression of full-length FANCA protein. Sequencing of DNA from these cells identified a missense mutation 2546C>T (Ser848Phe) instead of 2546delC, whereas the paternal mutation was unchanged. The altered protein (Ser848Phe) was confirmed to be functional when expressed in FANCA-null cells. Subsequently, the same reversion was detected in granulocytes and mononuclear phagocytes. Taken together, these results suggest that cells with the genetic reversion expanded predominantly in myeloid cells. The persistent hematopoiesis is attributed to the somatic reversion in myeloid cells. We found other FA-A patients with somatic reversion of FANCA, and these cases are under on-going study. Study of reversion mosaicism will provide a new viewpoint into the molecular pathogenesis of FA

4. Identification and Functional analyses of ABT1/ABTAP, a novel protein complex involved in transcriptional regulation.

T. Oda, T. Yamashita

Various TATA-binding protein (TBP)-associated proteins are involved in the regulation of gene expression through control of basal transcription directed by RNA polymerase (Pol) II. We recently identified a novel nuclear protein, activator of basal transcription 1 (ABT1), which binds TBP and DNA, and enhances Pol II -directed basal transcription. To better understand regulatory mechanisms for ABT1, we searched for ABT1-binding proteins using a yeast two-hybrid screening and isolated a cDNA clone encoding a novel protein termed ABT1-associated protein (ABTAP). Like ABT1, ABTAP is conserved from yeast to mammals. ABTAP formed a complex with ABT1 and suppressed the ABT1-induced activation of Pol II-directed transcription in mammalian cells. Furthermore, ABTAP directly bound to ABT1, disrupted the interaction between ABT1 and TBP, and suppressed the ABT1-induced activation of Pol II-directed basal transcription in vitro. These two proteins colocalized in the nucleolus and nucleoplasm and were concomitantly relocalized into discrete nuclear bodies at higher expression of ABTAP. Taken together, these results suggest that ABTAP binds and negatively regulates ABT 1. The ABT1/ABTAP complex is evolutionarily conserved and may constitute a novel regulatory system for basal transcription.

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Donation Laboratories and Research Units

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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 the functional linkage among proteins.

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 analysis of proteins from cells or tissues. Proteomics analyses are exemplified by large-scale 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 and comprehensive analysis of protein-protein interactions by mass identification of components in functional protein complexes, membrane domains and cellular organelles. Besides the 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 serve for functional proteomics. Using these technologies as analytical platforms, we are dissecting the signaling networks of proteins and their spatiotemporal regulation during fundamental cellular processes such as differentiation, growth, and apoptosis of cells.

1. Proteomic profiling of cell surface proteins expressed in mouse embryonic stem cells

Kazuto Nunomura[1], Chiharu Itagaki[1], Kohji Nagano[1], Nobuko Okamura[1,3], Tomonori Izumi[1], Toshiaki Isobe[1,2]: [1]Division of Proteomics Research (ABJ & Millipore), IMSUT, [2]Graduate School of Science, Tokyo Metropolitan University, [3]Nihon Millipore K.K

Cell surface proteins that are integral to or associated with plasma membranes play essential roles in regulation of a variety of cellular processes such as intercellular communication, cell adhesion and migration, and material transport. Because expression of a subset of these proteins may define the functional properties of individ-

ual cells, it is of great importance to profile surface proteins for understanding the molecular mechanisms of cellular functions and identifying valuable surface markers. In a previous study, we developed a method for the selective identification of cell surface proteins by nanoflow liquid chromatography (LC)-tandem mass spectrometry (MS/MS). Here we describe an application of the method to the large-scale analysis of embryonic stem (ES) cell surface proteins. Intact surfaces of D3 cells were biotinylated with membrane-impermeable reagent, fractionated, and subjected to tryptic digestion. Labeling of cell surface proteins was checked cytochemically and biochemically. Biotinylated peptides were affinity-purified and analyzed by multidimensional liquid chromatography-tandem mass spectrometry (2D LC-MS/MS) to determine both peptide sequences and labeled sites. In total, 324 proteins, including 50 CD markers, were identified with 965 peptide sequences carrying the biotin label. Of those 238 proteins were predicted to have signal sequences and/or transmembrane segments, common features for proteins integral to or associated with the plasma membrane. The number of predicted transmembrane segments in a protein molecule ranged from 1 (124 proteins) to 13 (7 proteins). Receptor (63 proteins), carrier and transporter (49 proteins), cell adhesion (44 proteins), and protease/inhibitor (25 proteins) were major functional categories assigned to proteins in the subset. Other functional categories included ribosome, structure, histone, chaperone and various enzymes, and 33 proteins were assigned to hypothetical or uncharacterized. The large-scale profiling revealed that the subset contained 57 receptors/signal transducers for signaling pathways including EGF, FGF, Eph, BMP, Wnt, Notch, Patched and LIF, 10 of their potential ligands, and 13 modulators. These proteins could be potential marker and target molecules for studying differentiation of ES cells. Although functional significance of individual molecules in ES cells has yet to be determined, our results provide a foundation for a more detailed understanding of stem cell biology.

2. Catalogue of proteins expressed in mouse embryonic stem cells toward construction of a mouse ES proteome database

Kohji Nagano[1], Yoshio Yamauchi[2], Chiharu Itagaki[1], Takashi Shinkawa[2], Kazuto Nunomura[1], Nobuko Okamura[1,3], Tomonori Izumi[1], and Toshiaki Isobe[1,2]: [1]Division of Proteomics Research (ABJ & Millipore), IMSUT, [2]Graduate School of Science, Tokyo Metropolitan University, [3]Nihon Millipore K.K.

A protein subset expressed in a mouse embryonic stem (ES) cell, E14-1, was characterized by mass spectrometry-based protein identification technology and data analysis. In total, 1,807 proteins including 370 potential nuclear and 257 membrane proteins were identified from tryptic digests of total cell lysates. The function and subcellular localization of the 1,807 proteins are widely distributed among the categories classified in the Gene Ontology (GO), suggesting that the identified proteins represented an unbiased set of proteins expressed in ES cells. Besides many housekeeping proteins found in common with other cell types, the subset contained a group of regulatory proteins that might determine the ES cell functions. Thus, we found 41 transcription factors including Oct-3/4, Sox-2, and UTF-1, 91 plasma membrane proteins including cell surface markers, such as CD9, CD 81, and alkaline phosphatase, 42 potential ligands for cell surface receptors including growth factors, cytokines, and hormones, and 96 cell-signal molecules. We also identified potential post-translational modifications in a number of ES-cell proteins including 8 phosphorylation and 11 lysine acetylation sites. The subset also contained 29 proteins that determine the "stemness" defined by Ramalho-Santos et al. We propose that this study provides a basic catalogue of major proteome set expressed in mouse ES cells.

3. Galectin-3 interferes with the interaction between Rb and c-Abl and contributes to survival of HeLa S3 cells

Kohji Nagano[1], Nobuko Okamura[1,2], Chiharu Itagaki[1], Kazuto Nunomura[1], Tadaomi Takenawa[3], Tomonori Izumi[1], and Toshiaki Isobe[1,4]: [1]Division of Proteomics Research (ABJ & Millipore), IMSUT, [2]Nihon Millipore K.K, [3]Department of Cancer Biology, Division of Biochemistry, IMSUT, [4]Graduate School of Science, Tokyo Metropolitan University

Cytoplasmic but not nuclear c-Abl tyrosine kinase has been suggested to play a role in resistance of cancer cells to apoptosis. The tumor suppressor protein Rb binds c-Abl through the C-terminal domain of Rb and inhibits the tyrosine kinase activity, although the physiological relevance in cancer cells is not fully understood. Here, we report that ectopic expression of the C-terminal region of Rb (amino acids 768-928) induces apoptosis of HeLa S3 cells and that apoptosis is suppressed by co-expression of a constitutively-active form of c-Abl. Co-immunoprecipitation studies followed by mass spectrometry analysis revealed that a galactose-

specific lectin, galectin-3, is associated with Rb. This interaction was also observed in the other human tumor cell line, MCF-7, but not A549. Galectin-3 also associated with endogenous c-Abl in HeLa S3 cells. Endogenous c-Abl protein was restricted to the cytoplasm of HeLa S3 cells, whereas Rb and galectin-3 were localized in both the nuclei and cytoplasm. Pull-down assays with GST-fusion proteins revealed that galectin-3 bound directly to c-Abl and the C-terminal region of Rb. Galectin-3 inhibited the interaction between Rb and c-Abl in vitro in a dose-dependent manner. When galectin-3 was overexpressed with Rb and c-Abl in 293-T cells, the interaction between Rb and c-Abl was also inhibited. Furthermore, co-expression of galectin -3 suppressed apoptosis induced by the C-terminal region of Rb. Taken together, our results suggest that the association of galectin-3 with Rb and c-Abl contributes to the survival of HeLa S3 cells by interfering with the interaction between Rb and c-Abl tyrosine kinase.

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Donation Laboratories and Research Units

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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 profile, 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 analyze phosphorylated proteins. This protocol is applied to identify ERK (extracellular signal-regulated kinase) substrates.

Proteomic investigation of signal transduction pathways

Kosako, H., Kobayashi, M., Machida, M., Ueda, K., Wakai, C., Yanagawa, S., Yamaguchi, N., Ushiyama, M.*, Inagawa, J.*, Hirano, J.*, Hattori, S.: Division of Cellular Proteomics (BML), Institute of Medical Science, University of Tokyo, *Amersham Biosciences, KK.

Recently the novel technology to analyze cellular proteome, proteomics, is emerging. This technology greatly depends on the information provided by human genome project. The protein in a single spot or single band on a gel is now identified rapidly by a mass spectrometer, by comparing the molecular weights of the 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 content 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? 2-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 Fe3 + 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 2-D fluorescent differential 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 are currently identifying 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 NIH3T 3 cells stimulated with PDGF in the presence or absence of wortmannin, a specific inhibitor for PI3-kinase, an upstream activator of Akt kinase.

Besides these proteomic projects, we also studied the function of an SH2-containing adaptor protein, Chat, which we identified previously.

A Novel Hematopoietic Adaptor Protein, Chat-H , Positively Regulates T-Cell Receptor-Mediated Interleukin-2 Production by Jurkat Cells

Akira SAKAKIBARA[1], Seisuke HATTORI[1,2], Shun NAKAMURA[1], and Takuya KATAGIRI[1,2]: [1]Division of Biochemistry, National Institute of Neuroscience, [2]Division of Cellular Proteomics (BML), Institute of Medical Science, University of Tokyo

Chat (Cas/HEF1-associated signal transducer) is a novel adaptor protein with an N-terminal SH2 domain and C-terminal Cas/HEF1 association domain. We report here the molecular cloning of Chat-H, the hematopoietic isoform of Chat. Chat-H has an extended N-terminal domain besides the known Chat domain structures, suggesting a unique function of Chat-H in hematopoietic cells. Jurkat transfectants overexpressing Chat-H show a marked increase in interleukin-2 (IL-2) production following co-stimulation of T-cell receptor and CD28. The degree of JNK activation is substantially enhanced in the Chat-H transfectants upon co-stimulation. The SH2 domain mutant of Chat-H loses this signal modulating activity. Expression of the Cas/HEF1 association domain mutant exhibits a dominant negative effect on both JNK activation and IL-2 production. We further found that Chat-H forms a complex with Pyk2H and enhances its tyrosine-402-phosphorylation, an up-regulator of the JNK pathway. These results suggest that Chat-H positively controls T-cell function via integrating the co-stimulatory signals.

The Active Cdc42-associated Tyrosine Kinase ACK1 Upregulates Integrin-mediated Adhesion Upon T-cell Receptor Engagement.

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We found that Ack1, a non-receptor tyrosine kinase which specifically binds to the activated Cdc42, was activated upon T-cell receptor (TCR) crosslinking. This activation was dependent on Cdc42 activation induced by TCR stimulation. PP2 but not piceatannol inhibited the activation of ACK1 and ACK1 was co-immunoprecipitated with Fyn but not Lck, suggesting that Fyn is involved in the activation of ACK1. The levels of Adhesion to both fibronectin and ICAM-1 were significantly increased in the Jurkat cells overexpressing ACK1 (J-ACK1) while they were decreased in those overexpressing kinase-inactive form of ACK1 (J-ACK1KI). Activation of Rap1 induced by the TCR engagement was enhanced in J-ACK1 clones while reduced in J-ACK1KI ones. Upon TCR ligation, we observed that tyrosine phosphorylation of C3G, a GEF for Rap1, was significantly promoted in J-ACK1 cells as compared with Jurkat cells. In addition, ACK1 was activated by cross-linking of b integrin. These results taken together, ACK1 may function as a modulator for the integrin-mediated signaling induced by the TCR engagement.

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Donation Laboratories and Research Units

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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 either 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 using mesenchymal stem cell

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Mesenchymal stem cells are known to differentiate into osteoblasts and induce new bone formation. A suitable carrier of the cells is critical for clinical application of the cells. We examined if a combination of fibrin glue, beta-tricalcium phosphate as a biodegradable (beta-TCP) and mesenchymal stem cells would provide three-dimensional templates for bone growth resulting in new bone formation. Growing stem cells and developing matrices, explanted from the rat femur, were fragmented and mixed with fibrin glue in a syringe. The cells/beta-TCP fibrin glue admixtures were injected into the subcutaneous space on the dorsum of the rat. Histological inspections showed newly formed bone structures in all admixtures, but none in the control groups. Mesenchymal

stem cells/beta-TCP fibrin glue admixtures can result in successful bone formation. This technique holds the promise of a minimally invasive means of generating autogenous bone to correct or reconstruct bony defects.

2. Bone induction by BMP

a. Prefabrication of vascularized bone flap induced by BMP-2

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An experimental model for the prefabrication of a vascularized bone flap was developed. To form vascularized bone in the desired configuration and to increase the survival rate of the grafted bone, a muscle vascularized pedicle was transformed into vascularized bone by the inducer recombinant human bone morphogenetic protein 2. The muscle flap raised on saphenous vessels in the rat thigh was sandwiched between same-size collagen sheets in the presence or absence of impregnated rhBMP-2. No evidence of muscle tissue transformation was found in control flaps, whereas all of the experimental flaps produced new bone. Saphenous vessels were observed to supply the new bone upon harvesting, and the newly formed vascularized bone showed good configuration with shape of the collagen sheet. The study indicates that this model of effective bone reconstruction could be potentially applied in a therapeutic setting.

We also succeeded in fabricating a bone flap using fat tissue, instead of muscle flap. Fat tissue connected to blood vessels was prepared to fit into the mold and implanted intramuscularly into the hind leg in rats. rhBMP-2 was applied in a collagen sheet previously placed on the inside surface of the mold. Our success in bone formation having a definite size, shape, and blood supply may lead to a therapeutic approach to effective bone reconstitution.

b. Effect of anabolic hormones on the bone induction induced by BMP

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BMP induces bone formation in young rodents, but aging causes a reduction in the bone-forming ability of BMP. Most patients who require bone reconstruction are relatively old. Accordingly, we examined whether anabolic hormones could restore the bone inductive activity of rhBMP-2 in aged rats. rhBMP-2 in a carrier pellet was implanted subcutaneously in both 4- and 50-week old female Wistar rats. PTH, PGE_2 or $1,25(OH)_2D_3$ was injected every day during the period of BMP implantation. Pellets in 50-week old rats showed a significant reduction in bone formation compared to pellets in 4-week old rats. However, daily injections of PTH into 50-week old rats restored both ALP activity and Ca content. $1,25(OH)_2D_3$ and PGE_2 also restored Ca content and stimulated ALP activity. These results show that the administration of these hormones restores bone-forming ability in aged rats. A combination treatment of these hormones with rhBMP-2 might be applicable to the reconstruction of bone defects in elderly patients.

3. Cultured mucosal cell sheet

a. Contraction of fibroblast-mediated collagen gel

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Grafting of cultured epithelium has become a useful technique for the treatment of epithelial defects, since grafted epithelial cells secrete factors promoting wound healing. We identified one such factor produced by cultured oral epithelial cells as thrombospondin-1 (TSP-1). Recently, TSP-1 was reported to act as an activator of transforming growth factor-beta1 (TGF-beta1). Accordingly, we examined the role of TSP-1 in wound healing and its mechanism in vitro and in vivo. The cultured oral epithelial cell-conditioned medium was harvested and proteins were analyzed. TSP-1 and the other factors were applied to fibroblasts-mediated collagen gel contraction assay. Collagen sponges were soaked with TSP-1 and implanted subcutaneously into rats. TSP-1 promoted collagen gel contraction activity. The diameters of the gels treated with LTGF and TSP-1 were reduced to a greater extent than those of gels treated with either factor alone. In vivo, increased numbers of fibroblasts were observed in the sponges treated with TSP-1. These findings suggested that TSP-1 causes collagen gel contraction by activation of LTGF. TSP-1 is expected to be especially suitable for regulating wound healing.

b. a double-layered mucosa membrane cultured on collagen sheet

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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 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.

4. Development of polymer scaffold for tissue regeneration

a. poly (L-lactide-co-(-caprolactone) scaffolds for condrocytes

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We examined the adhesion, proliferation, and morphology of chondrocytes on new scaffolds; and to examine these cells histologically for the ability of the chondrocytes to maintain chondrogenic properties after subcutaneous implantation into nude mice. Both 5 types of scaffolds were tested for chondrocytes. The cell growth on the scaffolds progressed with culture time in all scaffolds. Chondrocytes on every scaffold maintained a spherical shape. The hybrid-PLCs were superior to the PLCs with respect to the number of cells attached. The PLCs had an advantageous degradation characteristic in that they retained their original shape better than the collagen scaffold.

b. poly (lactic acid) composites containing calcium carbonate (vaterite).

Kasuga T, Maeda H, Kato K, Nogami M, Hata K, Ueda M*.: Department of Materials Science and Engineering, Nagoya Institute of Technology

A new type of ceramic-polymer biomaterial having excellent apatite-forming ability in simulated body fluid was prepared by hot-pressing a mixture of poly (-L-lactic acid) (PLA) and calcium carbonate (vaterite). After PLA dissolved in methylene chloride was mixed with calcium carbonate consisting of vaterite, the mixture was dried completely and subsequently hot-pressed uniaxially. When 30 wt% vaterite was introduced, the modulus of elasticity was effectively improved, which was about twice higher than the modulus of PLA. The composite showed no brittle fracture behavior and a comparably high bending strength. The composite containing 30 wt% vaterite formed a thin bonelike apatite layer on its surface after soaking in SBF.

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Donation Laboratories and Research Units

Human Genome Center Laboratory of Biostatistics (Biostatistics Training Unit)

バイオスタティスティクス人材養成ユニット

Professor	Katsuhisa Horimoto, Ph.D.	特任教授　理学博士	堀　本　勝　久
Research Associate	Sachiyo Aburatani, Ph.D.	特任助手　農学博士	油　谷　幸　代

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

0. Short History of Our Laboratory

Our laboratory was established in October, 2002; the member was only Professor Katsuhisa Horimoto. Now, our laboratory has 6 members: Dr. Makihiko Sato (FUJITSU) as a research scientist from December, 2002, Dr. Sachiyo Aburatani as a research associate from April, 2003, Dr. Nobuyoshi Sugaya as a research scientist from April, 2003, Drs. Hiroo Murakami as a research scientist from October, 2003, and Akira Imaizumi (AJINOMOTO) as a research scientist from October, 2003. All members investigate independently their own subjects as described below.

1. Network Inference

a. Deduction of a gene regulatory relationship framework from gene expression data by the application of graphical Gaussian modeling.

Sachiyo Aburatani, Hiroyuki Toh[1], Katsuhisa Horimoto: [1]Bioinformatics Center, Institute for Chemical Research, Kyoto University

Recently, we developed an automatic system for deducing a framework of regulatory relationships from gene expression data on a genomic scale. One of the merits of our system is that it simultaneously performs the gene classification and the relation inference from a large amount of gene expression data by combining the graphical Gaussian modeling with standard multivariate statistical techniques.

We describe our modifications of the previous system to estimate the cluster boundaries, by setting a user-defined threshold for measuring the linear relationship between the profiles of clusters. The modified system was applied to a whole set of yeast expression profiles of 6152 genes available at a web site, and the results obtained by the present analyses were statistically evaluated by a simulation that calculates the chance probability of the inferred framework of regulatory relationships, in addition to the statistical evaluation of the clusters by a previous method. In particular, the feasibility of the modified system is demonstrated by the com-

parison between two frameworks inferred from two sets of clusters that were estimated by distinctive thresholds.

b. ASIAN: A Web Site for Network Inference.

Katsuhisa Horimoto, Hiroyuki Toh[1], Sachiyo Aburatani, Nobuyoshi Sugaya, Hiroo Murakami, Makihiko Sato, Akira Imaizumi

Recently, we have developed a system, named "ASIAN", for inferring a network by the combination of clustering and graphical Gaussian modeling (GGM). The feasibility of the system was validated by the application of the system to the data of gene expression profiles.

The system is composed of five parts: the calculation of correlation coefficient matrix of raw data, the hierarchical clustering, the determination of cluster boundaries, the calculation of the average data from raw data based on the determined cluster number, and the application of GGM to the average data. Since the system was designed to apply the gene expression profiles, the first four parts were prerequisite to analyze the redundant data including many similar patterns of expression profiles by the last part, application of GGM. The five programs were performed in turn in the above order, being operated from the command line in UNIX machine. In this study, we present a web site to utilize widely the ASIAN system. In the ASIAN web site, the user can estimate a network only by input any data including the redundant information.

In the ASIAN web site, the user first pastes or uploads the data from the user's machine, and then, receives an ID number by e-mail. By the ID number, only user can see the results analyzed. We performed a benchmark test to estimate the computational time: expression data of 2467 genes measured under 79 conditions by a machine with the CPU of Intel Pentium III SSE1 996MHz cache 256KB and memory of 514MB under the LINUX operation system. By the test, the computational time was 9 min. and 38 sec.

c. Statistical Analysis of the Relationship between Gene Expression and Location.

Sachiyo Aburatani, Nobuyoshi Sugaya, Hiroo Murakami, Makihiko Sato, Katsuhisa Horimoto

DNA microarray data provide us with a large amount of information on gene expressions in the living cell. One of the important goals of such expression analysis is to estimate the relationships among genes in entire genome. The completion of bacterial genomes provided us the tendencies of genes with related functions to remain together across several genomes. Furthermore, the case of genes, whose protein products physically interact, conserved the gene order. The observation implies the possibility of correlation between the gene expression level and the gene location on the genome. In this study, we present a statistical approach to investigate the relationships between gene expression levels and their location on the genome.

We analyzed the data from 15 DNA microarray data performed on *E. coli*. We first identified the genes transcribed in the same direction of each replichore. Then, we calculated Pearson's correlation coefficients between the genes from their expression data according to their gene order on genome. Furthermore, we tested the correlation coefficients by t-test in the range from 5×10^{-3} to 10^{-11} significance probability.

Relative distance between the genes with a significance probability is evaluated. As a result, a large number of correlated clusters were found in the short relative distances with different probabilities. However, many correlated clusters were also found with the larger probabilities, regardless of relative distance. Furthermore, we counted the number of correlated genes with a significance probability for each gene (criterion gene), and the numbers of correlated genes with the criterion genes were mapped on each replichore. The numbers of correlated genes located within each 100000bp are averaged for distinctive replichores. The two patterns of the averaged numbers of correlated genes are mutually similar in the same strand of replichore. The correlated gene clusters in the same strand were analyzed in terms of their biological function, and the well-known regulons were well found among them. Similarity of correlated gene numbers between replichores might suggest the concerted transcription in the wider range on the genome.

2. Genome Comparison

a. Gene-Distribution Patterns on Cyanobacterial Genomes.

Nobuyoshi Sugaya, Hiroo Murakami, Makihiko Sato, Sachiyo Aburatani, Katsuhisa Horimoto

In the phylum Cyanobacteria, complete genome sequences have been already reported in seven species and strains. Very interestingly, only one speices of these cyanobacteria, *Anabaena* sp. PCC7120 (hereafter Anabaena), has considerably large genome size of approxi-

mately 6.4 Mb, while the remaining species have the genome sizes of 1.7-3.6 Mb to be in the range only from about a quarter to a half of the size of the Anabaena genome. In this study, we assess the genome-size difference in these cyanobacterial species by investigating patterns of gene distribution on the genomes of three cyanobacteria, Anabaena, *Synechocystis* sp. PCC 6803 (*ca*. 3.6Mb) (hereafter Synechocystis) and *Thermosynechococcus elongatus* BP-1 (*ca*. 2.6Mb).

In order to investigate patterns of gene distribution on three cyanobacterial genomes, we adopt a metric of gene-location distance to the gene-configuration comparison between the Anabaena genome and the genomes of Synechocystis and *T. elongatus*. Gene-location distance is a measure of the dissimilarity of a pair of genes, relative to the configuration of other pairs of genes on two circular genomes compared. For the present purpose, the Anabaena genome is divided into two halves and each gene configuration on a half of the Anabaena genome is compared with that on other two genomes.

The gene-location distances are calculated with respect to these ortholog pairs between a half region of the Anabaena genome and whole region of another genome. As a result, gene configuration on the region of 260°-80° in the Anabaena genome is similar to that on the Synechocystis genome, and that on the region of 80°-260° is similar to that on the *T. elongatus* genome. Interestingly, when the Anabaena genome is divided into two regions of 260°-80° and 80°-260°, a larger number of house-keeping protein genes involved in cell maintenance are encoded on the former region, and on the other hand, a number of Anabaena-specific protein genes involved in heterocyst differentiation and nitrogen fixation are on the latter region. These distribution patterns of functional categories are statistically significant ($P<10^{-6}$). The results obtained from the gene-location distances and the distribution patterns of functional categories imply that the contemporary Anabaena genome might have amplified the gene repertoire by an event such as whole-genome duplication and a fusion of two bacterial genomes during its course of evolution.

b. Identifying Obscure Periodic Patterns in Genomic DNA Sequence.

Hiroo Murakami, Sachiyo Aburatani, Nobuyoshi Sugaya, Makihiko Sato, Katsuhisa Horimoto

Genomic DNA sequence is very abundant in periodic patterns, which play important biological roles, such as gene expression, genome structural stabilization, and recombination. Tandem repeat is a type of periodic patterns and concerns several genetic diseases. Tandem repeat finder (TRF) is one of the widely used programs to find tandem repeats without a priori knowledge. Statistical models are used to identify an explicit tandem repeat in TRF. However, no other types of periodic patterns are recognized by TRF. In this study, we present a novel auto-correlation-based model to identify obscure periodic patterns, and apply it to eukaryotic chromosomes.

We developed a program named "STEP-STONE". The program outline is as follows:

1. Calculation of auto-correlation: Auto-correlation of whole *k*-oligomer (parameter: adjustable from 4-mer to 8-mer) in a query sequence is calculated by using hash. Correlated *k*-mers with periodicity that are counted above pre-simulated background by *z*-score (default is> 95%) are stacked for the following process. The process 1 requires only O (n+m) time. (n: sequence length, m: whole *k*-mer. *i.e*. m= 4096 at 6-mer)
2. Dot-plot analysis: The locations of one correlated *k*-mer in the query sequence are searched. Then, among the periodic locations, the periodic sequences are aligned as a presumable periodic unit (P.P.U.).
3. Dynamic Programming: To identify the actual periodic pattern unit (P.U.), each P.P.U. is computed by Smiss-Waterman-Goto type of dynamic programming. A DP score below the parameter (default: 40) and the exact multipliers of the period are filtered out. As a result, only one highest scored P.U. of the same period is chosen in the location that is abundant with periodic *k*-mers.

Our model's unique global auto-correlation algorithm enables us to find tandem-located periodic pattern with non-repeat sequence, as well as enough sensitivity and speed to recognize tandem repeat by TRF. Thus, our model identifies both tandem repeat and interspersed repeat. Indeed, the interspersed repeat of *Cyanobacteria Anabaena* genome was identified by STEP-STONE. In addition, interspersed repeats located within the Down's syndrome critical region in the human chromosome 21 were also identified.

3. Protein Structure Analysis

a. Profile-Profile Comparison based on Hidden Markov Model Profiles.

Makihiko Sato, Nobuyoshi Sugaya, Hiroo Murakami, Sachiyo Aburatani, Katsuhisa Horimoto

The detection of sequence similarity within the twilight zone has been a challenging problem in sequence analysis. Among various types of approaches for sequence similarity detection, the profile-profile comparison is one of the most reliable approaches. In this study, we design a profile, named match-node profile, which is generated from match nodes in the Hidden Markov Model (HMM) profiles. The profile in a representative HMM is composed of three types of nodes; match node, delete node, and insert node. The three nodes in the HMM profiles represent the probability distributions of amino acid residues, deletions, and insertions in each site of multiple alignment, respectively. Thus, the present match-node profiles for a multiple alignment can describe essential characteristics of the multiple alignments.

The PDB40D was adopted as a data set to perform a benchmark test for the feasibility of our approach. The sequences in PDB40D (4289 protein chains from one complete domain) corresponded with those in SCOP to evaluate the performance of our approach for fold recognition: 3327 chains in family level, 537 chains in superfamily level, 193 fold level, and 232 class level. To construct match-node profiles, each sequence in PDB40D was searched against KIND by PSI-BLAST. From the multiple alignments thus obtained, the HMM profiles were generated by HMMER, and then, the match-node profiles were extracted from them. Concomitantly, the frequency profiles were also generated from the alignment. As for the scoring of similarity between profiles, we used the log average scoring based on Bayesian theory, which is one of the most suitable scorings for the profile-profile comparison. Thus, we performed three methods in this study: PSI-BLAST, log average scoring for the frequency profile (FPLA), and log average scoring for the match-node profile (MPLA).

By a benchmark test, the fractions of proteins with the highest score that belongs to the same category of a query sequence are shown in each SCOP level. In summary, the MPLA shows the best performance among three methods in the three SCOP levels. In particular, in the fold level, the MPLA significantly outperforms (about 50% improvements) both other methods, although the relationships between the proteins are fairly weak.

b. Protein Structure Modeling from the Information of Distance Restraints Obtained by Chemical Cross-Linking

Katsuhisa Horimoto, Sachiyo Aburatani, Tatsuya Akutsu[1], Akira Kinjyo[2], Ken Nishikawa[2]: [2]Center for Information Biology, National Institute of Genetics

Recent progresses of mass spectrometry in accordance with chemical cross-linking experiments have provided us to new information of distance restraints between amino acid residues in the protein structure. Here, we elucidate the possibility of the protein structure modeling based on the information on distance restraints.

First, Intra-interactions by two cross-linkers, which are generally used in cross-linking experiments, are hypothetically generated in a known protein structure, and then the distances between the atoms with intra-interactions are calculated in consideration of steric hindrance of cross-linker and exposure area of amino acid residue. By the above procedure, the distance restraints of 35 structures that were tested in previous studies of *ab initio* structure modeling are generated and with the use of the restraint information, the structure models are constructed by a distance geometry method that have been developed to construct a protein structure model based on the distances by NMR experiments. Then, the accuracy of protein structure models is estimated by r.m.s.d. and folding pattern. As a preliminary result, the accuracy is less than 5.0A in all cases. The present results indicate that the information of distance restraints by chemical cross-linkers is useful for a high throughput modeling of protein structure.

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Donation Laboratories and Research Units

Division of Neural Signal Information (NTT-IMSUT)

神経情報シグナル(NTT−東大医科研)共同研究ユニット

Visiting Associate Professor Ichiro Fujimoto, Ph.D. | 客員助教授 理学博士 藤 本 一 朗

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, molecular and cellular biology. 1) Recently, it has been shown that structural change of certain membrane occurs by calcium ion concentration. We are normalizing the AFM microscope probe in the solution environment to observe the membrane protein structure changing without fixation. 2) We are focusing on Na^+/HCO_3^- co-transporter protein; 1) finding the binding sites of the binding protein, 2) identification of functional role of its binding protein by co-expression in oocyte and cells, and 3) identification of mRNA isoform specifically expressed in brain and protein expression patterns in developmental mouse brain.

Analysis of conformational change of inositol 1,4,5-trisphoshate receptor using atomic force microscope

Touichiro Goto[1], Wakako Suhara, Keiichi Torimitsu[1], and Ichiro Fujimoto: [1]Materials Science Laboratory, NTT Basic Research Laboratories, Nippon Telegraph and Telephone Corporation

The inositol 1,4,5-trisphosphate receptor (IP3 R) is a ligand-gated ion channel, the opening of which is controlled by both IP_3 and Ca^{2+}. This dual regulation is important in allowing IP_3 receptors (IP3Rs) to generate spatially and temporally complex intracellular Ca^{2+} signals. In addition, IP3R may also be directly involved in regulating Ca^{2+} entry into cells by providing a functional association between intracellular Ca^{2+} stores and plasma-membrane channels responsible for store-regulated Ca^{2+} entry. In mammals, three different IP3R isoforms are expressed; each is encoded by a distinct gene. The channel structure exhibits the expected 4-fold symmetry and comprises two morphologically distinct regions: a large pinwheel and a smaller square. The pinwheel region has four radial curved spokes interconnected by a central core. The IP_3-binding core domain has been localized within each spoke of the pinwheel region by fitting its x-ray structure into the reconstruction. A structural mapping of the amino acid sequences to several functional domains is deduced within the structure of the IP3R1 tetramer. We purified the membrane fraction from IP3R transfected cell lines. Biological samples were diluted to a concentration of 0.01 mg/ml in the dialysis buffer and adsorbed to freshly cleaved mica. Contact mode AFM topography were recorded at room temperature after adjusting the electrolyte concentration of the buffer to allow electrostatically balanced high-resolution imaging at a stylus loading force of<100 pN. The applied force was corrected manually to compensate for the ther-

mal drift of the microscope. No differences between topography recorded simultaneously in trace and in retrace direction were observed, indicating that the scanning process did not influence the appearance of the biological sample. The AFM used was a SPI3800N equipped with a J-piezoscanner (scan size 20μm) and a fluid cell. Calibration of the scanner in z-direction was performed on defects (large holes or steps) observed on layered solid-state crystals as described. The probe of cantilevers SN-AF01 was used, its nominal spring constant was 0.08 and 0.02 N/m. At low magnification (frame size>600 nm at 512×512 pixels) topography were recorded in the error signal mode acquiring the deflection and height signals. Optimizing gains and scan speed minimized the deflection signal. Fast Fourier transform was performed to the scan image to reduce the noise.

Analysis of sodium/bicarbonate co-transporter with its interacting protein

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The electrogenic sodium/bicarbonate co-transporter (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 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 1 Na^+: 2 HCO_3^- or 1 Na^+: 3 HCO_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 an intracellular protein that has not yet been published, suggesting that other proteins are probably involved in regulation of NBC1 function. Now we are confirming and investigating this interaction by pull down assay and immunocytochemical studies on transfected cells.

Functional study of sodium/bicarbonate co-transporter 1

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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. This will give detailed information about the activity changing caused to NBC1 by its binding protein in an *in vivo* system.

Developmental and regional expression of NBC isoforms in mouse brain

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

Publications

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