Division of Stem Cell Regulation (Amgen)

Our research interest is to characterize functional molecules of stem cells, particularly 1) signaling molecules that regulate proliferation and differentiation of stem cells, and 2) genetic manipulation of stem cells by appropriate gene transfer system. For this purpose, we are focusing on; 1) the mechanism of self-renewal in embryonic stem cells, 2) ex vivo expansion of hematopoietic stem cells using chimeric cytokine receptor transgenic mice, 3) identification of master regulator(s) for kidney formation using animal caps of Xenopus embryos and knockout mice, and 4) identification of self-renewal molecule(s) for neural stem cells.

Functional cells of blood, immune system, nervous system and skin have a relatively short life compared to an individual. To supply these cells, functional cells are maintained and generated from stem cells of various lineage during embryonic development or adult, resulting in the formation, maintenance, repair and regeneration of tissues and organs. Stem cells are defined by virtue of their functional attributes as (a) undifferentiated cells (i.e. lacking certain tissue-specific differentiation markers), (b) capable of proliferation, (c) able to self-maintain the population (self-renewal), (d) able to produce a large number of differentiated, functional progeny, (e) able to regenerate the tissue after injury, and (f) flexible use of these options.

A major impetus here has been the desire to identify these stem cells in tissues so that strategies for targeting these permanent lineage ancestor cells for gene therapy may be developed. Also there has been an increased interest in manipulating the tissue stem cells in various clinical situations including, for example, the normal tissue stem cells during cancer therapy. It is still not possible to identify stem cells in most tissues by either their morphological characteristics or by the use of a specific marker. Numerous attempts have been made to find stem cell specific markers, but the problem here may be that what characterizes these cells is more likely to be the absence of specific features than the presence of something that can be identified by a marker or probe. So far, there are no established stem cell lines and self-renewal factors for stem cells, except embryonic stem (ES) cells and leukemia inhibitory factor (LIF), respectively. Therefore, identification, establishment and *in vitro* expansion of stem cells is essential not only for analyzing their self-renewal mechanism, but also for a variety of clinical applications, such as bone marrow transplantation, tissue regeneration, and gene therapy.

We have been investigating (1) the mechanism of self-renewal in ES cells using chimeric cytokine receptor and STAT3ER, (2) identification of essential genes for kidney formation using animal caps of *Xenopus* embryos and knockout mice, (3) identification and establishment of neural stem cells from mouse fetal brain, and (4) the mechanism of growth and differentiation of muscle stem cells.

1. Self-renewal mechanism of embryonic stem cells

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The pluripotent phenotype of ES cells is maintained in the presence of LIF. LIF binds to a cell surface receptor complex composed of LIF receptor β and gp130, through which several signaling molecules including MAP kinase and STAT3 are activated. We have previously constructed a fusion protein composed of the entire region of STAT3 and the ligand binding domain of estrogen receptor. This fusion protein (STAT3ER) is dimerized and functions as an active STAT3 in the presence of 4-hydroxytamoxifen (4HT). Using this construct, we have demonstrated that STAT3 activation is sufficient for self-renewal of ES cells. Moreover, from ES cells transfected with STAT3ER, we have generated transgenic mice expressing STAT3ER.

In this study, we tried to understand the molecular events downstream of STAT3 by employing following two strategies. One is to identify factor(s) whose expression is regulated by STAT3, and the other is to elucidate the relationship between STAT3 and Oct-3/4, a transcription factor indispensable for maintenance of pluripotent ES cells. We also examined whether IL-6 family-dependent various biological responses are induced by 4HT in STAT3ER transgenic mice. In addition, we have started a search for a serum factor(s) that is essential for survival of ES cells.

a. STAT3ER transgenic mice

In this study, we focused on following IL-6 family-dependent biological responses: a) IL-6-dependent production of acute phase proteins in the adult liver, b) Oncostatin M-dependent fetal liver development, c) LIF-dependent establishment of ES cells from blastocysts, and d) CNTF/LIF-dependent astrocyte differentiation of fetal neuroepithelial cells. Addition of 4HT induced gene expression of some acute phase proteins in hepatocytes prepared from STAT3ER transgenic mice. Fetal liver development was also promoted by 4HT in STAT3ER transgenic mice. However, 4HT had no effects on establishing ES cells from blastocysts and on promoting differentiation of fetal neuroepithelial cells into astrocytes. These results suggest that dimerization of STAT3 is sufficient for some, but not all, IL-6 family-dependent biological responses.

b. Targets of STAT3

To identify factor(s), downstream of STAT3, responsible for self-renewal of ES cells, we performed cDNA subtraction and cDNA array analyses using mRNAs prepared from undifferentiated and differentiated ES cells. We identified more than 30 clones whose expression levels are down-regulated upon inactivation of STAT3.

We are now planning to examine whether forced expression of these clones affects self-renewal and differentiation of ES cells.

c. Oct-3/4-interacting proteins

Oct-3/4, a pluripotent stem cell-specific transcription factor, plays a critical role in maintenance of pluripotent ES cells, and is one of the candidates that function downstream of STAT3. Since several evidences suggest that stem cell-specific gene expression via Oct-3/4 requires unidentified cofactor(s), we tried to identify Oct-3/4-interacting protein(s) by yeast two-hybrid screening. We obtained more than 100 clones that interact with Oct-3/4 fused to the Gal4 DNA binding domain, and sequences of 53 clones were determined. We plan to examine whether these clones modulate the function of Oct-3/4, and whether these clones are under the control of STAT3.

d. Survival factor in serum

Although known as a self-renewal factor of ES cells, LIF alone is not sufficient to support proliferation of ES cells, and ES cells undergo apoptosis in the absence of serum. Thus, we are now trying to identify a serum factor(s) that supports survival of ES cells. For this, we first examined several chemical properties of the factor, and found that the survival activity of the factor is resistant to treatment with either acid, heat, or organic solvent.

2. A zinc-finger protein *Sall1* is essential for kidney development

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Animal caps of *Xenopus* embryos, which are treated with activin and retinoic acid, give rise to pronephric tubules. By differential display using the animal caps, we cloned a new transcription factor homologous to *Drosophila spalt* (*sal*). This gene (*Xsal-3*) has multiple double-zinc finger motifs characteristic to the *sal* gene family and is expressed in the central nervous system and pronephric duct in *Xenopus* embryos. We also isolated a new mouse gene which belongs to the *sal* family. It is expressed in metanephric mesenchyme, a subventricular region of the brain, olfactory bulb,

and limb buds. This new gene is likely to be a murine homologue of human *SALL1* and we generated mice lacking this *SALL1* homologue(*Sall1*) to examine its roles in kidney formation.

Homozygous mutant mice died within 24 hours after birth. They had either no or small remnant kidneys. At day 11.5 of gestation, *Sall1* homozygosity resulted in an absence of ureteric bud outgrowth and a subsequent failure of metanephric induction. Most of mesenchymal genes involved in kidney development were downregulated in the mutant kidneys. These results indicate that *Sall1* is essential for early inductive signaling in kidney development and analysis of upstream and downstream molecular events will lead to better understanding of kidney formation.

3. Neural stem cells

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EGF and FGF-2 are important factors to maintain neural stem cells isolated from specific regions of the embryonic and adult brain. Neural stem cells can proliferate in response to EGF and FGF-2, exhibit self-maintenance, and retain their multipotency to differentiate into neurons, astrocytes and oligodendrocytes. In preliminary experiments, we found that EGF and FGF-2 have different effects on proliferation and differentiation of neural stem cells.

In primary cultures of mouse embryonic day 14 striatum maintained *in vitro* for 5 days, FGF-2 induced cell proliferation, whereas EGF showed little response. However, after growth factor treatment for 8 days, most cells responded to both factors. It is suggested that neural stem cells initially respond to FGF-2 only, and then acquire EGF responsiveness during the *in vitro* culture. Next, we examined the effects of EGF and FGF-2 on differentiation of neurosphere. Neurosphere was attached onto polyethylenimine-coated dish and cultured in the presence of 1% FBS and no growth factors. Compared with FGF-2-generated neurosphere, EGF-generated neurosphere showed accelerated differentiation to glial cells, suggesting that EGF induces the proliferation of not only neural stem cells, but glial cells. Using neural marker, we confirmed that FGF-2 generated neurosphere differentiated into neurons, astrocytes and oligodendrocytes. Rat neurosphere also differentiated into neurons and glia.

The lineage relationship between the EGF- and FGF-2-responsive neural stem cells is unknown. Moreover, a major drawback to study of neural stem cells is that the specific cell surface marker is not found. Several specific molecules expressed in neural stem cell, such as nestin and musashi, are reported. However, since these molecules are localized within cells, antibodies against those can not recognize intact cells. To characterize living neural stem cells, we intend to make monoclonal antibodies that recognize the cell surface molecules of neural stem cells.

4. Muscle stem cells

Hiroshi Koide, Manabu Watanabe, Takashi Yokota

Satellite cells, adult skeletal muscle stem cells located between the sarcolemma and the basal lamina of the muscle fiber, possess high myogenic potential and participate in muscle regeneration. They are usually stay at the resting state and, upon muscle injury, proliferate, migrate and differentiate to repair the damaged fiber. Since satellite cells can be easily obtained from adult tissues, satellite cells could be a powerful tool for cell therapy.

In order to manipulate satellite cells, it is important to understand the molecular mechanism that regulates their self-renewal and differentiation. Although extensive studies have identified several myogenic regulatory factors (MRFs), including MyoD and myogenin, little is known about signaling pathways which connect extracellular stimuli to the MRFs. LIF is shown to be produced in injured muscle and stimulate the growth of satellite cells. Moreover, significant reduction of skeletal muscle regeneration is observed in LIF knockout mice. These findings suggest that LIF is a crucial factor for muscle regeneration. Thus, as a first approach to study satellite cells, we are going to identify the essential downstream signaling molecules of LIF receptor for growth of satellite cells using the GM-CSFR/gp130 chimeric receptors.

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Division of Cell Processing (Asahi Chemical and Nissho)

Division of Cell Processing studies cell purification, modification of cellular functions by immunological and genetic means, expansion, cryopreservation and transfusion. We have established Tokyo Cord Blood Bank in IMSUT on September 1997 and our division is responsible for cord blood collection, processing and storage. Sixty-five transplantations have been done and the clinical results indicate that placental/ umbilical cord blood transplantation is quite promising. We support the clinical departments through cell therapy and gene therapy.

Division of Cell Processing was established in the Institute of Medical Science, the University of Tokyo (IMSUT) on September 1995 in response to the requirements of the Institute, particularly the clinical research departments. The task of our division is to support departments through cell therapy and gene therapy. Our efforts at *ex vivo* processing of human cells will be directed towards to developing methods for collection, separation, isolation, advanced processing, expansion, genetic manipulation and cryopreservation of a variety of cell populations, including hematopoietic progenitor cells, lymphocytes, monocytes, dendritic cells, and marrow stromal cells. These methods will be applied to IMSUT clinical trials of autologous and allogeneic hematopoietic stem cell transplantation, adoptive immunotherapy, cellular vaccine immunotherapy and gene therapy. Among these projects, we have first focused on cord blood stem cell processing and cryopreservation. Placental/ umbilical cord blood (PCB) has recently been found to be an efficacious alternative source of hematopoietic stem and progenitor cells for engrafting. PCB transplantation has been performed in 2000 unrelated through the world. In Japan 336 unrelated cord blood transplantations were reported until December 2000. Nine local cord blood banks have been established in Japan. We have developed the technology for cord blood collection, nucleated cell separation from red blood cells, cryopreservation, and washing out of cryoprotectant. The methods we developed worked well and we have started collection cord blood and established Tokyo Cord Blood Bank in IMSUT as a model of a national cord blood bank in Japan that started on April 1999. Sixty-five transplantations have been done until January 2001 using our stocked cord blood units and the clinical results indicate the placental/umbilical cord blood transplantation is quite promising. Dendritic cells (DCs) are professional antigen presenting cells (APCs) that initiate primary T cell dependent immune response. DC-based immunotherapy is rapidly emerging in the treatment of cancer. We have started both basic and clinical studies of DC immunotherapy.

1. Effect of washing procedures on cord blood transplantation: Engraftment of human CD45$^+$ cells in NOD/SCID mice transplanted with cord blood stem cells with or without washing out of DMSO after cryopreservation

Ken-ichi Saito, Norihisa Sasayama, Tomohiro Sato, and Tsuneo A. Takahashi

In cord blood (CB) transplantation, washing out of DMSO (dimethyl sulfoxide) from cryopreserved CB unit has been recommended to insure the early engraftment. To prove the benefit of washing out of DMSO on transplantation, we did the following experiments using NOD/SCID mice. Leukocyte concentrates (LC) were prepared from cord blood by the HES method. The LCs were loaded with cryoprotectants (final concentration, 10% DMSO and 1% dextran 40), and cryopreserved in liquid nitrogen for 3 months. After rapid thawing in a water bath, the thawed cells were directly infused to the mice to

mimic the situation of cord blood transplantation without washing out of DMSO, or washed stepwise with 10% dextran 40 and 5% human serum albumin following our clinical protocol. The recovery of cells after stepwise washing was higher than 95%. One million of cryopreserved, thawed and washed or not washed cells were intravenously injected into 6-8-week-old female NOD/SCID mice irradiated with 300cGy of total body irradiation. The recipient mice were injected intraperitoneally with anti asialo GM1 antibody immediately before the cell transplantation. Identical treatments were performed biweekly after infusion of experimental cells. At 2, 4, 6,and 8weeks after transplantation, mice were sacrificed and human $CD45^+$ cells were assessed in femurs, tibias and humeri. Cells were stained with monoclonal antibody specific for human CD45 and analyzed by FACS .In the mice infused with the washed cells, human $CD45^+$ cells were recognized in 1.32% (average, n=3) and 0.57% of leukocytes at 4 and 8 weeks, respectively. In the mice which cells were infused directly after thawing, i.e., without washing out of DMSO, the human $CD45^+$ cells were recognized in 1.88% and 2.9% at 4 and 8 weeks, respectively. These results indicate that the procedure of washing out of DMSO did not injure the cells, but there was no difference of engraftment of human $CD45^+$cells between transplantation of washed and non-washed cells in this animal model.

2. SCID-repopulating activities of human cord blood cells separated by stem cell collection filter (SCF): A comparative study of filter method and HES method

Yasuo Tokushima, Mie Midorikawa, and Tsuneo A. Takahashi

Cord blood(CB) is an important source of hematopoietic stem/progenitor cells for transplantation. The Hydroxyethyl starch (HES) method developed by Rubinstein et al. (PNAS, 92: 10119,1995) has been the standard method for red cell removal and volume reduction of CB unit. Recently, a new approach has been developed to reduce both volume and the number of red blood cells of CB unit with simple process, i.e., stem cell collection filter (SCF) (Asahi Medical Co. Ltd., Tokyo, Japan). We have demonstrated that the SCF gives high recovery rates of total nucleated cells, $CD34^+$ cells and colony-forming cells (CFC) with short operation time (mean operation time was 11.3min.). Also, we have shown that hematopoietic system can be repopulated in NOD/SCID mice by CB cells, which was separated by SCF. The aim of this study is to compare directly the SCID-repopulating activities of CB cells separated by SCF or HES method using two-separated identical CB units. CB units were divided into two aliquots and the one was applied to the SCF and the other was to the HES method. After the separation, both CB samples were cryopreserved in liquid nitrogen for more than one week, and infusions were performed after thawing and washing with 2.5% dextran 40/1.25% human serum albumin. We infused $1x10^6$or $5x10^6$ CB cells into NOD/SCID mice irradiated with 350cGy through tail vein. Mice were sacrificed after 60 days after transplantation and bone marrow was examined for the engraftment of human cells after flow cytometric analysis (CD45, CD3, CD8, CD13, CD14, CD19, CD33 and CD34) and hematopoietic progenitor assay. Survival rate was not different between the two separation methods. All survived mice had detectable human $CD45^+$ cells. Mice transplanted with CB cells, separated by SCF, showed similar degrees of engraftment of human $CD45^+$ cells as compared with mice transplanted with CB cells separated by HES method. All engrafted mice showed evidence of human B cells, myeloid/monocytic cells and $CD34^+$ cells. There was no difference of the lymphohematopoietic reconstitution pattern within the $CD45^+$ population, total numbers of CFC recovered from BM between the two separation methods. These results indicate that the SCF can recover the similar levels of hematopoietic stem cell as compared with HES method. These data assure the safety of the SCF for cord blood processing for clinical use.

3. Phase I clinical study of autologous monocyte-derived dendritic cell therapy for STAGE IV malignant melanoma patients in Japan

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Object; To clarify the feasibility and efficacy of administering autologous monocyte-derived dendritic cells (DCs) for stage IV malignant melanoma patients. Methods; 10 eligible patients were entered to our study after signed informed consent and recommendation of Institutional Review Board. Autologous monocytes were harvested from 15l of pheresis products, and cultured with GM-CSF and IL-4 for 7days, then pulsed with autologous tumorlysate. After terminal differentiation using TNF-a for 4days, 107 of DCs were weekly vaccinated for ten times intradermally. Results; Only 1 patient was re-

sponded to our therapy, other two of ten patients were stable disease, and 7 patients showed disease progression. *In vitro* examination of cultured DCs revealed dysfunctional surface expression of CD1a, CD86, and HLA-DR molecules compared to normal volunteer donor-derived DCs. Conclusion; Further evaluation is needed for dysfunctional differentiation capacities of monocyte-derived DCs from melanoma patients.

4. Dendritic cells derived from umbilical/placental cord blood monocytes have more potential functions than those derived from peripheral blood monocytes

Makoto Enomoto and Tsuneo A. Takahashi

Dendritic cells (DCs) are professional antigen presenting cells that are required for the initiation of the immune response. DCs are generated from peripheral blood (PB) monocytes, or hematopoietic stem cells of bone marrow (BM), umbilical/placental cord blood (CB), or PB. However, little is known about the functional properties of CB monocyte-derived DCs (CBMo-DCs). In this study we examined the functional properties and activities of CBMo-DCs as compared with PBMo-DCs. Immature CBMo-DCs were generated in cultures containing granulocyte/macrophage colony stimulating factor (GM-CSF; 50ng/ml) and interleukin-4 (IL-4; 50ng/ml). These cells have a typical dendritic morphology and high endocytosis capacity for fluorescein-conjugated dextran (FITC-Dextran), and migration activity to inflammatory chemokines at the same levels of immature PBMo-DCs. On the other hand, the expression levels of CD86 and HLA-DR on the immature CBMo-DCs revealed high levels (CD86; 910±31, HLA-DR; 1150±28 at each MFI volume) compared to the expression levels of these markers on PBMo-DCs (CD86; 430±51, HLA-DR; 916±36). Furthermore, immature CBMo-DCs have a significant increase ($p<0.05$) of allogeneic T cell stimulatory capacity in comparison to the capacities of immature PBMo-DCs. These immature CBMo-DCs treated with tumor necrosis factor-alpha (TNF-a; 50ng/ml) for 4 days resulted in a slightly higher average expression of CD86 and HLA-DR than mature PBMo-DCs. Otherwise, mature CBMo-DCs further increased allogeneic T cell stimulatory capacity significantly than mature PBMo-DCs. These results indicate that CBMo-DCs appear to have more active and functional properties as compared with PBMo-DCs.

5. Signaling events following chemokine receptor ligation in human dendritic cells at different developmental stages

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Responsiveness of dendritic cells (DC) to inflammatory CC chemokines is down-regulated during their maturation. We analyzed the mechanism underlying these events. Cell-surface expression of CC chemokine receptor (CCR)-1, -3 and -5 was increased during differentiation of immature DC (iDC) from monocytes. In contrast, these expressions were decreased during development of iDC into mature DC (mDC) to levels similar to those of monocytes. Transcriptional expression of CCR-1, -3 and -5 was increased during differentiation of iDC from monocytes, while the expression was decreased during development of iDC into mDC. Expression of CCR-7 transcript was detected in mDC, but not in monocytes or iDC. Both monocytes and iDC, but not mDC, migrated in response to inflammatory CC chemokines such as regulated on activation normal T cell expressed and secreted (RANTES)/CCL5, whereas mDC, but not monocytes or iDC, migrated to macrophage inflammatory protein (MIP)-3ss/CCL19. Receptor engagement of monocytes or iDC by RANTES (for CCR-1, -3 and -5) resulted in protein tyrosine phosphorylation events including activation of focal adhesion kinase as well as mitogen-activated protein kinase, whereas this stimulation induced little activation of these molecular events in mDC when compared with monocytes or iDC. On the other hand, stimulation with MIP-3ss (for CCR-7) induced tyrosine phosphorylation events in mDC, but not in monocytes or iDC. These results suggest that the down-regulation of cell-surface expression of CCR and of their downstream signaling events may be involved in the reduced chemotaxis of DC to inflammatory CC chemokines during their maturation.

6. Chemokine Receptor Expressions and Responsiveness of Cord Blood T Cells

Katsuaki Sato, Hiroshi Kawasaki[6], Hitomi Nagayama, Makoto Enomoto, Chikao Morimoto[6], Kenji Tadokoro[7], Takeo Juji[7] and Tsuneo A. Takahashi

Chemokines and their receptors play a critical role in the selective attraction of various subsets of leukocytes. We examined the chemokine receptor expressions and responsiveness of cord blood (CB) T cells. Flow-cytometric analysis revealed that peripheral blood (PB) T cells expressed CCR-1, CCR-2, CCR-5, CCR-6, CXC chemokine receptor-3 (CXCR-3), and CXCR-4, while CB T cells expressed only CXCR-4 on their surface. Chemotactic migratory re-

sponse of CB T cells to macrophage-inflammatory protein (MIP)-1α, monocyte chemoattractant protein-1, RANTES, MIP-3α, monokine induced by IFN-γ, and IFN-γ -inducible protein-10 was significantly impaired compared with those of PB T cells. In contrast, the ability of CB T cells to migrate to MIP-3β, 6Ckine, and stromal cell-derived factor-1α was greater than that of PB T cells, and these events were correlated with the expression levels of CCR-7 and CXCR-4, respectively. Engagement of CD3 and CD28 specifically up-regulated CXCR-3 expression and chemotaxis to monokine induced by IFN- and IFN-γ-inducible protein-10, whereas this stimulation down-regulated CCR-7 expression and chemotaxis to MIP-3β and 6Ckine in PB T cells, but not in CB T cells. These results suggest that PB T cells and CB T cells exhibit distinct chemokine responsiveness via different chemokine receptor repertoire.

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Division of Hematopoietic Factors (Chugai)

Our major projects are (1) investigation of signal transduction of cytokine receptors, and (2) identification and characterization of novel cytokines, cytokine receptors, and transcription factors.

Division of Hematopoietic Factors was established in September 1996. In the lab, we apply a retrovirus-mediated expression screening system in a variety of ways to study hematopoiesis, signal transduction and etc. Our goals are (1) to identify and characterize oncogenic forms of signaling molecules and to relate these to *in vivo* leukemogenesis, (2) to clone novel cytokines, cytokine receptors, and transcription factors using retrovirus-mediated expression cloning strategies, and (3) to develop various retrovirus vectors and packaging cell lines.

1. Characterization of constitutively active forms of signaling molecules

Tetsuya Nosaka, Kouichi Ariyoshi, Toshiyuki Kawashima, Kazuhide Misawa, Koji Yamada, Ken Murata, Fumi Shibata, Mayumi Onishi[1], Atsushi Miyajima[2], and Toshio Kitamura: [1]Third Department of Internal Medicine, The University of Tokyo, and [2]The Institute of Molecular and Cellular Biosciences, The University of Tokyo

We combined retrovirus-mediated screening system and PCR-driven random mutagenesis to identify activating mutations in cytokine receptors, kinases, and other signaling molecules. As a first target, we chose the receptor for thrombopoietin MPL. We introduced random mutations into the MPL sequence followed by retrovirus-mediated screening for constitutively active forms of MPL. In this way, we identified the mutant MPL which can induce autonomous proliferation of IL-3-dependent cell lines. The mutant MPL harbored a point mutation in the transmembrane domain, and constitutively activated the Ras-Raf-MAPK and Jak (Janus kinase)-STAT (signal transducer and activator of transcription) pathways. In addition, Ba/F3 cells expressing the mutant MPL were oncogenic in syngeneic mice. Using the same method, we also identified the constitutively active form of STAT5A and STAT5B. Two mutations, one (H298R) in the DNA binding domain and the other (S710F) in the effector domain were required for the constitutive activity of STAT5s. The active STAT5 mutants showed constitutive tyrosine phosphorylation, nuclear localization, and transcriptional activation, and were able to induce factor-independency of IL-3-dependent cell lines. Interestingly, the mutant STAT5s 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 STAT5s by expressing each target gene of STAT5 using a bicistronic retroviral vector having IRES-GFP. Pim-1, $p21^{WAF1/Cip1}$, and SOCS1 (suppressor of cytokine signaling 1) were found to be responsible for inducing proliferation, differentiation, and apoptosis, respectively. In addition, we have identified another constitutively active STAT5 mutant which harbors a single point mutation in the SH2 domain (N642H) that shows the same phenotype as the STAT5 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 STAT5 as was the case for the wild type. These findings demonstrate that different mutations rendered STAT5 constitutively active, through a common mechanism, which is similar to that of physiological activation.

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

Tetsuo Kojima, Keishi Fujio, Takuya Sugiyama, Hiromichi Tsuruga, Kazuhide Misawa, Yuichi Ikeda, Sumiyo Morita, Tsuguteru Ohtsubo, Fumi Shibata, Hidetoshi Kumagai[3], Wing-Chun Bao[3], Sizhou Feng[3], Kaori Tamitsu[3], Yoshihiro Morikawa[4], Tetsuya Nosaka, and Toshio Kitamura:[3]Takada Research Labs, Chugai Pharmaceutical Co., Ltd., [4]Department of Neurobiology and Anatomy, Wakayama Medical School of Medicine

Secreted and cell-surface proteins play essential roles in cell-cell interaction. So far, two methods to isolate cDNAs with signal sequences that encode such proteins have been established. We have recently 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 receptor for thrombopoietin (MPL) were introduced 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; infection of 5 ml of library retroviruses readily detected 150 known and 48 novel cDNA clones, and all the known cDNA clones were found to encode secreted and cell-surface proteins. In addition, type II membrane proteins, which had never been isolated by the previous SST methods, were also obtained by our SST-REX.

We are now searching for novel secreted proteins such as cytokines and membrane proteins such as cytokine receptors in various cells and tissues including hemopoietic stem or progenitor cells, Th1 and Th2 cells, neuronal cells, adipocytes, mast cells, and cardiocytes. Three novel receptors (one type I cytokine receptor similar to common γ chain, designated as δ1; one TNF receptor-like molecule; and one novel low-density lipoprotein receptor-related protein) and a member of TNF superfamily have been identified by this method and functionally characterized. We are also making the δ1-knockout mice.

3. A novel method FL-REX to identify cDNAs by the localization of their protein products

Kazuhide Misawa, Tetsuya Nosaka, Sumiyo Morita, Azusa Kaneko[5], Tatsutoshi Nakahata[5], Shigetaka Asano[6], and Toshio Kitamura:[5]Department of Clinical Oncology, Institute of Medical Science, The University of Tokyo, [6]Department of Hematology-Oncology, Institute of Medical Science, The University of Tokyo

We previously established a high-efficiency retrovirus-mediated expression cloning method. Using this system, we have now developed a novel expression cloning method (FL-REX; fluorescence localization-based-retrovirus mediated expression cloning) in which cDNAs can be isolated based on the subcellular localization of their products. We make cDNAs using random hexamer and fuse them to 5' end of the cDNA of green fluorescent protein (GFP) in the pMX retrovirus vector to construct a cDNA-GFP fusion retrovirus library. The derived retroviruses are then infected to NIH3T3 cells to identify a cDNA of interest based on the subcellular localization of its protein product such as nucleus, nucleoli, Golgi apparatus, and cell surface. Using this strategy, we have identified a novel zinc finger protein from fetal mouse liver cells which contain hematopoietic progenitor cells.

4. Development of retrovirus vectors and packaging cell lines

Sumiyo Morita, Tetsuo Kojima, Fumi Shibata, Tetsuya Nosaka, Carol Stocking[7], Wolfam Ostertag[7], and Toshio Kitamura:[7]Heinrich-Pette Institute, Hamburg University

We previously developed an efficient retrovirus vector pMX which is suitable for library construction, and was based on MuLV. 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 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 derived from the packaging constructs. We also compared the strength of various promoters including MuLV LTR, SV40, SRα, CMV, TK, and EF-1α in 293T cells and 293 cells, and found that the CMV and the EF-1α promoter worked best in 293T cells, and the EF-1α promoter worked best in 293 cells. We chose the EF-1α promoter 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 (RCRs) 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 are currently establishing a new packaging cell line for efficient infection to human hematopoietic stem cells by using *env* gene of feline endogenous retrovirus RD114.

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

Tetsuya Nosaka, Sumiyo Morita, Fumi Shibata, Yuichi Ikeda, Yuki Kataoka[8], Issei Komuro[9], Nobuaki Yoshida[8], and Toshio Kitamura: [8]Laboratory of Gene Expression & Regulation, Center for Experimental Medicine, Institute of Medical Science, The University of Tokyo and [9]Department of Cardiovascular Medicine, University of Tokyo Graduate School of Medicine

In the search for soluble factors which are expressed in the mouse AGM (aorta-gonad-mesonephros) 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 are generating TSG-deficient mice. We are also studying the role of TSG in cardiogenesis by using the system of *in vitro* differentiation of embryonic carcinoma cells into beating cardiomyocytes.

6. Molecular cloning and functional characterization of a human novel type I cytokine receptor related to Delta1

Yukio Tonozuka, Keishi Fujio, Takuya Sugiyama, Yuichi Ikeda, Tetsuya Nosaka, Momoki Hirai[10], and Toshio Kitamura:[10]Department of Integrated Biosciences, Graduate School of Frontier Sciences, Tne University of Tokyo

We have isolated a novel human type I cytokine receptor similar to δ1, based on the sequence homology search, and tentatively named this gene CRLF-2. CRLF-2 was expressed in liver, kidney, heart, and skeletal muscle. The gene for *crlf-2* was mapped to the pseudoautosomal region, Xp22.3 and Yp11.3 on which α chains of the IL-3 receptor and GM-CSF receptor are also located. We are now doing the functional cloning of the gene for the ligand to CRLF-2.

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

Toshiyuki Kawashima, Koichi Hirose, Takaya Satoh[11], Yoshito Kajiro[11], Tetsuya Nosaka, and Toshio Kitamura:[11]Faculty of Bioscience and Biotechnology, Tokyo Institute of Technology

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.

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

Ken Murata, Hidetoshi Kumagai[3], Tetsuya Nosaka, and Toshio Kitamura

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 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. We are currently testing the effect of the newly identified molecule on the leukemic cell growth *in vivo* using mice.

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Division of Genetic Diagnosis (Otsuka) (1)

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 identify susceptibility genes for common or otherwise clinically relevant diseases of metabolism such as asthma, hypertension, and intracranial aneurysm and analyze the molecular causality.

Although genetic and environmental factors play equally crucial roles in the pathogenesis of the common diseases of civilization, genetic factor is directly involved in the causality and molecular mechanism. The elucidation of molecular etiology provides specific molecular targets for therapeutic drugs even at the individual level. Thus our priority in basic research is analysis of the molecular causality of the common metabolic disorders of civilization, in other word identification of the causal gene. We will identify individual and group polymorphisms in the genome relevant to the treatment of individual patients closely related to susceptibility to disease, prognosis of disease, and responses to drugs. Our laboratory should establish personalized medicine in which prevention, diagnosis, prognosis, and treatment of a patient is determined by the patient's individualized genomic information. Our diseases of current interest are asthma, essential hypertension, subarachnoid hemorrhage, and ossification of the posterior longitudinal ligament of the spine. To determine genetic susceptibilities we are using genetic approaches such as linkage studies with affected sib-pairs, association studies using SNP data-base, which are currently being established in the Institute of Medical Science. The etiological heterogeneity and multifactorial nature of these common diseases challenge our understanding at all levels. We are currently in a gene-discovery stage.

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

Shingo Maeda, Kozo Furushima, Toshihiro Tanaka, Kazuki Shimo-onoda, Takahiro Nobukuni, Toshiaki Nakajima, Ituro Inoue

Ossification of the posterior longitudinal ligament of the spine (OPLL) is a common disorder among Japanese and other Asian populations, with a reported prevalence of 1.9-4.3 % of the general population over age 30 years. Heterotopic ossification of the spinal ligament is the specific feature of OPLL, which compresses the spinal cord and leads to various degrees of myelopathy. In spite of the late-onset nature of the disease, it is well known that genetic determinants play an important role in OPLL. The possibility that OPLL has strong genetic determinants is supported by several lines of evidence, including the high rate of concordance among MZ /DZ twins and the increased recurrence risk in siblings, 10 times the risk in the general population. OPLL is considered to be a state of hyperostosis. Previously, we have provided genetic linkage evidence in Japanese affected sib-pairs showing that the genetic locus for OPLL is within or near the HLA region of chromosome 6. Two genes in the region, the collagen α2(XI) (*COL11A2*) and retinoic X receptor β (*RXRb*) genes are considered candidate genes for OPLL, and the patients have been extensively screened for molecular variants. In *COL11A2*, a strong evidence of allelic association to OPLL was observed with a T to A nucleotide substitution at position -4 from the acceptor site in intron 6 (denoting intron 6 (-4A)), suggesting a certain functional role of this polymorphism in the pathogenesis of OPLL. In the *RXRβ*, three polymorphisms were detected, two of them showing positive associations with OPLL. However, these polymorphisms were in linkage disequilibrium with the intron 6 (-4A) of the *COL11A2*, and showed less significant allelic association to OPLL than did the intron 6 (-4A). Together, it is strongly suggested that

the intron 6 (-4A) of the *COL11A2* constitutes a genetic determinant of OPLL. Despite these genetic findings, determining the involvement of the *COL11A2* in the etiology of OPLL is hampered by the lack of evidence regarding the functional significance of the *COL11A2* polymorphisms. We also identified a polymorphism in exon 6, which is in linkage disequilibrium with the intron 6 (-4A). We examined to find if the polymorphism in either intron 6 or exon 6 has potential impact on alternative splicing of the *COL11A2* transcript that may be part of the molecular etiology of OPLL. We report here functional impact of the intron 6 polymorphism of the *COL11A2* that results in altered splicing in the region containing exons 6-8. These exons encode an acidic subdomain, also called a variable region because of the complicated alternative splicing that depends on cell type or developmental stage. The consequence of the altered splicing due to the intron 6 (-4A), especially the existence of exon 7, may be part of the biological mechanism by which individual differences in the *COL11A2* are protective in the development of ectopic ossification in the cervical ligament.

OPLL is a complex disorder, thus *COL11A2* might not be the sole genetic causality. Currently we perform genome-wide linkage analysis with 140 sib-pairs of OPLL to systematically detect the genetic loci. Evidence of linkage was observed with several chromosomes, and precise mapping by linkage disequilibriium analysis with SNPs are underway.

2. Subarachnoid hemorrahge (SAH) due to rupture of intracranial aneurysm (IA)

Taku Yoneyama, Toshiaki Nakajima, Ituro Inoue

The rupture of an intracranial aneurysm (IA) causes a subarachnoid hemorrhage (SAH) that is a catastrophic event with high morbidity and mortality. Large autopsy studies of unruptured incidental IA among adults showed the prevalence of 0.8 to 6 percent and angiographic studies of them demonstrated the prevalence of 0.5 to 2.7 percent. The annual incidences of aneurysmal SAH were 10 to 20 per 100,000 people. Approximately 10 percent of patients died before receiving medical attention and 40 percent of hospitalized patients died at one month after the event. Aneurysmal SAH is still a serious public health problem despite diagnostic, medical, and surgical advances over past several decades. The etiology and pathogenesis of IA have not been elucidated and thought to be multifactorial. Because the incidence of aneurysmal SAH increased with age and IA was very rare in children, acquired factors, such as cigarette smoking and hypertension, play an important role in the etiology of IA. On the other hand, the role of genetic factors is considerable because IA is associated with heritable connective-tissue diseases and their familial aggregation. The heritable connective-tissue disorders related with IA are autosomal dominant polycystic kidney disease, Ehlers-Danlos syndrome type IV, and Marfan's syndrome, but the mechanisms of the aneurysmal formation are not known in these diseases. The risk of a ruptured IA in first-degree relatives of patients with aneurysmal SAH is four times higher and the risk in siblings is six times higher than the general population. A segregation analysis revealed that the inheritance pattern of IA does not follow simple mendelian model and genetic heterogeniety was important feature. The purposes of our research are to specify chromosomal regions linked to IA by a genome-wide search using an affected sib-pairs linkage analysis and to identify the gene associated with IA by case-control design together with haplotype analysis among Japanese subjects.

3. Molecular variants and haplotype analysis in the human angiotensinogen gene

Toshiaki Nakajima Ituro Inoue

For most common diseases having a complex multifactorial etiology, research is still in the gene-discovery stage. One recent suggestion has been to develop a dense single nucleotide polymorphism (SNP) map, because of their high frequency within the genome and ease of genotyping. Gene mapping by association studies explicitly depends upon the persistence of linkage disequilibrium (LD). The ability to detect association between marker alleles and disease depends critically on the nature of LD between disease alleles and surrounding markers and the strength of LD will affect the magnitude of an association. However, little information is available on the distribution of LD in the human gene. To understand the organization of LD in the human gene promises to have a great impact on the strategies for identifying candidate genes for common diseases. Linkage and association studies have shown that molecular variants in the human angiotensinogen gene (*AGT*) constitute inherited predisposition to essential hypertension. We have reported that a molecular variant in the proximal promoter of *AGT*, an adenine instead of a guanine at six nucleotide upstream from the transcription initiation site, was significantly more common in hypertensive subjects than in normotensive controls and associated with a 10%-20% increase in plasma angiotensinogen. We determined the complete genomic sequence of human *AGT* and performed the contiguous scan (14.4 kb) for sequence variations in human *DNA*. Forty-two SNPs and a dinucleotide repeat were identified. We evaluated the distribution of LD and performed haplotype analysis based on fourty-two SNPs. Those data revealed that the complexity of the organization of LD in the human *AGT*.

Haplotype analysis provided an evidence that one main possible cause of the complexity of the organization of LD in the human *AGT* is due to admixture of populations sharing same haplotype in the prehistoric age.

4. Association Studies of Single Nucleotide Polymorphisms (SNPs) in Candidate Genes for Bronchial Asthma

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Bronchial asthma (BA), one of the most common of all chronic inflammatory diseases in human populations, is considered to be 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. While genetic variations involve insertion, deletion or base substitution, we chose to examine single-nucleotide polymorphisms (SNPs) of one-base substitution because they are the most abundant genetic variation in the human genome. 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.

5. Characterization of the NOA mouse, an animal model for atopic dermatitis

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The NOA (Naruto Research Institute Otsuka Atrichia) mouse is an animal model for allergic or atopic dermatitis, a condition characterized by ulcerative skin lesions with an accumulation of mast cells and increased serum IgE. The mode of inheritance was autosomal recessive with incomplete penetrance. We reported earlier that a major gene responsible for dermatitis of the NOA mouse locates in the middle of chromosome 14, and that the incidence of disease clearly differed according to parental strain. We searched for loci that might modify the NOA phenotype, and identified two candidate loci that appeared to contain genes capable of suppressing atopic or allergic dermatitis; one in the middle of chromosome 7 (χ^2 =14.66, P=0.00013 for D7Mit62) and the other in the telomeric region of chromosome 13 (χ^2=15.34, P=0.000089 for D13Mit147). These loci correspond to regions of synteny in human chromosomes where linkages to asthma, atopy, or related phenotypes such as serum IgE levels have been documented.

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Division of Genetic Diagnosis (Otsuka) (2)

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 will lead to development of myeloid disorders such as bone marrow failures and myeloid malignancies. To approach this subject, we study pathogenesis of "Fanconi anemia", a genetic disease characterized by chromosome instability and myeloid disorders.

1. Studies of molecular pathogenesis of Fanconi anemia

T. Yamashita, T. Oda, H. Yagasaki, and D. Adachi

Fanconi anemia (FA) is an autosomal recessive disease characterized by congenital anomalies, bone marrow failure, and susceptibility to malignancies, especially leukemia. FA cells show chromosome instability and hypersensitivity to DNA cross-linking agents such as mitomycin C. Recent studies indicate that there are at least eight genetically distinct groups (A, B, C, D1, D2, E, F, G). To date, six genes (for groups A, C, D2, E, F and F) have been cloned. Increasing evidence indicates that the multiple FA proteins cooperate in a biochemical pathway and/or a multimer complex (Fig. 1).

We previously reported that FANCA was phosphorylated in cells from normal controls, whereas the phosphorylation was defective in cells from multiple FA patients. Our recent studies revealed that wild-type FANCA protein, exogenously expressed in FANCA(-) cells, was phosphorylated, whereas phosphorylation of patient-derived forms of FANCA, containing missense mutations in the carboxy terminal region, was abrogated. A physiological protein kinase for FANCA (FANCA-PK) is likely to bind and phosphorylate the substrate. Furthermore, FANCA-PK as well as phosphorylated FANCA was contained in the FANCA/FANCG complex. Taken together, these results suggest that FANCA-PK is another component of the FA protein complex and regulates the function of FANCA. We characterized FANCA-PK as a cytoplasmic serine kinase sensitive to wortmannin. To identify phosphorylation sites of FANCA, we are trying to make a number of deletion mutants and examine phosphorylation of these mutant proteins. At the same time, we are testing possibilities that two protein kinases, Sgk and Mnk, which were found to interact with FANCA in a yeast two-hybrid system.

A downstream component of the FA pathway, FANCD2, has recently been shown to be ubiquitinated in response to DNA damage, and translocates to nuclear foci containing BRCA1, a breast cancer susceptibility gene product, suggesting a role for this protein in DNA repair functions. Importantly, the ubiquitination and subnuclear localization of FANCD2 seem to depend on a complex formation of other multiple FA proteins, thus providing a specific functional test for the FA pathway. It is possible that dysfunction of the FA pathway is involved in development of acquired hematopoietic diseases and solid tumors. To address this question, we are now analyzing FANCD2 ubiquitination in multiple leukemia and tumor cells.

2. Cloning and functional analyses a novel SH2-containing adapter protein predominantly expressed in hematopoietic cells

T.Oda, M. Muramatsu[1], T. Isogai[1], Y. Masuho[1] and T. Yamashita :[1] Helix Research Institute

We cloned cDNA encoding a novel SH2 domain-containing protein from a human placenta cDNA library. Northern blot analysis and RT-PCR analysis revealed that its transcript was predominantly expressed in hematopoietic cells, so this protein was termed hematopoietic SH2 protein (HSH2). This protein is composed of 352 amino acids (Mr=47 kD) and has four proline-rich motifs.

The SH2 domain of HSH2 fused to GST (GST-SH2/HSH2) pulled down several Tyr-phosphorylated proteins from cell lysates of IL-3 stimulated cells. Among these proteins, we identified 130 kD protein as the beta common subunit of the IL-3/GM-CSF receptor, and 70kD protein as a tyrosine phosphatase SHP2. In addition, the GST-SH2/HSH2 pulled down a number of Tyr-phosphorylated proteins including BCR-ABL from lysates of K562 cells. Furthermore, the SH2 domain of HSH2 binds to BCR-ABL but not to kinase-deficient BCR-ABL in a yeast two-hybrid assay. These results suggest that the SH2 domain of HSH2 has a potential to bind to the activated IL-3/GM-CSF receptor complex and BCR-ABL.

We found that HSH2 interacts with two tyrosine kinases, ACK1 and c-FES in a yeast two-hybrid system and confirmed these interactions in mammalian cells. ACK1 is a non-receptor tyrosine kinase belonging to the focal adhesion kinase family and binds to a GTP-bound form of Cdc42, a Rho-family GTPase protein, which is important for the regulation of cytoskeletal reorganization and membrane ruffling. An HSH2 mutant lacking a proline rich region at the N-terminus failed to bind to ACK1, indicating that HSH2 interacts with the SH3 domain of ACK1 through the proline-rich motif. c-FES is primarily expressed in myeloid cells and is likely to regulate differentiation, proliferation, and function of these cells. The C-terminus of HSH2, which does not contain the SH2 domain binds to c-FES more strongly than the N-terminus containing the SH2 domain.

Taken together, these results suggest that HSH2 appears to be an adapter protein which regulates tyrosine kinases signaling and the Cdc42 machinery. Thus, this protein may play an important role in the regulation of cell growth, locomotion, adhesion and transformation in hematopoietic cells.

3. Cloning and functional analyses of ABTAP, a novel repressor of basal transcription

T. Oda, Y. Masuho[1], M. Muramatsu[1] and T. Yamashita

We recently cloned cDNA encoding a novel TATA-binding protein-binding protein, ABT1 and showed that this protein activates basal transcription. In the current study, we cloned cDNA encoding a novel nuclear protein binding to ABT1 (ABT1-asso-

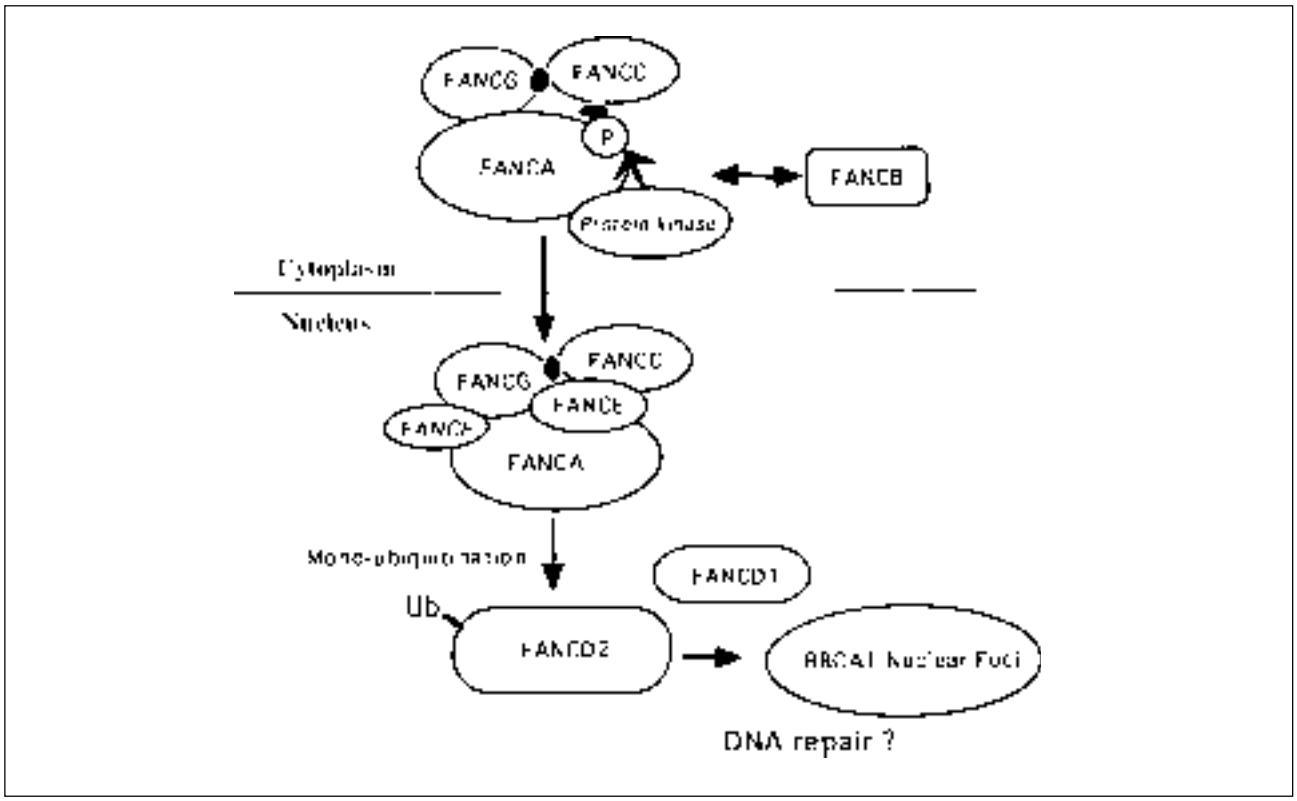

Fig. 1 A hypothetical model of the Fanconi anemia pathway

ciated protein, ABTAP) by yeast two hybrid screening from rat brain cDNA library. ABTAP mRNA was ubiquitously expressed in rat tissues as an approximately 4.0 kb transcript. Sequence analysis and searching in the public database showed that ABTAP had homologous counterparts in yeast, nematode and fly. A yeast ABTAP homolog as well as an ABT1 homolog is essential for growth. An ABT1-binding region of ABTAP localizes between a.a.284 and a.a.585, which is strikingly conserved among different species. Co-expression of ABT1 and ABTAP fused with distinct fluorescent proteins in COS7 cells showed that these two co-localized in the nuclei. Finally, a luciferase reporter gene assay showed that ABTAP repressed the transcriptional activation caused by ABT1. These results suggest that ABT1 and ABTAP form a complex in the nuclei and that the complex is evolutionary conserved to regulate transcription in eukaryotes.

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Division of Gene Regulation

The goal of Division of Gene Regulation is to elucidate the molecular mechanisms of gene expression involved in cellular proliferation, differentiation and transformation as well as development and tumor formation. We design proteins that enhance or suppress specific steps in signal transduction pathways and also develope several new types of high-titer retrovirus vectors, which are used to estimate the biological activities of these designed proteins in cell cultures, organ cultures, embryos or adults of experimental animals and in the cell lines originated from human solid tumors.

1. Functional analysis of endogenous AP-1 -Discovery of mechanistic link between AP-1 and chromatin remodeling

The transcriptional factor, AP-1 (Activator Protein-1) complex comprises members of the Fos (c-Fos, Fra-1, Fra-2 and FosB) and Jun (c-Jun, JunB and JunD) protein families. Jun family members can form low-affinity dimers among themselves and high-affinity heterodimers with the Fos family components, whereas Fos-related proteins do not form stable dimers among themselves. Although these hetero- and homodimers bind to similar DNA binding sites (TGAC/GTCA, AP-1 binding sites), we have previously shown that each kind of heterodimer has distinct the transcriptional activity. For example, transcriptional activity of c-Fos/c-Jun complex is much higher than Fra-2/c-Jun complex. While functional interactions between some members of Fos/Jun family proteins and adaptor proteins such as CBP or TBP were reported crucial proteins that recognize the dimer specificity and/or facilitate the induction of transcriptional initiation were largely unknown. This year, we have demonstrated that BAF60a, a component of SWI/SNF chromatin remodeling complex can select specific Fos/Jun dimers and function as a major determinant of transcriptional activation via AP-1 binding sites (**a**).

We previously showed that the activation of endogenous AP-1 is important for transformation induced by v-*src*, as well as v-*yes*, v-*fps*, c-Ha-*ras*, activated c-*raf*. We further indicated that positive autoregulatory loop of *fra*-2 is triggered by activation of endogenous MAP kinase in v-*src* transformed CEF (chicken embryo fibroblasts) and this autoregulatory loop is mainly responsible for the activation of endogenous AP-1 in these cells. This year, we indicated that activation of endogenous AP-1 is also important for the oncogenic potential in several cell lines originated from human solid tumors by showing that a dominant negative mutant, *supjun*D-1, which we previously designed, efficiently suppresses the anchourage-independent growth (**b**). For the introduction of this designed anti-oncogene, we used a VSV-G pseudotype retrovirus vector system, which is developed by our group in these four years.

a. Identification of SWI/SNF complex subunit BAF60a as a determinant of transactivation potential of Fos/Jun dimers

Taiji Ito, Mai Yamauchi, Mitsue Nishina, Nobutake Yamamichi, Taketoshi Mizutani, Motoyasu Ui, Masao Murakami, and Hideo Iba

Fos family proteins form stable heterodimers with Jun family proteins and each heterodimer shows distinctive transactivating potential regulating cellular growth, differentiation, and development via AP-1 binding sites. However the molecular mechanism underlying dimer specificity and the molecules that facilitates transactivation remain undefined. Here

we show BAF60a, a subunit of SWI/SNF chromatin remodeling complex, is a determinant of transactivation potential of Fos/Jun dimers. BAF60a binds to a specific subset of Fos/Jun heterodimers using two different interfaces for c-Fos and c-Jun, respectively. Only when functional SWI/SNF complex is present, c-Fos/c-Jun (high affinity to BAF60a) but not Fra-2/JunD (no affinity to BAF60a) can induce the endogenous AP-1-regulated genes such as collagenase and c-met. These results indicate that a specific subset of Fos/Jun dimers recruits SWI/SNF complex via BAF60a to initiate transcription.

b. Endogenous AP-1 levels necessary for oncogenic activity are higher than that sufficient to support normal growth

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We investigated the role of endogenous AP-1 in human tumor cell lines by introducing SupJunD-1, a dominant-negative mutant of AP-1, using vesicular stomatitis virus G protein (VSV-G)-pseudotyped retrovirus vectors. Single inoculation of six human tumor cell lines, originating from osteosarcomas, non-small lung cell carcinomas or cervical carcinomas, with recombinant SupJunD-1 virus at a high multiplicity of infection readily inhibited colony formation in soft. We detected no significant changes in expression levels of AP-1 components c-Jun or Fra-1, adhesion molecules CD44 or E-cadherin, or cell cycle regulator p53, which are encoded by genes previously reported to be under the control of AP-1 in some mouse or human cell lines. By varying the dosage of VSV-G-pseudotyped retrovirus, we were able to change the proviral copy number of *supjun*D-1 from 1 to approximately 10 and monitor suppression of endogenous AP-1 function as assessed by growth characteristics of the tumor cell lines, we found a SupJunD-1 dosage which significantly suppressed anchorage-independent growth without affecting the cellular growth in monolayer cultures at all. We conclude that endogenous AP-1 levels necessary for oncogenic activity are much higher than that sufficient to support normal growth.

2. Analysis of cell-cell or tissue-tissue interactions using unique culture systems and retrovirus vectors

We could regard tumor formation and metastasis as "cellular escape from normal tissue-tissue interactions that is accompanied with their transition of cell type specificity". For the analysis of cell-cell or tissue-tissue interactions, we have currently developed new biological assay systems that involves the induction of ectopic expression in a specific region of embryonic tissues, micromass cultures (**a**) or organ cultures (**b**). The discoveries from these experiments as well as the results of the biological effects of the ectotic expression in adult would enclose our understanding on the formation and maintenance of tissues and organs in a feed back manner and would provide new concept on the tumor formation.

a. Developmental patterning in chondrocytic cultures by morphogenic gradients: BMP-2 induces expression of *Indian hedgehog*, *Noggin* and subsequently BMP-6

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The *Indian Hedgehog* (*Ihh*) gene is expressed in prehypertropic chondrocytes and has been proposed to regulate chondrocyte maturation. While such secretary factors as PTHrP and BMP are thought to be involved in *Ihh* expression, the mechanism of the restricted expression of *Ihh* is not clear. Using primary chondrocytes, we have developed a modified micromass culture (MM-C) system that allows the formation of concentration gradients of secreted factors, expressed either autonomously or retrovirally, from each of plural micromass cultures on a single plate. Using this system, we detected autonomous inhibitory factors for *Ihh* induction. We also showed that retrovirally induced BMP-2 induces expression of both *Ihh* and *Noggin* (encoding BMP-inactivating protein), and we further present evidence that a negative-feedback loop involving Noggin might account for the precise localization of BMP signaling for *Ihh* induction.

Furthermore we show that, upon longer culture, secreted BMP-2 subsequently promotes the maturation step as judged from the induction of t*ype X collagen* and *BMP-6* expression, which are known to be detectable in the later phase of cartilage maturation. Induction of all of these genes by secreted BMP-2 was not inhibited by ectopic expression of PTHrP induced by retrovirus vector infection, though the same virus vector showed strong inhibitory effects on the expression of type X collagen gene or alkaline phosphatase activity in mature chondrocytes. These results suggest that the maturation-promoting activity exhibited by BMP-2 is dominant over the suppressive effect of PTHrP in immature chondrocytes. When the *BMP-6* gene was introduced into the same virus vector as that used for BMP-2, it induced the same sets of genes (*Ihh*, *Noggin*, type X collagen and endogenous BMP-6) as BMP-2 did. These results also suggest that BMP-6 would autonomously maintain and/or promote the later of chondrocytic maturation.

b. Epithelial-mesenchymal interaction in embryonic digestive tract examined by tissue-restricted ectopic gene expression using retrovirus vectors

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We established here a method to retrovirologically introduce exogenous genes specifically into either epithelium or mesenchyme of organ cultures of chicken digestive tract. This method consists of reconstitution of digestive organs by recombining epithelial and mesenchimal tissues dissected from either virus-sensitive embryos (C/O) or virus-resistant embryos (C/AB) which lack the virus receptors of both A and subgroup B avian retrovirus. The subsequent infection with replication-competent avian-retrovirus into the entire reconstituted organ cultures would allow strictly restricted infection onto tissues originated from C/O embryos.

Virus-mediated BMP-2 overexpression in mesenchyme resulted in increase in numbers of the glands formed, demonstrating that BMP-2 is involved in the gland formation. Moreover, ectopic expression of Noggin, which antagonizes the effect of BMPs, in the proventricular messenchyme led to the complete inhibition of the gland formation, indicating that BMP signals from mesenchyme to epithelium are necessary for the proventricular gland formation. These findings suggest that BMPs are primarily important mesenchymal signals for inducing proventiricular glands. Using this system, we are currently introducing several oncogenes specifically into epithelium to establish model systems for epithelial-mesenchymal transition.

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