Donation Laboratories

Division of Stem Cell Regulation(Amgen)

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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) identification of master regulator(s) for kidney development using animal caps of Xenopus embryos and knockout mice, and 3) 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 working on (1) the mechanism of self-renewal in ES cells, (2) identification of essential genes for kidney development, and (3) identification and establishment of neural stem cells from mouse fetal brain.

1. Self-renewal mechanism of embryonic stem cells

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The self-renewal of ES cells is maintained in the presence of LIF. LIF binds to a cell surface receptor complex composed of LIF receptor and gp130, through which several signaling molecules including ERK and STAT3 are activated. We previously demonstrated that STAT3 activation is sufficient for the self-renewal of ES cells, using a fusion protein composed of the entire region of STAT3 and the ligand binding domain of estrogen receptor (STAT3ER).

To understand the molecular mechanism of the STAT3-dependent self-renewal, we are now searching for a target molecule(s) of STAT3 in ES cells. For this cDNA subtraction and cDNA array analyses were performed 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. Of them, we focused our attention on two proteins, zinc finger protein (Zfp) 57 and embryonic ectoderm development (Eed).

a. Zfp57

Embryonic stem (ES) cells can be maintained in the presence of leukemia inhibitory factor (LIF). Two transcription factors, STAT3 and Oct-3/4, play a crucial role in the self-renewal. To clarify the molecular mechanism of the self-renewal, we performed DNA chip analysis to screen a gene(s) whose expression was decreased upon differentiation and yeast two-hybrid screening to isolate Oct-3/4-interacting proteins.

By DNA chip analysis, we previously obtained zinc finger protein (Zfp) 57 and confirmed by Northern- and Western-blot analyses that this protein is expressed specifically in the undifferentiated state. In the present study, we examined if the expression of Zfp57 is regulated by STAT3 and/or Oct-3/4. Forced expression of a dominant-negative STAT3 mutant in ES cells decreased the level of Zfp57 mRNA. On the other hand in ZHBTc4 ES cells where tetracycline (Tet) leads to the down-regulation of Oct-3/4, the Tet induced no drastic change of Zfp57 mRNA expression. These results indicate that Zfp57 is not a target gene of Oct-3/4, but of STAT3. In addition, we showed that the POU domain of Oct-3/4 interacted with the zinc finger region of Zfp57. Next, to perform a reporter assay using a promoter region of Rex-1, a target gene of Oct-3/4, we constructed reporter plasmids carrying five repeats of the RoxOct element that consists of binding sites for Oct-3/4 and uncharacterized Rox-1. Transcriptional activity of Oct-3/4 was enhanced in the presence of Zfp57, and this enhancing activity was not affected even when a mutation was introduced into Rox-1 binding. These results suggest that Zfp57 is not Rox-1 and acts as a co-factor of Oct-3/4.

From yeast two-hybrid screening, we obtained a nuclear hormone receptor DAX-1 as an Oct-3/4-interacting protein. The interaction was confirmed by co-immunoprecipitaion assay with HEK293 cells. It was also found that the expression of DAX-1 mRNA was specific for the undifferentiated state in ES cells. To clarify whether STAT3 and/or Oct-3/4 regulate the expression of DAX-1, the expression of DAX-1 was determined in two cell-lines, STAT3ER expressing ES cell line, in which 4-hydroxytamoxifen (4HT) stimulation leads to activation of STAT3, and ZHBTc4 ES cell line. As a result, either an inactivation of STAT3 following removal of 4HT or a decrease of Oct-3/4 by Tet stimulation induced a drastic decrease in DAX-1 mRNA expression. These results indicate that the expression of DAX-1 is regulated by both STAT3 and Oct-3/4. It has been reported that the expression of DAX-1 is regulated by nuclear hormone receptor SF1 in gonad and adrenal tissue. However, since expression of SF1 was not detected in ES cells, there may be another mechanism for regulation of DAX-1 expression in ES cells.

We are now trying to disrupt Zfp57 and DAX-1 genes in ES cells.

b. Eed

While it is well-established that the undifferentiated state of mouse embryonic stem (ES) cells can be maintained by LIF, its molecular mechanism is not fully understood. By using Atlas^{TM} cDNA array, we searched for genes that show higher expression in the undifferentiated state of ES cells than the differentiated state. We found that expression of a polycomb group gene, embryonic ectoderm development (eed), was not only reduced during differentiation induced by removal of LIF, but also increased by LIF stimulation. It is well known that the polycomb group genes repress expression of various genes at the organogenesis stage. In this study, we therefore tried to identify a gene(s) whose expression is reduced by eed. Althouth preliminary, we found that ehox might be downregulated by eed. ehox is a novel homeobox gene which facilitates ES cell differentiation into hematopoietic, endothelial or cardiac cells. When we induced the differentiation of ES cells by removal of LIF, expression of eed was reduced, while that of ehox was increased. Moreover, eed-overexpressing ES cells did not produce cardiac cells when their differentiation was induced by forming embryoid body. We are now confirming these results, as well as searching for other genes whose expressions are suppressed by eed. We are also establishing eed-/- ES cells to clarify the role of eed in the maintainance of undifferentiated state of ES cells.

2. Organogenesis – Molecular mechanisms of kidney development

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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 also 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.

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.

3. Neural stem cells

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Neural stem cells can proliferate in response to EGF and FGF-2, exhibit self-maintenance, they retain their multipotency to differentiate into neurons, astrocytes and oligodendrocytes. Our previous study showed that EGF and FGF-2 have different effects on proliferation and differentiation of neural stem cells. Neural stem cells initially responded to FGF-2 only, and then acquire EGF responsiveness during the in vitro culture. Compared with FGF-2-generated neurosphere, EGF-generated neurosphere showed accelerated to differentiation to glial cells, suggesting that EGF induces the proliferation of not only neural stem cells, but glial cells.

The lineage relationship between the EGF- and FGF-2-responsive neural stem cells is unknown. Moreover, the specific cell surface marker for neural stem cell is little found. Several specific molecules expressed in neural stem cell, such as nestin and Musashi have been reported. However, since these molecules are localize within cells, antibodies against those cannot recognize that intact cells. Recently CD133, which is a five transmembrane protein and have been used to enrich for hematopoietic stem cells, was reported to recognize neural stem cells. To identify the membrane molecules on the neural stem cells involved in the maintenance of self-renewing of neural stem cells, we generated a monoclonal antibody by immunizing mouse with neurosphere from rat striatum as an antigen.

One antibody specifically recognized neural stem (-like) cells in the neurosphere, although it did not bind to neuron and astrocyte *in vitro*. The localization of this antigen was restricted on the ventricular zone in fetal rat brain. Western blotting of neurosphere homogenates showed that this antibody recognized 170 kDa protein, but not recognized EGFR. Using this antibody and FACS, the cells possessed sphere-initiating activity could enrich in neurosphere cells. Moreover, this antibody could enhance proliferation of neurosphere. It was concluded that this antibody may recognize a specific antigen on the neural stem cells, and will be useful tool for the direct isolation of neural stem cells.

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Division of Cell Processing was established in IMSUT on September 1995 to support three projects. The first one is to establish the Tokyo Cord Blood Bank. Our bank now is the biggest cord blood bank in Japan. In addition of enlargement of banking scale, we acquired ISO 9002 as the cell processing and cryopreservation facility in March 2001. Upon the request of cord blood from abroad, we are now proceeding procedure to join international NetCord, and also AsiaCord. The clinical evaluation has been performed about the affective factors on engraftment and survival post cord blood transplantation. Homing ability of cord blood was also studied for the understanding of engraftment. The second one is to manage cell therapy. We have been supporting the clinical departments through dendritic cell therapy for patients with malignancies, such as melanoma and thyroid cancer. Since 2001, we started to investigate the expansion of NK/T cells derived from CB as the immunotherapy. The third project is regenerative medicine. In spring, 2001, we started research for placenta-derived mesenchymal cells and we are now getting several new findings.

1. Quality management and internationalization of Tokyo Cord Blood Bank: International Standardization and Organization (ISO) 9002 (JIS Z9902), NetCord, AsiaCORD and Eurocord

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Tokyo Cord Blood Bank collected 4,500 units of cord blood cells and has registered 2,450 units in Network (Japan cord blood bank organization) and also NetCord (one of the international cord blood bank organizations) by December 2002. Out of them, we have shipped 140 units for 134 patients with hematopoietic malignancies, congenital immunodeficiencies and congenital metabolic disorders including 7 units to foreign countries by December 2001. In Japan there are now 10 CB banks and more than 700 CB transplantations have been performed by the end of last year. For these rapid expanding requests of cord blood and the need to show the quality assurance, Tokyo Cord Blood Bank adopted the international quality assurance system, ISO (International Organization for Standardization and Organization) 9002, and the international CB networks; NetCord, AsiaCORD, and Eurocord. To establish the quality assurance system, we applied our processing and cryopreservation system based on "Guidelines for the Practice of Umbilical Cord Blood Transplantation, 1998" developed by Ja-

pan Umbilical CBT Working Group (Japan Ministry of Health, Labor and Welfare) and Network, and got certified as ISO 9002 reviewed by BVQi on March 16, 2001. We a re now working in this system and preparing to renewal ISO 9001,2000 series in this spring, 2003. In addition to the ISO quality assurance system, we joined NetCord, which requires very high cord blood bank processing standards (FAHCT-NetCord standards) and bypasses the search requests from transplantation centers and releases of the cord blood worldwide. Another international cord blood network, we have established AsiaCORD to get more advantage to search the appropriate donor in Asia in July 2001. In relation to AsiaCORD, we have supported to establish the first national CB bank in Vietnam. In another aspect of analyzing CBT results, we have been participating in Eurocord (European Research on Cord Blood Banking and Use for Transplantation) and reporting CBT results for the collaboration of clinical study. Tokyo Cord Blood Bank keeps the great effort to grow with high quality valid to the world.

2. CBT clinical analysis from the aspect of Cord Blood Bank: Effect of wash-out of DMSO and dose of nucleated cells number, CD34+ cells, colony counts on 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

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The number of adult patients who receive unrelated cord blood transplantation (CBT) has been rapidly increasing. We analyzed the factors influencing the incidence and speed of engraftment in 46 adult patients compared with 55 children who received CBT from September 1998 to March 31, 2002 with units shipped from the Tokyo Cord Blood Bank, Japan. All adults had hematopoietic malignancies. In the adults, the mean±SD of nucleated cells (NC) was $2.6\pm0.6\times10^7$/kg, that of colony-forming cells (CFC) $5.3\pm2.7\times10^4$/kg, and that of CD34+ cells $0.89\pm0.6\times10^5$/kg, all less than half of those of the children. NC dose showed no significant difference of the engraftment in adults. The patients who received CB of CFC>5×10^4/kg showed median neutrophil recovery of 22 days with cumulative incidence of engraftment of 85.0%, and for the patients with CB of CFC<5×10^4/kg 29 days and 69.6%, respectively ($P<0.01$). The patients infused with CD34+ cell dose > 0.8×10^5/kg had significantly faster neutrophil recovery (median 21 days) than those with <0.8×10^5/kg (median 29days)($P<0.02$). We also analyzed the effect of wash-out of DMSO by the two-fold dilution method, on the engraftment in the above adult patients. Twenty-four patients were directly infused (non-washed) and 22 were patients infused with washed CB units (washed). Median neutrophil recovery was not different, being 24 days in the non-washed group and 25 days in the washed group. In conclusion, we suggest that the doses of CFC and CD34+ cells, but not wash-out of DMSO are predictive factors for the incidence and speed of engraftment in adult patients.

3. Expansion of NK/NKT/T cells

a. Different proportional expansion of umbilical cord blood mononuclear cell derived natural killer cells and T cells dependent on the dose of Interleukin 15 with Flt3L

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We investigated the effect of Interleukin-15 (IL15) with Flt3 ligand (Flt3L) on the expansion and activation of NK cells derived from umbilical cord blood (UCB) mononuclear cells (MNCs). *Materials and Methods*. UCB-derived MNCs were cultured at various dose of IL15 plus Flt3L (10ng/ml) or 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 NK. *Results*. After 2 weeks-culture with 5ng/ml and 10ng/ml of IL15 plus 10ng/ml of Flt3L, the fold inductions of NK cells vs. T cells were 17.0±9.3 folds vs.10.0±4.1 folds that of the number of NK cells of day0, while with 50ng/ml and 100ng/ml of IL15, the inductions of NK cells vs. T cells were 4.1±2.2 folds vs. 32.4±9.9 folds and 3.3±2.9 folds vs. 38.9±21.2 folds, respectively (ratio of NK/T cells: 5ng/ml vs. 50 or 100ng/ml of IL15; $P<0.005$, 10ng/ml vs. 50 or 100ng/ml of IL15; $P<0.05$)(n=6). On the other hand, the various dose of IL2 with Flt3L did not show this phenomenon. 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 Il2 and Flt3L. Cytotoxic activity against K562 of cultured NK cells displayed the same level as adult peripheral blood (PB)-derived NK cells, although that of pre-cultured UCB-derived-NK cells was extremely low. *Conclusions*. Low, but not high dose of IL15 with Flt3L showed higher induction of UCB-MNCs into NK cells with cytotoxic activity.

b. Expansion and characterization of Va24+Vb11+ NKT cells derived from umbilical cord blood mononuclear cells

Natural killer T (NKT) cells, a novel T cell popula-

tion firstly defined as NK1.1 positive and TCRab positive cells in mice act as one of the innate immune mechanism, interacting with NK cells and T cells. We succeeded in expanding $Va24^+Vb11^+$ NKT cells from cord blood-derived mononuclear cells (CBMNCs) 2,300 fold in the presence of IL-15, IL-7 and Flt3-L with a-Galactosylceramide (KRN7000). The addition of 100ng/mL of aGalCer on day 0 compared to day 8 and day 15 induced a significantly more proliferative potential of $Va24^+Vb11^+$NKT cells. A control experiment with a vehicle of aGalCer showed no induction of $Va24^+Vb11^+$NKT cells. The maximal induction was observed after 16 days of culture, when aGalCer was added on day 0. Then the absolute number of NKT cells decreased slowly. This decrease seemed to correspond to the disappearance of CD1d positive cells. According to the depletion experiment of $CD14^+$ monocytes, $CD19^+$ B cells and $CD1a^+$ dendritic cells, there was a trend that $CD14^+$ monocytes were responsible for the NKT expansion in our system. We observed that the expanded $Va24^+Vb11^+$NKT cells were CD4 positive and produced both IL-4 and IFN-g. In this system, $CD3^+$T cells and $CD3^-CD56^+$ NK cells were also expanded. While NK cells of the innate immune system can be directly activated without prior sensitization, and T cells are more specific and efficient for targeted responses, NKT cells seem to play an important role in immunoregulation and as a mediator between innate and adaptive immunity. Our method for expanding NKT cells derived from CBMNCs is simple and these expanded cells might be considered for future clinical applications.

4. Different expressions of adhesion molecule and chemokine receptor repertoire among umbilical cord blood-, mobilized peripheral blood-, and bone marrow-derived $CD34^{bright}$ cells

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The cause(s) for delayed hematopoietic reconstitution after umbilical cord blood transplantation (UCBT) remains controversial. We hypothesized that hematopoietic stem/progenitor cells (HS/PCs) from UCB have some defects of the homing-related molecules responsible for their slow engraftment. Using flow cytometry, we compared expression levels of homing-related molecules on HS/PCs from UCB, mobilized peripheral blood (mPB) and bone marrow (BM). Expression levels of CD49e, CD49f, CD54 and CXCR-4 on UCB-derived $CD34^{bright}$ cells were significantly lower than those on mPB- and BM-derived (except CD54) $CD34^{bright}$ cells. Cryopreserved UCB-derived $CD34^{bright}$ cells expressed significantly further lower levels of CD49e, CD49f, CXCR-4 and CD62L, accompanying lower *ex vivo* transmigration. mPB-derived $CD34^+$ cells exhibited significantly higher *ex vivo* transmigration than UCB- and BM-derived $CD34^+$ cells, which were largely blocked by neutralizing antibodies to CD49e or CD49f. Only mPB-derived $CD34^{bright}$ cells expressed the matrix metalloproteinases MMP-2/-9. Recombinant human tumor necrosis factor (rHuTNF)-a treatment resulted in significantly higher expression levels of MMP-2/-9 and enhanced *ex vivo* transmigration of $CD34^+$ cells from UCB and BM, the latter was completely blocked by MMPs inhibitor KB8301. Short-term stimulation of UCB-derived $CD34^+$ cells with rHu-stem cell factor (rHuSCF) up-regulated their expression levels of the homing-related molecules with increased *ex vivo* transmigratory potential. Furthermore, rHuSCF treatment accelerated human hematopoietic recovery in xenotransplantated NOD/SCID mice. Our results indicated that defective transmigratory behavior of HS/PCs from UCB, which might partly explain the cause(s) of delayed reconstitution after UCBT, could be reversed by an *ex vivo* manipulation.

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

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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 litters of pheresis products and culture with GM-CSF (50 ng/ml) and IL-4 (50 ng/ml) for 7 days to generate immature DCs. Immature DCs were then pulsed with autologous tumor lysate (100 mg/ml) and subsequently cultured with TNF-alpha (50 ng/ml) for another 4 days for maturation. $1x10^7$ of loaded DCs were injected intradermly once a week for four times and subsequently once in two weeks for four times. This clinical trial is now in progress.

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

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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 flow cytometric analysis, immunohistochemical analysis or reverse transcription-PCR (RT-PCR). In flow cytometric 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^{-7}M) for 24 hours resulted in decrease (>30%, n=3) 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. Moreover, CRF-treated immature DCs were down regulated production of mRNA for IL-12p35 and IL-12p40. In conclusion, 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.

7. Human placenta-derived mesenchymal stem cells differentiate into neural cells

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Mesenchymal stem cells (MSCs) are primary, multipotent cells derived from different tissues, capable of differentiating to at least osteocytic, chondrocytic, adipocytic lineages when stimulated under appropriate conditions. Neural lineages developed from MSCs derived from bone marrow stromal cells include neurons, astrocytes, oligodendrocytes, and Schwann cells *in vivo* and *in vitro* in recent reports. Here, we present that different unique tissue; human placenta-derived MSCs could differentiate into neural lineages. Human placenta consists of fetal part and maternal part. The fetal part of the placenta includes amnion and chorion, umbilical cord and chorionic villi with the covering trophoblast, while maternal part consists of the deciduas and the intervillous space. Placentae were obtained from the mothers whose cord blood cells were also donated for Tokyo Cord Blood Bank with informed consent. We isolated adherent cells from fetal part (chorionic villi) and maternal part (deciduas) of placenta using the explants method. The cellular isolates were cultured in DMEM (low glucose) plus 10% fetal bovine serum (FBS). To generate neural differentiation, both of the MSCs from fetal and maternal part in placenta were cultured on poly-L-lysine coated culture dishes in DMEM/F12 (1:1) plus B27 supplement with 100 mM BHA/0.5 mM IBMX/1mM dbcAMP/1.5% DMSO and 20 ng/ml bFGF for 24 h. We could expand the MSCs expressing CD13 from both fetal and maternal part in placenta. To confirm the origin of MSCs, we used the placenta from male and investigated XY chromosome by FISH analysis. Both fetal and maternal MSCs in placenta could differentiate into neural lineage cells with a typical dendritic morphology. Characteristic markers of neural cells were positive for neuron specific enolase (NSE) and glial fibrillary acidic protein (GFAP), but negative for 2'3'-cyclic nucleotide 3'-phosphodiesterase (CNPase). These cells also expressed tyrosine hydroxylase, which is seen in dopamine secreting neurons. These data suggest that human placenta-derived MSCs have a plasticity differentiating into neurons and astrocytes under the conditions of increased intracellular cAMP level and antioxidant properties. Placenta should be an attractive source for clinical use with several reasons including non-harmful burden for donors.

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Donation Laboratories

Division of Hematopoietic Factors(Chugai)

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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 STAT5A 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 responsible 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 have recently 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 are being 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], Katsutoshi Ozaki, Yasuhide Hayashi[6], Tomohiko Taki[6], Yuki Kataoka[5], Nobuaki Yoshida[5], 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 are generating 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

Sumiyo Morita, Fumi Shibata, Yuko Gotoh-Koshino[1], Dan Wang[1], Toshihiko Oki[1], Tetsuo Kojima, Hideaki Nakajima[1], Kohichiro Tsuji[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, and the efficiency of infection of the viruses produced by PLAT-F, into human CD34 positive cells, is being investigated by a long term reconstitution assay in SCID mice.

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

Toshiyuki Kawashima, Yukinori Minoshima[1], Koichi Hirose, Yukio Tonozuka[1], Ying Chun Bao[1], Tetsuya Nosaka, Kentaro Semba[7], Takaya Satoh[8], Masaki Inagaki[9], and Toshio Kitamura[1]: [7]Department of Cellular and Molecular Biology, Institute of Medical Science, The Univertsity of Tokyo, [8]Division of Molecular Biology, Department of Molecular and Cellular Biology, Kobe University Graduate School of Medicine, Kobe, [9]Laboratory of Biochemistry, Aichi Center Cancer Research Institute, Nagoya

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*

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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.

9. A critical role for IL-21 in regulating immunoglobulin production

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The cytokine interleukin-21 (IL-21) is closely related to IL-2 and IL-15, and their receptors all share the common cytokine receptor gamma chain, γ_c, which is mutated in humans with X-linked severe combined immunodeficiency disease (XSCID). We demonstrate that, although mice deficient in the receptor for IL-21 (IL-21R) have normal lymphoid development, after immunization, these animals have higher production of the immunoglobulin IgE, but lower IgG1, than wild-type animals. Mice lacking both IL-4 and IL-21R exhibited a significantly more pronounced phenotype, with dysgammaglobulinemia, characterized primarily by a severely impaired IgG response. Thus, IL-21 has a significant influence on the regulation of B cell function *in vivo* and cooperates with IL-4. This suggests that these γ_c-dependent cytokines may be those whose inactivation is primarily responsible for the B cell defect in humans with XSCID.

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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 the complexity of human genome by studying linkage disequilibrium and haplotype structure. Accordingly, 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.

As the Human Genome Project is close to accomplish its goal of a completed human DNA sequence, research interests rapidly shift from the common nucleotide sequences to nucleotide diversities in human populations. The nucleotide diversities represented by single nucleotide polymorphisms (SNPs), and their linkage disequilibrium and haplotype structures in the human genome provide important implications for gene-mapping underlying complex disorder and for understanding of human population history or human evolution. Especially, a great deal of recent attention to LD in humans is due in large to the prospect of large-scale association studies to localize susceptibilities underlying complex diseases. Our priority is analyses of the molecular causality of the common metabolic disorders of civilization, in other word, identification of the causal gene for complex trait. 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. Elucidations of the molecular etiology provide specific molecular targets for therapeutic drugs even at the individual level. 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. Diseases of current interests are asthma, essential hypertension, subarachnoid hemorrhage, and ossification of the posterior longitudinal ligament of the spine.

1. Linkage disequilibrium analysis and mapping of disease gene

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

Linkage disequilibrium (LD) has recently become the focus of interest in the mapping of complex disease loci through genome-wide association studies. To better understand the pattern of LD in the human genome, the refined structure of LD in a 25.6 kb region covering the entire human interleukin 13 gene (*IL-13*) and interleukin 4 gene (*IL-4*) was evaluated among African, Caucasian, and Japanese, based on 104 single nucleotide polymorphisms (SNPs). *IL-13* and *IL-4* locate adjacently on chromosome 5q31 where susceptibility loci for several allergic diseases were reported. Two LD blocks, one spanning 0.8 kb

in *IL-13* and the other spanning at least 20 kb in *IL-4*, were identified in Caucasian and Japanese, but not in African. Within LD blocks at *IL-13* and *IL-4*, very limited number of distinct haplotypes, which were shared between Caucasians and Japanese, were observed. Neutrality test was performed on the regions within and outside LD blocks, which allowed us to infer that a selective sweep could be one of factors to generate and maintain LD block. Understanding the structure and composition of LD in whole genome takes on importance increasingly for genotype-phenotype association study and human population genetics.

2. Natural selection of the human angiotensinogen gene

Toshiaki Nakajima and Ituro Inoue

The human angiotensinogen gene (*AGT*) represents an important locus whose variation is involved in the predisposition to essential hypertension. *AGT* might be a target for natural selection, because of the extreme pattern of the geographic distribution of protective G-6/M235 allele, associated with less sodium retention, for essential hypertension. The relationships between genetic distance and frequencies of G-6/M235 allele showed that the more distant from African, the higher frequencies of G-6/M235 occur in the Middle East and Europe. Under modern conditions of high salt intake, the protective G-6/M235 allele may have been selectively advantageous outside Africa. To evaluate natural-selection signature we determined the structure of linkage disequilibrium (LD) and haplotype of *AGT* based on 334 complete sequences of 14.4-kb genomic regions covering *AGT* from worldwide samples. The relationships between G-6/M235 and CA-repeats alleles located downstream of exon 5 showed the tight linkage disequilibrium between G-6/M235 and a specific CA-repeats allele-197 in non-African populations. Thirty G-6/M235 homozygotes in 167 worldwide samples also have the low nucleotide diversity in analyzed genomic regions (π=2.02 x 10^{-4} and θ=4.20 x 10^{-4} in thirty G-6/M235 homozygotes). These observations indicate that the recent bottleneck might cause the predominance of G-6/M235 allele before modern humans were geographically spread outside Africa and the recent expansion of G-6/M235 allele might occurred in the Middle East and Europe, that cannot be explained by random genetic drift. The estimated age for the origin of G-6/M235 allele based on inter-haplotype variation, 20,000~40,000 years ago, was consistent with the age when modern humans were present throughout most of Europe. In addition, Fay and Wu *H* test for the promoter region of *AGT* showed the large difference between θ_π and θ_H in the sample from the Middle East and Europe, suggesting the directional selection of *AGT*. Other neutrality tests, such as Tajima's D, Fu and Li's *D**, Fu and Li's *F**, and HKA tests, was consistent with the directional selection of *AGT*. Taken together, these results support the directional selection signature of *AGT* in the human evolution, especially in the Middle East and Europe.

3. Subarachnoid hemorrhage due to rupture of intracranial aneurysm

Taku Yoneyama, Toshiaki Nakajima, and Ituro Inoue

Rupture of intracranial aneurysms (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. We extensively studied the associations between IA and the candidate-genes located around chromosome 5q22-31, 7q11, and 14q22, where positive evidence of linkage were observed based on 104 Japanese affected sib-pairs analysis. The genes for either vascular components or vascular formation, such as Lysyl oxidase (*LOX*), fibrillin 2 (*FBN2*), fibroblast growth factor 1 (*FGF1*), collagen type 1 alpha-2 (*COL1A2*), and transforming growth factor beta-binding protein (*LTBP2*), were analyzed as the candidate-genes. We have now identified a common variant at the 459 amino acid position of the collagen type1 alpha-2, in which an alanine is replaced by a proline, in significant association with IA. Circular dichroism analysis suggested that this amino acid substitution causes the structural and biochemical change of a triple helix of type 1 collagen. These observations provide some biological insights about the possible mechanism of a genetic predisposition to IA.

4. Genetics of ossification of the posterior longitudinal ligament of the spine

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

Ossification of the posterior longitudinal ligament of the spine (OPLL) is the predominant myelopathy among Japanese, and is usually diagnosed by ectopic bone formation in the paravertebral ligament in Japanese and other Asians. In order to detect genetic susceptibilities associated with OPLL, we performed an extensive non-parametric linkage study with 126 affected sib-pairs using 420 microsatellite markers, which cover the entire genome at 8 cM interval in average. Non-parametric linkage was evaluated by two distinct analyses, SIBPAL and GENEHUNTER. A positive evidence of linkage was observed on chro-

mosome 21 with peak lod score of 3.6 at D21S263. The linkage region, spanning 10 cM, was extensively screened for causality by use of SNPs for association study.

5. Association studies of single nucleotide polymorphisms in candidate genes for bronchial asthma

Takuro Sakagami, Nobuyoshi Jinnai, 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. 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.

6. Genetic approach to dissect 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 produces suitable assay system of replication initiation. Because commonly applied knocking out of an essential gene only resulted in cell mortality, we have developed a domain specific disruption method through homologous recombination in human cell line. We obtain a hypomorphic or minimal functional domain disrupted mutant cells that restrain DNA replication of chromosome and of viral episomes in cultured cells. We have focused on several new mammalian proteins implicated in replication initiation and are examining their roles in DNA replication and regulation throughout the cell cycle.

As a first example, targeting disruption of the destruction box of Geminin, an inhibitor of the mammalian DNA replication initiation complex, in a human cancer cell line through homologous recombination stabilizes Geminin protein levels during the G1-S phase. The diminished level of the chromatin loading of MCM complex resulted in inhibition of DNA replication and the cells accumulated in G1 phase of the cell cycle. *In vivo* tumorigenicity as well as *in vitro* cell growth was suppressed by endogenous level of truncated Geminin. Cells carrying this mutation failed to support the replication of a plasmid bearing the oriP replicator of Epstein Barr virus but not the origin of human papilloma virus. Activation of the DNA damage/checkpoint signaling pathway was manifested in the mutated cells with the increased level of p53 protein accompanying the phosphorylation of Ser 20 and p21 protein, and this was rescued by overexpression of Cdt1. Therefore, a functional mutation that alters the cell cycle dependent regulation of Geminin induces the DNA damage/checkpoint pathway in human cells.

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Division of Genetic Diagnosis(Otsuka) (2)

ゲノム情報応用診断(大塚製薬)研究部門臨床分野

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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 will lead to development of hematopoietic disorders such as bone marrow failure, myelodysplastic syndrome and leukemia. To approach this subject, we are studying molecular pathogenesis of Fanconi anemia (FA), an autosomal recessive disease with progressive bone marrow failure, leukemia susceptibility and chromosomal instability. FA has at least eight different complementation groups (FA-A to FA-G). Growing evidence indicates that multiple FA gene products function in a common pathway, termed the FA pathway. In a current model, a nuclear multiprotein complex including FANCA, C, E, F and G converts FANCD2 into an active form, which regulates DNA damage response in collaboration with the BRCA1 machinery. Furthermore, FANCD1 was recently shown to be identical with BRCA2. We are studying the regulatory mechanism of the FA pathway. In addition, we have identified and are studying functions of novel molecules:(i)HSH2, an SH2 domain-containing protein which is likely to regulate cytoine signaling in hematopoietic cells, and (ii) ABT1/ABTAP, a nuclear protein complex localized in nucleoli and Cajal bodies, which is likely to regulate RNA metabolism.

1. Stable complex formation of the Fanconi anemia (FA) proteins is not essential for activation of the FA pathway

D Adachi, T Oda, H Yagasaki, T Yamashita

In a current model of the FA pathway, nuclear formation of multiprotein complex including FANCA/C/E/F/G is critical for activation of FANCD2 into a monoubiquitinated form, which is linked to normal cellular response to MMC. This model is mainly based on the following observations: (i) FANCA, C, G, E and F assemble into a stable nuclear complex, detected by means of co-immunoprecipitation; and (ii) deficiency and point-mutations of either of FANCA, C, E, F or G abolish nuclear complex formation of the FA proteins, monoubiquitination of FANCD2 and normal cellular sensitivity to MMC. Exceptionally, the FANCA/FANCG binding is not affected by these pathogenic mutations. FANCG directly binds to the N-terminal region (amino acids 1-35) of FANCA, which includes a bipartite nuclear localization signal (NLS) constituting of two separate sequences rich in Arg and Lys, NLS1 ($R^{18}R^{19}R^{20}$) and NLS2 ($R^{29}K^{31}R^{32}K^{34}$). However, functional significance of the FANCA/FANCG binding has not been fully un-

derstood. To address this question, we characterized FANCA mutants with amino acid substitution in the N-terminal region, shown in Table, in comparison with patient-derived dysfunctional mutants H1110P and delF1263. The results have demonstrated that, contrary to the current model of the FA pathway, stable complex formation is not essential for nuclear localization of FANCA, FANCD2 monoubiquitination or correction of MMC sensitivity. Unstable interaction among FA proteins, which is not detectable in co-immunoprecipitation assays, may be enough to activate the FA pathway. Dysfunction of patient-derived mutants is not attributed simply to disruption of the FA protein complex. Our present findings provide novel insights into regulatory mechanisms of the FA pathway.

2. Characteristics of FANCA and FANCG mutations in Japanese Fanconi anemia (FA) patients

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Extensive mutational analyses of FA genes in Western countries revealed that subgroup prevalence and mutational spectrum vary depending on the ethnic background. However, much less is known about Asian populations. We therefore screened 45 unrelated FA families in Japan for FANCA and FANCG mutations. We identified 29 carrying FANCA mutations and 10 carrying FANCG mutations, and further characterized these mutations .

To detect FANCA mutations, six overlapping cDNA fragments obtained by RT-PCR and each exon amplified from genomic DNA were sequenced. Since such routine analyses are inadequate for detection of large deletions which were reported to be frequent in the FANCA gene, we employed a real-time quantitative PCR assay to determine copy numbers of each exon of the gene. This technique gave highly reliable results to identify deleted exons. As a result, we identified 42 mutant alleles (13 frameshifts, 12 splice mutations, 9 missense mutations, 7 large deletions, 1 nonsense mutation, and 1 in-frame deletion) including 24 novel mutations. About 60% of the mutations were detected in a single patient, i.e. 'private mutation', while some mutations were recurrent. A frameshift mutation 2546delC was detected in 14% of the mutant alleles but has not been reported in Western countries, thus it may be a founder mutation in the Japanese population. Missense variants were functionally inactive and identified breakpoints of two large deletions, one of which was presumably generated by Alu-mediated recombination.

Mutations of FANCG were screened by direct sequencing of cDNA, with subsequent confirmation by analysis of genomic DNA. We identified 10 unrelated FA-G families. A splice mutation IVS3+1G>C was detected in 9 Japanese families, (4 homozygous and 5 heterozygous) accounting for 65% of mutant alleles. Among the heterozygotes, three carried 1066C>T in the second allele. Of note, a patient homozygous for 1066T>C was of Korean ethnicity. Haplotype analysis using 9 polymorphic microsatellite markers spanning the FANCG locus showed that IVS3+1G>C and 1066T>C were associated with distinct founder haplotypes. Thus, the two founder mutations derived from distinct ancestries cause most of FA-G patients in Japan. Our results suggest that IVS3+1G>C arose in the Japanese population, whereas 1066T>C migrated from Korea.

To our knowledge, this is the first report of FANCA and FANCG mutations in a single ethnic group. The FANCA gene shows a wide spectrum of mutations, consistent with previous results obtained in Western countries. By contrast, most of FANCG mutant alleles are derived from a few founders.

3. ABT1-associated protein (ABTAP), a novel nuclear protein conserved from yeast to mammals, represses transcriptional activation by ABT1

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Table

FANCA mutant	FANCG-binding	FANCC and F-binding	Nuclear localization	FANCD2-Ubiquitination	MMC resistance
WT	+++	+++	++	+++	+++
L25P	-	-	++	++	++
LL25/26AA	-	-	++	++	++
NLS1-mut (RRR>AAA)	+	+	+	+++	+++
NLS2-mut (RKRK>AAAA)	-	-	+	+	+
NLS1/NLS2-mut	-	-	-	-	-

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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 from a rat brain cDNA library and we isolated a cDNA clone encoding a novel protein of 842 amino acids, termed ABT1-associated protein (ABTAP). Like ABT1, ABTAP has homologous counterparts in humans, yeast, *Caenorhabditis elegans*, and *Drosophila melanogaster*. In *Sacchaaromyces cerevisiae*, the ABTAP homolog was, like the ABT1 homolog, essential for cell growth, and the interaction between these two homologs was conserved. In mammalian cells, ABTAP formed a complex with ABT1 and suppressed the ABT1-induced activation of Pol II-directed transcription. Furthermore, ABT1 and ABTAP colocalized in the nucleoli 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 ABT1. The ABT1/ABTAP complex is evolutionarily conserved and may constitute a novel regulatory system for basal transcription.

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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 and mass spectrometry, our laboratory is equipped with advanced liquid chromatography 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 analysis of leukemia inhibitory factor signaling in mouse embryonic stem cells

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Embryonic stem (ES) cells are cultured cells derived from the inner cell mass of blastocysts. ES cells display two cardinal features. First, they are maximally pluripotent in that they can differentiate into cells of all lineages. Second they are capable of dividing to produce maximally pluripotent daughter cells, a process referred to as renewal. Undifferentiated mouse ES cells can be maintained in the presence of the cytokine, leukemia inhibitory factor (LIF), while removal of LIF can cause cell differentiation. This shows that under certain conditions, LIF signaling is important to maintain the pluripotency of the ES cells. However, the molecular mechanism of LIF function remains unknown. We have addressed this problem by proteomic analyses of

mouse ES cells using two-dimensional gel electrophoresis and mass spectrometry (MS) to identify LIF-dependent proteins. In this way, 22 ES-cell-derived protein spots have been identified whose intensity clearly correlated with growth in the presence or absence of LIF. Reduction of the Oct-3/4 level upon LIF withdrawal was confirmed by Western blotting. Of these 17 were identified by MS. For example, we found that the expression of glia maturation factor β and nuclear autoantigenic sperm protein were reduced to a significant extent upon removal of LIF, whereas importin β, dihydrolipoamide S-acetyltransferase, succinate dehydrogenase Fp subunit, myo-inositol 1-phosphate synthetase A1, leukotriene A_4 (LTA_4) hydroxylase, ER-60 protease, Y-box binding protein, and phospholipase A_2 (PLA_2) were increased upon LIF withdrawal. Notably, this group includes a subset of enzymes involved in the leukotriene B_4 metabolism, such as PLA_2, LTA_4 hydroxylase, and myo-inositol 1-phosphate synthetase A1, suggestive of a role for inositol phospholipid biogenesis, membrane homeostasis or phosphoinositide signaling in the differentiation of mouse ES cells.

2. Large-scale identification of proteins in mouse embryonic stem cells by multi-dimensional liquid chromatography-tandem mass spectrometry

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Embryonic stem (ES) cells are uniquely pluripotent, although studies of the underlying molecular nature of this trait have focused on specific molecules such as leukemia inhibitory factor (LIF) and its receptor, LIFR, STAT3, and Oct3/4. However, little is known about global protein expression in ES cells. We have addressed this shortfall by attempting to catalog mouse ES cell proteins. To this end, we performed proteomic profiling of ES cells by an integrated liquid chromatography (LC)-based protein identification system (LC-MS). This comprised microscale two-dimensional LC coupled with electrospray ionization mass spectrometry on a high-resolution hybrid mass spectrometer and an automated data analysis system. Whole cell extracts were digested with trypsin, and the resulting complex peptide mixture was subjected to this system. We identified 1,233 and 987 proteins in ES cells respectively grown in the presence or absence of LIF. These include more than 500 cytosolic enzymes and other proteins, cytoskeletal proteins, and transmembrane and membrane-associated proteins such as cell surface receptors and cell adhesion molecules. Semi-quantitative analysis of the LC-MS data suggested that among the identified proteins, approximately 100 showed notable differences in the level of expression between the cells grown in the presence or absence of LIF. These results imply that the expression and/or modification of a subset of proteins in ES cells is regulated by LIF signaling; such proteins are candidate determinants of ES cell pluripotency.

3. Specific labeling and LC-MS-based protein identification for the analysis of cell surface proteins

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A variety of cellular events are triggered or regulated by extracellular stimuli *via* cell surface proteins. Because these proteins play important roles in fundamental cellular processes such as proliferation and differentiation, the protein profiling of the surfaces of certain cells should provide valuable information about the underlying mechanisms. To date, two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) has been widely used for the large-scale analysis of proteins expressed in a cell. Despite its utility in the analysis of thousands of cellular proteins, 2D-PAGE often fails to identify membrane proteins due to their hydrophobicity and/or large size. We therefore developed an alternative method to analyze the cell-surface membrane proteins using a specific labeling reaction coupled with liquid chromatography-tandem mass spectrometry (LC-MS/MS). The method consists of (1) biotinylation of intact cell surfaces, (2) subcellular fractionation of the cell lysate, (3) tryptic digestion of the membrane fraction and affinity purification of biotinylated peptides, and (4) protein identification by LC-MS/MS. To evaluate this approach, we applied to identifying cell-surface proteins in mouse embryonic stem (ES) cells. More than 80% of peptides identified by LC-MS carried the biotin-label and most were derived from transmembrane or membrane-associated proteins. These included alkaline phosphatase, cadherin 1, CD9, CD98 (solute carrier family 3), CDw92 antigen, c-kit, embigin, Eph receptor A2, integrin α and β chains, laminin α1, and transmembrane protein 2. The advantages of this approach are manifold. First, the biotinylation of intact cells prior to lysis favours specific labeling of cell surface proteins, because the labeling reagent cannot permeate the plasma mem-

branes of viable cells. Second, subcellular fractionation enriches membrane proteins, thereby reducing contamination from biotinylated soluble proteins. Third, tryptic digestion enhances the recovery of soluble peptides from hydrophobic insoluble proteins. And fourth, LC-based MS/MS analysis identified biotin modifications that corroborated the localization of proteins within membranes. Cell surface labeling together with LC-MS-based identification should therefore prove a powerful combination in the comprehensive analysis of cell surface proteins such as cell surface receptors and cell adhesion molecules.

4. Characterization of phosphotyrosine-containing protein complexes in malignant tumor cells during anchorage-independent growth

Kohji Nagano[1], Nobuko Okamura[1,2], Chiharu Itagaki[1], Uchiko Hamaguchi[3], 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]Division of Biochemistry, IMSUT, [4]Graduate School of Science, Tokyo Metropolitan University

Anchorage-independent growth is characteristic of malignant tumor cells. Specific inhibition of this process potentially triggers apoptosis in detached, malignant tumor cells without affecting non-malignant cells, and is thus an attractive target for cancer therapy. Using a nano flow liquid chromatography system coupled with high-resolution mass-spectrometry (MS) recently developed in this laboratory, we seek to explore the protein complexes that play essential roles in this process. It has been reported that a variety of tyrosine kinases are constitutively activated in malignant tumor cells, suggesting that phosphotyrosine-containing protein complexes persist in malignant tumor cells, but not non-malignant ones. We therefore focused our study on phosphotyrosine-containing complexes existing in malignant tumor cells during anchorage-independent growth. First, we analyzed alterations in tyrosine phosphorylation in highly metastatic melanoma cells grown in suspension. Phosphotyrosine-containing proteins were captured by affinity purification with anti-phosphotyrosine antibody and subjected to SDS-PAGE, and MS identification. Retinoblastoma protein (Rb), a cell cycle inhibitor, was highly phosphorylated at tyrosine in metastatic melanoma cells during anchorage-independent growth as compared to levels of phosphotyrosyl Rb in its weakly metastatic parental cell line. The phosphorylation of Rb was also observed in another metastatic cell line, HeLa S3 cells grown in suspension. Immunoprecipitation studies revealed that galectin-3, a galactose-specific lectin, was endogenously associated with Rb in HeLa S3 suspension cultures. Moreover, c-abl, a non-receptor tyrosine kinase known to bind Rb, was also present in the protein complex. Both galectin-3 and c-abl co-localized to the cytoplasm of HeLa S3 cells and both were able to bind the C-terminal domain of Rb *in vitro*. Together, these findings strongly suggest a physiological role for the Rb-galectin-c-abl complex. Studies are now in progress to test this and elucidate the mechanism of the putative complex in anchorage-independent malignant tumor cell growth.

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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 concentration. To overcome this difficulty, we are currently establishing the protocols to isolate transcriptional machinery and phosphorylated proteins.

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 Two-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 membrane raft fraction. The second is to immunoprecipitate the protein of interest with associating proteins. The third approach is to affinity purify transcriptional complex formed on specific recognition sequence by means of oligonucleotides attached to matrices. The fourth approach is to purify phosphorylated proteins. We are currently focus our efforts to isolate transcriptional complex and phosphorylated proteins.

Immobilized metal affinity column using Fe^{3+} has been shown to be useful to purify phosphopeptides. We applied this simple method to isolate phosphoproteins and got preliminary 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 ten-fold purification of these phosphoproteins was achieved.

Besides these proteomic projects, we are studying the role of an SH2-containing adaptor protein, Chat, in T-cell signaling (Ref. 1, 2). Following are the abstracts of the first two papers published in this year.

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

A. Sakakibara, S. Hattori, S. Nakamura, and T. Katagiri

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.

2. Chat activates the small GTPase Rap1 via up-regulation of the Cas-Crk signaling pathway

A. Sakakibara, Y. Ohba, K. Kurokawa, M. Matsuda, and S. Hattori

Chat is a novel signaling molecule with an N-terminal SH2 domain and C-terminal Cas/HEF1 association domain that is implicated in the regulation of cell adhesion. The Cas/HEF1 association domain also shows a sequence similarity with guanine nucleotide exchange factors for Ras family small GTPases. In this study, we found significant activation of Rap1 in Chat-overexpressing cells. Myr-Chat, a membrane-targeted Chat, activated Rap1 more efficiently. Certain Cas and Crk mutants suppressed Rap1 activation by Myr-Chat, indicating the involvement of the Cas-Crk signaling pathway in Chat-induced Rap1 activation. We also confirmed the ternary complex formation consisting of Chat, Cas and Crk. We further demonstrated that Myr-Chat expression induced a highly spread cell morphology, and this activity depended on the Cas-Crk pathway and Rap1 activity. Moreover, expression of Myr-Chat enhanced integrin-mediated cell adhesion. These findings suggest that Chat-induced Rap1 activation was mediated by up-regulation of the Cas-Crk signaling pathway rather than direct guanine nucleotide exchange factor activity of Chat, and that Chat-Cas complex plays an important role in controlling cell adhesion via the activation of Rap1.

Publications

A. Sakakibara, Y. Ohba, K. Kurokawa, M. Matsuda, and S. Hattori. Novel function of Chat in controlling cell adhesion via Cas-Crk-C3G-pathway-mediated Rap1 activation. J Cell Sci. 115:4915-4924, 2002

A. Sakakibara, S. Hattori, S. Nakamura, and T. Katagiri: A novel hematopoietic adaptor protein, Chat-H, positively regulates T-cell receptor-mediated interleukin-2 production by Jurkat cells. J. Biol. Chem. in press

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