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) 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). In addition, we searched for a serum factor(s) that is essential for survival of ES cells.

a) Zfp57

Oct-3/4, a pluripotent stem cell-specific transcription factor, plays a critical role in the self-renewal of ES cells. Several evidences suggest that gene expression via Oct-3/4 requires an unidentified co-factor(s). We found that Zfp57 is expressed during the self-renewal of ES cells, while its expression is reduced upon differentiation. By co-immunoprecipitation assay, it was also found that Zfp57 interacts with Oct-3/4. Furthermore, Zfp57 enhanced the transcriptional activity of Oct-3/4 towards the promoter of *rex-1*, an Oct-3/4 target gene, in human embryonic kidney 293 cells. Our data suggest that Zfp57, a target of STAT3, is one of the Oct-3/4 co-factors, and raise the possibility that this protein may cooperate with Oct-3/4 in induction of Rex-1, which in turn may play an important role in the self-renewal of ES cells.

b) Eed

Recent studies revealed that gene expression is tightly associated with acetylation of histone, which is controlled by histone acetyltransferases and histone deacetylases (HDACs). Eed, a member of the polycomb family, is known to bind with HDAC. We found that Eed is one of the downstream molecules of STAT3. Treatment of ES cells with trichostatin A, an HDAC inhibitor, led to the initiation of differentiation, suggesting that histone acetylation is involved in regulation of the self-renewal. Interestingly, we found that Eed forms a complex also with Rex-1. Since deacetylation of histone generally results in repression of gene expression, these results suggest the possibility that the Eed•Rex-1•HDAC complex may maintain the undifferentiated state of ES cells by suppressing the expression of a differentiation-inducing gene(s).

c) 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 have searched for a serum factor(s) that supports survival of ES cells. When serum was fractionated by gel filtration or ion-exchange chromatography, the survival-supporting activity was eluted with bovine serum albumin (BSA). Furthermore, addition of BSA into the medium inhibited the apoptosis of ES cells upon serum deprivation, indicating that serum can be substituted by BSA. On the other hand, lipid-free BSA could not replace serum. These results suggest that a lipid that binds to BSA is the survival factor for ES cells.

2. Murine homolog of SALL1 is essential for ureteric bud invasion in 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. Here we report cloning, expression patterns and loss-of-function studies of mouse Sall1. Our data show that murine Sall1 is essential for initial inductive events for kidney development.

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 regard-

ing epithelial differentiation. Therefore Sall1 is essential for ureteric bud invasion, the initial key step for metanephros development.

We are currently examining molecular functions of Sall1, in addition to upstream and downstream molecular events of Sall1. We are also generating Sall2 KO, and eventually mice lacking all the Sall genes. In addition, we are establishing an induction system of kidney progenitors from a variety of cell sources, and in vitro and in vivo assays for kidney progenitors. Our final goal is to understand molecular mechanisms underlying kidney development and to utilize the knowledge for manipulating kidney progenitors for cell therapy of kidney diseases.

3. Neural stem cells

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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. Our previous study showed that EGF and FGF-2 have different effects on proliferation and differentiation of neural stem cells. Neural stem cells initially respond 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 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 localized within cells, antibodies against those cannot recognize intact cells. Recently CD133, which is a five transmembrane protein and have been used to enrich for hematopoietic stem cells, was reported to recognized neural stem cells. To characterize intact neural stem cells, we intend to make monoclonal antibodies that recognize the surface antigen of FGF-2 generated neural stem cells.

BALB/c mice were immunized in the footpad with rat neurosphere for a month. The popliteal lymph node cells were fused with mouse PAI myeloma cells. Approximately 230 hybridomas were selected, expanded, and further tested. 122 independent clones recognized neurosphere cells prepared from rat fetal brain. Of them, 51 clones recognized adherent neural stem (-like) cells maintained in the presence of FGF-2, and 52 clones stained differentiated cells, which contained neurons, astrocytes, and oligodendrocytes. Among the former 51 clones, two clones showed characteristic staining pattern of embryonic brain section for neural stem cells. These antibodies recognized 180 kDa proteins in the membrane fraction of neurosphere. Moreover, these could synergistically enhance proliferation of neurosphere with FGF-2. These antibodies will be useful tool for the direct isolation of neural stem cells using FACS and the identification of cell surface markers of neural stem cells.

Publications

Nakayama, N., Han, C., Nishinakamura, R., Scully, S., He, C., Zeni, L., Yamane, H., Chang, D., Yu, D., Yokota, T. and Wen, D. A novel chordin-like protein inhibitor for bone morphogenetic proteins, expressed preferentially in mesenchymal cell lineages. Dev. Biol. 232: 372-387, 2001.

Nishinakamura, R., Matsumoto, Y., Nakao, K., Nakamura, K., Sato, A., Copeland, N.G., Gilbert, D.J., Jenkins, N.A., Scully, S., Lacey, D.L., Katsuki, M., Asashima, M. and Yokota, T. Murine homolog of SALL1 is essential for ureteric bud invasion in kidney development. Development 128: 3105-3115, 2001.

Ishibashi, H., Hihara, S., Takahashi, M., Heike, T., Yokota, T. and Iriki, A. Tool-use learning selectively induces expression of brain-derived neurotrophic factor, its receptor trkB, and neurotrophin 3 in the intraparietal cortex of monkeys. Cognitive Brain Research in press.

Yamazaki, Y., Kaziro, Y. and Koide, H. Ral promotes anchorage-independent growth of human fibrosarcoma, HT1080. Biochem. Biophys. Res. Commun. 280:868-873, 2001.

Mizutani, S., Inouye, K., Koide, H. and Kaziro, Y. Involvement of B-Raf in Ras-induced Raf-1 activation. FEBS Lett. 507:295-298, 2001.

松田孝彦，赤木紀之，臼田雅幸，横田崇，ES細胞の未分化状態維持機構．実験医学，羊土社．19(3): 330-338, 2001.

横田崇，松田孝彦，高木峰生，平家俊男，造血幹細胞の維持機構．分子細胞治療，先端医学社．2(2): 3-10, 2001.

横田崇，平家俊男，高木峰生，キメラレセプタートランスジェニックマウスを用いた造血幹細胞の分化･増殖の制御．造血幹細胞研究のいまと臨床応用．中内啓光編，羊土社．P92-102, 2001.

松平忠弘，荒井好裕，横田崇，造血系に作用するサイトカインの開発の方向性と将来．造血幹細胞移植の進歩．峯石真編，組織培養工学，ニュー・サイエンス社．27(3): 36-39, 2001.

西中村隆一，横田崇，再生医療の可能性･ES細胞による再生医療の現状．生物の科学･遺伝，裳華房．別冊13: 130-136, 2001.
西中村隆一，横田崇，幹細胞研究の最前線･体性幹細胞と組織特異的幹細胞･腎臓の発生と分化－腎臓に幹細胞はあるか？実験医学，羊土社．増刊 19 (15): 99-105, 2001.
西中村隆一，器官発生のメカニズム．医学の歩み，医歯薬出版株式会社．199 (13): 929-934, 2001.
水野憲一，横田崇，神経幹細胞の分子機構・神経幹細胞の自己再生メカニズム．臨床神経科学，中外医学社．20 (1): 37-40, 2002.

Division of Cell Processing (Asahi Kasei and Nipro)

Division of Cell Processing was established in IMSUT on September 1995 to support the institute through cell therapy and gene therapy. After establishment of Tokyo Cord Blood Bank on September 1997, more than 3,000 units of cord blood (CB) have been stored in this bank by the end of 2001. In addition of enlargement of banking scale, we have made two progresses; quality management and internationalization. Our bank acquired ISO 9002 on March 2001, and are proceeding procedure to join international NetCord. We are also supporting the clinical departments through dendritic cell therapy for patients with malignancies. Expansion of NK cells and homing of stem cells are studying to improve the results of CB transplantation. In addition, we have started research for regenerative medicine using placenta-derived mesenchymal cells.

Cord blood (CB) has recently been found to be an efficacious alternative source of hematopoietic stem and progenitor cells for transplantation. In Japan 500 unrelated cord blood transplantation (CBT) were reported by the end of 2001. According to increasing number of transplantation, two problems in CBT are becoming clear; relatively weak graft versus of leukemia response (GVL) and delayed hematopoietic reconstitution following CBT. In 2001, we started new projects to dissolve these problems; NK/NKT cell expansion for induction of GVL, and analysis of homing ability of CB-derived hematopoietic stem cells for induction of early hematopoietic reconstitution following CBT. Another new project, regenerative medicine, was also started using placenta-derived mesenchymal cells in last year to investigate the possibility to expand variety of cell source for future tissue/organ bank.

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 3,352 units of cord blood cells and has registered 1,565 units in Network (Japan cord blood bank organization) and also NetCord (one of the international cord blood bank organizations) by December 2001. Out of them, we have shipped 102 units for 96 patients with hematopoietic malignancies, congenital immunodeficiencies and congenital metabolic disorders including 4 units to foreign countries by December 2001. In Japan there are now 9 CB banks and more than 500 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. 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 Japan 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 have passed the follow-up review in September 2001 and we are now working in this system. 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 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. Effect on the Rate of Engraftment of the Removal of Cryoprotectant from Thawed Cord Blood Units

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In CB transplantation, washing out of DMSO (dimethyl sulfoxide) from cryopreserved CB unit has been recommended to insure the early engraftment. We showed previously, direct exposure of the 10% DMSO containing unit against large volume of saline after thawing *in vitro* resulted in the lower recovery of colony forming unit, although no difference of engraftment of human $CD45^+$ cells in mice model. The removal of cryoprotectant from thawed cord blood unit by the step-by dilution method (wash) is originally recommended by New York Blood center to reduce the osmotic injury of cells by DMSO. However, none of the clinical follow-up studies has been done about it. To prove the effect of washing out DMSO in clinic, we analyzed eighty-five patients aged from 0.5 to 58 years old, who received CBT from September 1998 to September 30, 2001 released from Tokyo Cord Blood Bank. Thawed CB cells were washed with a 2.5% human albumin and 5% Dextran 40 solution. In thirty five adult patients, 14 patients were directly infused (non-washed) after thawing with mean nucleated cells (NCs)/kg; 2.6±0.8/kg and twenty one patients infused the step-by diluted unit (washed) after thawing with mean NCs/kg; 2.6±0.5/kg. In fifty children, 34 patients received non-washed CB with NCs 5.6±3.8/kg and 16 patients were infused washed CB with mean NCs/kg; 6.8±3.2/kg, respectively. We did not see the significant difference of the incidence of engraftment and speed of myeloid and platelet recovery among the two groups in adults. Over all survival and event free survival (EFS) was also not significantly different in non-washed vs. washed group. Only we could see some tendency of the effect of total colony forming counts (CFC)/kg on the incidence and engraftment speed, although not significant yet. In children, the speed of recovery was not significant different between two groups. But the incidence of engraftment in children aged from 2 to 5 years including high-risk group showed 40%, significant lower than other ages. We found also neutrophil recovery in children was delayed in high-risk leukemia group (Standard group was 26 days of median recovery vs. high risk group was 34 days respectively). Overall we found that the status of leukaemia at CBT strongly affects on the EFS. Conclusively our data suggested

that two-step dilution washing method did not affect the speed of engraftment itself clinically, although follow-up studies are necessary because of multiple backgrounds.

3. Activation and expansion of natural killer cells in umbilical cord blood with interleukin-15 in combination with Flt3-ligand

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Graft versus leukemia/tumor (GVL/T) effects after stem cell transplantation has been focused on recently, because of high incidence of relapse in high-risk leukemia patients. Unlike cytotoxic T cell, natural killer (NK) cells are known as non-HLA restricted, non-tumor specific cytotoxic activity. Expansion and activation of NK cells might greatly contribute to GVL/T effects and engraftment after stem cell transplantation. On the other hands, gene targeted mice of Flt3 ligand (Flt3L) and interleukin-15 (IL-15) reported to have the low number of NK cells, while the targeted mice of interleukin-2 (IL-2) known as activator of NK cells showed normal NK cells. We have studied the effect of IL-15 and Flt3L on NK cell expansion and activation. CB-derived mononuclear cells (MNCs) or NK cells sorted by depletion of non-NK cells (NK cell Isolation kit, Miltenyi Biotec, ISA) using AUTOMACS were cultured for 2 to 3 weeks with RPMI containing various dose of Flt3L and IL-15. After 15 days and 22days, we counted the viable cell number and performed flow cytometric analysis. RT-PCR was done for the expression of functional molecules after sorting. MNCs rather than purified NK cells responded to proliferate more efficiently, showing attachment to the bottom of the culture dishes. Maximal folds induction of NK cell are 5 to 10 in 2 weeks culture 40 to 80% of total viable cells. Surface markers after culture with IL-15 and Flt3L are

strong CD56 positive, LFA-1 positive, VLA-4 positive and perforin positive. RT-PCR showed the induction of FasL and Granzyme B. Cytotoxic activity against K562 and Jurkat was also recognized, which was inhibited by Concanamycin A (perforin inhibitor). These results indicated that CB-derived NK cells may contribute to the clinical use for anti-leukemia therapy.

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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Both adhesion molecules and chemokine receptors play a critical role in hematopoietic stem/progenitor cells (HS/PCs) engraftment and hematopoietic reconstitution. We herein hypothesized that some defects of adhesion molecules and chemokine receptors expressed on HS/PCs in umbilical CB might account for delayed hematopoietic reconstitution of CB transplantation (CBT) recipients. So we compared levels of the following adhesion molecule and chemokine receptor expressions on CB-, mobilized peripheral blood (mPB) and bone marrow (BM)-derived CD34^{bright} cells using four-color FACS analysis. CB-, mPB- and BM-derived CD34^{bright} cells expressed strongly CD44, CD11a, CD18, CD62L, CD31 and CD49d; meanwhile, significantly lower expressions of CD49e, CD49f and CD54 on UCB-derived CD34^{bright} cells, compared with those of mPB-, and BM-derived CD34^{bright} cells, were observed as follows: CD49e, 21.1±12.7% vs 81.5±19.8% ($p<0.001$, compared to the former, same for follows) vs 75.0±25.5% ($p<0.005$); CD49f, 4.2±6.5% vs 72.5±13.3 ($p<0.001$) vs 64.5±20.2% ($p<0.001$); and CD54, 26.0±21.4% vs 75.1±11.5% ($p<0.005$) vs 37.2±16.1% ($p<0.05$). Different chemokine receptor expression patterns were revealed: CB (but not for mPB- and BM)-derived CD34^{bright} cells expressed CCR-3, CCR-2 and CCR-1; the most striking finding is the significantly different expressions of CXCR-4 among them: 5.4±5.3% vs 20.7±11.9% ($p<0.05$) vs 67.6±5.8% ($p<0.001$). None of them expressed CCR-5, CXCR-1, CXCR-2, CXCR-3, and CXCR-5. Our results indicate that different adhesion molecule and chemokine receptor expression patterns among CB-, mPB- and BM-derived CD34^{bright} cells might partly explain the cause(s) of delayed recovery after CBT.

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

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To clarify the feasibility and efficacy of administering autologous monocyte-derived dendritic cells (DCs) for patients with malignant melanoma and thyroid carcinoma, we used tumor lysate-pulsed DCs as tumor vaccine in ten and five stage IV patients, respectively. Autologous monocytes were harvested from 15 liter of pheresis products and cultured with GM-CSF and IL-4 for 7 days to generate immature DCs. Immature DCs were then pulsed with autologous tumor lysate (10 mg/ml) and subsequently cultured with TNF-α for another 4 days for maturation. $1x10^7$ of mature DCs were injected intradermly once a week for ten times. These clinical trial are now in progress.

6. Phenotypic and functional characterization of umbilical/placental cord blood monocyte-derived dendritic cells

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Dendritic cells (DCs) are important antigen-presenting cells required for initiation of the immune response and potentially valuable tools for immunotherapy. We compared phenotypic and functional characteristics of cord blood monocyte-derived DCs (CBMo-DCs) and peripheral blood monocyte-derived DCs (PBMo-DCs) to clarify whether CBMo-DCs are available for DC therapy. Immature CBMo-DCs generated from adherent monocytes cultured for 7 days in the presence of 50 ng/ml GM-CSF and 50 ng/ml IL-4 showed that the expression levels of CD1a, CD11c and CD40 were lower (CD1a; 98±50, CD11c; 323±38, CD40; 43±25 at each MFI value) than immature PBMo-DCs (CD1a; 130±25, CD11c; 463±41, CD40; 56±28), while the levels of CD86 and HLA-DR revealed higher (CD86; 910±31, HLA-DR; 1150±28) than immature PBMo-DCs (CD86; 430±51, HLA-DR: 916±36). Mature CBMo-DCs subsequently cultured

with 50 ng/ml TNF-α for another 4 days had similar intensities of these two molecules to immature CBMo-DCs and mature PBMo-DCs. Immature CBMo-DCs had almost the same capacity of endocytosis for FITC-Dextran and LY as immature PBMo-DCs. The chemotactic migratory responses of CBMo- and PBMo-DCs to RANTES or MIP-3β indicated the same activities. The ability of mature CBMo-DCs to stimulate T cells in an allogeneic mixed lymphocyte reaction was significantly higher ($p<0.05$) than that of mature PBMo-DCs. Furthermore, this result was also observed in immature stage. Determination of cytokine mRNA levels by RT-PCR revealed that both immature CBMo- and PBMo-DCs stimulated by LPS induced the production of IFN-γ, GM-CSF, IL-6 and IL-12 p40. These results suggested that CBMo-DCs appear to function as well as PBMo-DCs and possibly to be an acceptable source for DC therapy.

7. Multileneage potential of Human Placenta-Derived Mesenchymal Cells

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We cultured freshly isolated mesenchymal cells from human placenta, and analyzed their cell biological features *in vitro*. Cells separated using trypsin/EDTA were cultured for 10-14 days, then stained by fluorescence-conjugated antibodies for flow cytometric analyses. Placenta-derived cells were separated into 3 populations; $CD34^-CD45^+$ cells, $CD34^+CD45^{dull}$ cells, and $CD34^-CD45^-SH2^+$ cells. $CD34^-CD45^-SH2^+$ cells showed the fibroblastic shapes after sorting following by few days culture. Most of these $CD34^-CD45^-SH2^+$ cells were stained by Hoechst 33342 (G0/G1) and less than 1% of these cells belonged to Hoechst Red (660/20)/Hoechst Blue (424/20 nm) double negative cells (side population, SP). These $CD34^-CD45^-SH2^+$ cells could be introduced to the neuronlike cells. RT-PCR analysis showed that these placenta-derived cells expressed constitutionally mRNA of some growth factor receptors; bone morphogenic protein 4 receptor type II (BMP4RII), c-met, and retinoic acid receptor type α, in addition to rennin mRNA. Taken together, we showed that there were mesenchymal progenitor cells with multilineage potential in human placenta. These cells must be very useful as the mesenchymal cell source for regenerative medicine in addition to CB hematopoietic stem cells with known information about HLA type and infectious disease.

Publications

De Rosa, S.C., Mitra, D.K., Watanabe, N., Herzenberg, L.A., Herzenberg, L.A. and Roederer, M. Vδ1 and Vδ2 γδ T cells express distinct surface markers and might be developmentally distinct lineages. J. Leukoc. Biol. 70: 518-526, 2001.

Enomoto, M., Nagayama, H. and Takahashi, T.A. Enhancement of migratory and aggregate activities of human peripheral blood monocyte-derived dendritic cells by stimulation with RANTES. Microbiology and Immunology. 2002 (in press)

Igura, K., Ohta, T., Kuroda, Y. and Kaji, K. Resveratrol and quercetin inhibit angiogenesis *in vitro*. Cancer Letters, 171: 11-16, 2001.

Kashiwakura, I., Murakami, M., Inanami, O., Hayase, Y., Takahashi, T.A., Kuwabara, M. and Takagi, Y.
Effects of amifostine on the proliferation and differentiation of megakaryocytic progenitor cells. Eur. J. Pharmacol. 2002 (in press)

Kashiwakura, I., Murakami, M., Takagi, Y., Inanami, O., Kuwabara, M. and Takahashi, T.A. Radioprotective synergistic action of cytokine on the clonal growth of X-irradiated human megakaryocytic progenitor cells. Blood, 98, Suppl. 1: 117b, 2001.

Murakami, M., Kashiwakura, I., Hayase, Y., Takahashi, T.A. and Takagi, Y. Inhibitory Effect of Murine Kidney Extracts on Human Mast Cells derived from Human Placental and Umbilical Cord Blood. Renal Failure. 2002 (in press)

Nagamura, F. and Nagamura-Inoue, T. Autologous bone marrow transplantation for CML. Nippon Rinsho 59: 2369-74, 2001.

Nagamura-Inoue, T., Shioya, M., Sugo, M., Cui, Y., Kikuchi, H., Takahashi, A., Kodo, H., Asano, S. and Takahashi, T.A. Effect on the rate of engraftment of the removal of cryoprotectant from thawed cord blood units. Blood, 98, Suppl. 1: 199a, 2001

Nagamura-Inoue, T., Tsujimura, H., Tamura, T., Takahashi, T.A. and Ozato, K. ICSBP-dependent induction of macrophage differentiation and inhibition of granulocytes differentiation augmented by interferon-γ. Blood, 98, Suppl. 1: 287a, 2001

Nagamura-Inoue, T., Tamura, T. and Ozato, K. Transcription factors that regulate growth and differentiation of myeloid cells. Int. Rev. Immunol. 20: 83-105, 2001.

Watanabe, N., Tomizawa, M., Tachikawa-Kawana, A., Goto, M., Ajisawa, A., Nakamura, T. and Iwamoto, A. Quantitative and qualitative abnormalities in HIV-1-specific T cells. AIDS, 15: 711-715, 2001.

Watanabe, N., De Rosa, S.C., Chao, N.J., Herzenberg, L.A., Herzenberg, L.A. and Roederer, M. Dynam-

ics of cytokine and perforin expression in T cells after allogeneic bone marrow transplantation: possible predictors of GVHD. Blood, 98, Suppl. 1: 661a, 2001.

Zhang, F., Romano, P.R., Nagamura-Inoue, T., Tian, B., Dever, T.E., Mathews, M.B., Ozato, K. and Hinnebusch, A.G. Binding of double-stranded RNA to protein kinase PKR is required for dimerization and promotes critical autophosphorylation events in the activation loop. J. Biol. Chem. 276: 24946-58, 2001.

Division of Hematopoietic Factors (Chugai)

Our major projects are (1) investigation of signal transduction of cytokine receptors, (2) identification and characterization of novel cytokines, cytokine receptors, soluble factors, and transcription factors, (3) study on the roles of small GTPases and GAPs, (4) analysis on the molecular mechanism of leukemogenesis, particularly in leukemias resulted from chromosomal translocations, 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, cytokine receptors, and transcription factors using retrovirus-mediated expression cloning strategies, (2) to identify and characterize oncogenic forms of signaling molecules 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. 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, and 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

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Secreted and cell-surface proteins play essential roles in cell-cell interaction. 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 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.

3. Development of retrovirus vectors and packaging cell lines

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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.

4. 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 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.

5. Molecular cloning and functional characterization of a human novel type I cytokine receptor related to delta1/TSLPR

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In a search for a human sequence related to a recently identified type I cytokine receptor *delta1*, which turned out to be a receptor subunit for a cytokine called TSLP (thymic stromal lymphopoietin), we have now identified a novel human type I cytokine receptor from a human T lymphocyte cDNA library. The deduced amino acid sequence of 371residues has a typical signal sequence and a membrane-spanning region. The mature protein is predicted to have a molecular mass of 39,698 Da. It has common characteristics of type I cytokine receptor family members, and we tentatively termed this protein CRLF2, which stands for cytokine receptor-like factor 2. Northern blot analysis revealed *CRLF2* mRNA in liver, kidney, heart, and skeletal muscle. The fetal liver also expresses *CRLF2* transcripts. The gene for CRLF2 was mapped to the pseudoautosomal region, Xp22.3 and Yp11.3 by FISH analysis, a region where genes encoding the IL-3 receptor α and the GM-CSF receptor α chains are also located. CRLF2 has recently been found to constitute the receptor subunit for human TSLP, together with the α subunit of the IL-7 receptor.

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

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In the search for key molecules that prevent murine M1 leukemic cells from undergoing IL-6-induced differentiation into macrophages, we isolated an antisense cDNA that encodes full-length mouse MgcRacGAP through functional cloning. In human HL-60 leukemic cells, overexpression of the human MgcRacGAP induced growth suppression and macrophage differentiation. Analysis using the mutants revealed that the GAP activity was dispensable, but the myosin-like domain and the cysteine-rich domain were indispensable for growth suppression and macrophage differentiation. Interestingly, MgcRacGAP localized to the nucleus in interphase, accumulated to the mitotic spindle in metaphase, and was condensed in the midbody during cytokinesis. Overexpression of an N-terminal deletion mutant resulted in the production of multinucleated cells in HeLa cells. This mutant lost the ability to localize in the mitotic spindle and midbody. MgcRacGAP was also found to bind α-, β-, and γ-tubulins through its N-terminal myosin-like domain. These findings indicate that MgcRacGAP dynamically moves during cell cycle progression probably through binding to tubulins and plays critical roles in cytokinesis. Furthermore, using a GAP-inactive mutant, we have disclosed that the GAP activity of MgcRacGAP is required for cytokinesis, suggesting that inactivation of Rho family GTPases may be required for normal progression of cytokinesis. We have recently found that MgcRacGAP is phosphorylated by some of the kinases that are known to work in the midbody.

7. 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 was dispensable for cell growth.

8. 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, in press). 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*.

9. 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, Yasuhide Hayashi[12], Tomohiko Taki[12], Yuki Kataoka[7], Nobuaki Yoshida[7], Toshio Kitamura, 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* knockout mice.

10. STAT5 induces macrophage differentiation of M1 leukemia cells through activation of IL-6 production mediated by NF-kB p65

Toshiyuki Kawashima, Ken Murata, Yukio Tonozuka, Yukinori Minoshima, Tetsuya Nosaka, and Toshio Kitamura

Using a constitutively active STAT5A (STAT5A1*6), we have shown that STAT5 induces macrophage differentiation of mouse leukemic M1 cells through a distinct mechanism, autocrine production of IL-6. The supernatant of STAT5A1*6-transduced cells contained sufficient concentrations of IL-6 to induce macrophage differentiation of parental M1 cells, and STAT3 was phosphorylated on their tyrosine residues in these cells. Treatment of the cells with anti-IL-6 blocking antibodies profoundly inhibited the differentiation. We have also found that the STAT5A1*6 transactivated the IL-6 promoter, which was mediated by the enhanced binding of NF-κB p65 (RelA) to the promoter region of IL-6. These findings indicate that STAT5A cooperates with Rel/NF-κB to induce production of IL-6, thereby inducing macrophage differentiation of M1 cells in an autocrine manner. In summary, we have shown a novel mechanism by which STAT5 induces its pleiotropic functions.

11. A novel secreted form of immune suppressor factor with high homology to vacuolar ATPases identified by a forward genetic approach of functional screening based on cell proliferation

Edgardo E Tulin, N Onoda[13], M Maeda[13], M Hasegawa[13], Hideaki Nakajima, Atsushi Urano, Fumi Shibata, Tetsuya Nosaka, H Nomura[13], Shigetata Asano[11], and Toshio Kitamura:[13]Chugai Research Institute for Molecular Medicine

In the search for stromal-derived growth factors, we have identified a novel secreted short form of immune suppressor factor (ISF) using a combination of a genetic approach and retrovirus-mediated functional screening. This protein, which we termed ShIF, was isolated based on its ability to support proliferation of a mutant clone S21, which was established from Ba/F3 cells that are usually interleukin-3-dependent but became dependent on a stroma cell line ST2 after chemical mutagenesis. ISF, a membrane protein harboring six transmembrane domains, was reported to have immunosuppressive functions. The coding region of ShIF started from the third transmembrane domain of ISF. Biochemical analysis demonstrated that ShIF was expressed in both the secreted and membrane-bound forms of 27-kDa protein, which was supposed to have an internal ATG present in the third transmembrane domain of ISF as a start codon. In addition to the full-length form of ISF, a major protein with a molecular size of

27 kDa was also expressed through the proteolytic process of ISF. ShIF resembles this naturally occurring short form of ISF (sISF). Deletion analysis of the major domains of ISF cDNA revealed that ShIF is an active functional domain of ISF with a capability to support proliferation of S21 cells. Enforced expression of ShIF in MS10 cells, bone marrow stroma cells that do not express endogenous ShIF or ISF, conferred on the cells an ability to support the growth of S21 cells as well as bone marrow cells. Interestingly, ShIF shows a high sequence homology to the C-terminal part of a 95-kDa yeast vacuolar H (+)-ATPase subunit, Vph1p (39%), and a 116-kDa proton pump (VPP1) (54%) of the rat and bovine synaptic vesicle. Therefore, it is possible that ShIF also acts as a proton pump and somehow prevents the cells from undergoing apoptosis. We are currently examining the effects of ISF and ShIF on the growth of hematopoietic progenitor cells from bone marrow, and also trying to identify the molecules that interact with these factors.

12. Genetic approach and phenotype-based complementation screening for identification of stroma cell-derived proteins involved in cell proliferation

Edgardo E Tulin, N Onoda[13], M Hasegawa[13], Tetsuya Nosaka, H Nomura[13], and Toshio Kitamura

The functional capacities of stromal cell lines to support stem cell activity are heterogeneous and the mechanism of how they support bone marrow cultures remains unclear. Recently, we reported a strategy of functional analysis in which a genetic approach is combined with phenotype-based complementation screening to search for a novel secreted growth factor from mouse bone marrow stroma called ShIF that supported proliferation of bone marrow cells. To investigate the role of stromal cells in hemopoiesis, we extended this strategy to search for stroma-derived proteins that induce cell proliferation by establishing stroma-dependent Ba/F3 mutants of three stroma cell lines from two mouse tissues. Seven stroma-dependent Ba/F3 mutants were used as responder cells to identify cDNAs from stroma cell lines whose products supported proliferation not only to the mutant cells but also to hemopoietic progenitor cells *in vitro*.

13. SF20/IL-25, a novel bone marrow stroma-derived growth factor that binds to mouse thymic shared antigen-1 and supports lymphoid cell proliferation

Edgardo E Tulin, N Onoda[13], Y Nakata[13], M Maeda[13], M Hasegawa[13], H Nomura[13], and Toshio Kitamura

Using a forward genetic approach and phenotype-based complementation screening to search for factors that stimulate cell proliferation, we have isolated a novel secreted bone marrow stroma-derived growth factor, which we termed SF20/IL-25. This protein signals cells to proliferate via its receptor, which we have identified as mouse thymic shared Ag-1 (TSA-1). Enforced expression of TSA-1 in IL-3-dependent Ba/F3 cells that do not express endogenous TSA-1 rendered cells to proliferate in a dose-dependent manner when stimulated with SF20/IL-25. FDCP2, a factor-dependent hemopoietic cell line that expresses endogenous TSA-1, could also be stimulated to proliferate with SF20/IL-25. Binding of SF20 to TSA-1 was blocked by anti-TSA-1 antibody and SF20-induced proliferation of TSA-1-expressing cells was inhibited by anti-TSA-1. *In vitro* assay revealed that SF20/IL-25 has no detectable myelopoietic activity but supports proliferation of cells in the lymphoid lineage.

14. Role of co-repressors in STAT5-dependent transcription

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STAT5 is a latent transcription factor activated by a variety of cytokines including IL-3, GM-CSF and erythropoietin. To gain more insight into the molecular mechanism how STAT5 regulates the variety of cytokine responses, we set out to explore the proteins that interact with STAT5 *in vivo*. We employed yeast two-hybrid screening to look for STAT5-interacting molecules, and identified silencing mediator for retinoid and thyroid hormone receptor (SMRT) as a potential binding partner. SMRT interacted with both STAT5A and 5B, and the association was detected both *in vitro* and *in vivo*. Interestingly, SMRT strongly repressed STAT5-dependent transcription both on heterologous and native promoters in reporter assays. To further clarify the physiological role of this interaction, we created a stable cell line overexpressing SMRT. In clear contrast to parental cell line, expression of STAT5 target genes in this cell line was not sustained and was quickly suppressed within 2 hours after normal initial phase of induction. Conversely, histone deacetylase inhibitor, Trichostatin A effectively enhanced and prolonged induction of STAT5-target genes in a parental cell line. Extensive mutational and binding analysis revealed that this interaction was mediated through N-terminal coiled-coil region of STAT5. Surprisingly, previously identified point mutation in the coiled-coil region that makes STAT5 hyperactive, disrupted Stat5-

SMRT interaction, suggesting overall transcriptional activity of STAT5 is determined by the balance of co-activators and corepressors bound to it. In addition, above data predicts that naturally occurring dominant negative mutant of STAT5 lacking carboxyl-transactivation domain interacts only with SMRT, but not with CBP, suggesting that it acts as dominant negative by actively repressing target genes through SMRT. Together, this study illuminates the potential role of SMRT in negative regulation of STAT5-dependent transcription, and reveals a novel crosstalk between nuclear receptor and JAK-STAT signaling pathways.

15. Granulocyte colony-stimulating factor regulates myeloid differentiation through CCAAT/enhancer-binding protein ε

Hideaki Nakajima and James N Ihle[14]

Granulocyte colony-stimulating factor (G-CSF) is a major cytokine that regulates proliferation and differentiation of myeloid cells, although the underlying mechanisms by which G-CSF controls myeloid differentiation are largely unknown. Differentiation of hematopoietic cells is regulated by lineage-specific transcription factors, and gene-targeting studies previously revealed the critical roles of CCAAT/enhancer-binding protein C/EBPα and C/EBPε, respectively, in the early and mid-late stages of granulocyte differentiation. The expression of C/EBPε in 32Dcl3 cells and FDCP1 cells expressing mutant G-CSF receptors was examined and it was found that G-CSF up-regulates C/EBPε. The signal for this expression required the region containing the first tyrosine residue of G-CSF receptor. Dominant-negative signal transducers and activators of transcription 3 blocked G-CSF-induced granulocytic differentiation in 32D cells but did not block induction of C/EBPε, indicating that these proteins work in different pathways. It was also found that overexpression of C/EBPε greatly facilitated granulocytic differentiation by G-CSF and, surprisingly, that expression of C/EBPε alone was sufficient to make cells differentiate into morphologically and functionally mature granulocytes. Overexpression of c-myc inhibits differentiation of hematopoietic cells, but the molecular mechanisms of this inhibition are not fully understood. In 32Dcl3 cells overexpressing c-myc that do not differentiate by means of G-CSF, induction of C/EBPε is completely abrogated. Ectopic expression of C/EBPε in these cells induced features of differentiation, including changes in nuclear morphologic characteristics and the appearance of granules. The data show that C/EBPε constitutes a rate-limiting step in G-CSF-regulated granulocyte differentiation and that c-myc antagonizes G-CSF-induced myeloid differentiation, at least partly by suppressing induction of C/EBPε.

16. Identification of factor(s) supporting self renewal of primate embryonic stem cells

Takuya Sugiyama, Atsushi Urano, Tetsuya Nosaka, and Toshio Kitamura

Dissection of molecular nature of embryonic stem (ES) cells may promote our understanding of cellular pluripotency and inner cell mass (ICM) development, and also can assist ES-based tissue engineering. Both mouse and human ES cells require feeder layer cells to retain the undifferentiated state. Whereas mouse ES cells were reported to remain undifferentiated without feeder cells in the presence of leukemia inhibitory factor (LIF), primate ES cells without feeder cells do differentiate even in the presence of LIF. Our goal is to identify the feeder-derived factor(s) supporting undifferentiated state and growth of the primate ES cells, through cDNA expression cloning. This project is collaboration with Drs. Suemori and Nakatsuji at Kyoto University.

Publications

Hirose, K., Kawashima, T., Iwamoto, I., Nosaka, T., and Kitamura, T. MgcRacGAP is involved in cytokinesis through associating with mitotic spindle and midbody. J. Biol. Chem. 276: 5821-5828, 2001.

Kawashima, T., Murata, K., Akira, S., Tonozuka, Y., Minoshima, Y., Feng, S., Kumagai, H., Tsuruga, H., Ikeda, Y., Asano, S., Nosaka, T., and Kitamura, T. STAT5 induces macrophage differentiation of M1 leukemia cells through activation of IL-6 production mediated by NF-kB p65. J. Immunol. 167: 3652-3660, 2001.

Kamizono, S., Hanada, T., Yasukawa, H., Minoguchi, S., Kato, R., Minoguchi, M., Hattori, K., Morita, S., Kitamura, T., Kato, H., Nakayama, K., and Yoshimura, A. The SOCS box of SOCS-1 accelerates ubiquitin-dependent proteolysis of TEL-JAK2. J. Biol. Chem. 276: 12530-12538, 2001.

Kagami, S., Nakajima, H., Suto, A., Hirose, K., Suzuki, K., Morita, S., Kato, I., Saito, Y., Kitamura, T., and Iwamoto, I. Role of Stat5a in T Helper Cell Differentiation. Blood 97: 2358-2365, 2001.

Tonozuka, Y., Fujio, K., Sugiyama, T., Nosaka, T., Hirai, M., and Kitamura, T. Molecular cloning of a human novel type I cytokine receptor related to Delta1. Cytogenet. Cell Genet. 93: 23-25, 2001.

Ishido, M., Yamamoto, A., Kitamura, T., Nagayama,

K., and Ohashi, M. Cholesterol requirement for receptor transport from multivesicular late endosomes to the Golgi. J. Cell Sci. 114: 1965-1976, 2001.

Tulin, E.E., Onoda, N., Maeda, M., Hasegawa, M., Nosaka, T., Nomura, H., Asano, S., and Kitamura, T. A novel secreted form of immune suppressor factor with high homology to vacuolar ATPase identified by forward genetic approach of functional screening based on cell proliferation. J. Biol. Chem. 276: 27519-27526, 2001.

Kimura, M., Koseki, Y., Yamashita, M., Watanabe, N., Shimizu, C., Katsumoto, T., Kitamura, T., Taniguchi, M., Koseki, H., and Nakayama, K. Regulation of Th2 cell differentiation by mel-18, a mammalian polycomb group gene. Immunity 15: 275-287, 2001.

Senga, T., Iwamoto, T., Kitamura, T., Miyake, Y., and Hamaguchi, M. JAK/Stat3 dependent activation of RalGDS/Ral pathway in M1 mouse myeloid leukemic cells. J. Biol. Chem. 276: 32678-32681, 2001.

Frasor, J., Park, K.-S., Byers, M., Telleria, C., Kitamura, T., Yu-Lee, L., Djiane, J., Park-Sarge, O.-K., and Gibori, G. Differential roles for Stat5α and Stat5β in prolactin stimulation of estrogen receptor a and b transcription. Mol. Endcrinol. 15: 2172-2181, 2001.

Yamamoto, S., Nagao, M., Kitamura, T., Nakatomi, H., Sugimori, M., Kosako, H., Yamamoto, N., Takebayashi, H., Weinmaster, G., Nakamura, K., and Nakafuku, M. Transcription factor expression and Notch-dependent regulation of neural progenitors in the adult rat spinal cord. J. Neurosci. 21: 9814-9823, 2001.

Yamamoto, S., Yamamoto, N., Kitamura, T., Nakamura, K., and Nakafuku, M. Proleferation of parenchymal neural progenitors in response to injury in the adult rat spinal cord. Exp. Neurol., 172: 115-127, 2001.

Ye, S.K., Agata, Y., Lee, H.C., Kurooka, H., Kitamura, T., Shimizu, A., Honjo, T. and Ikuta, K. The IL-7 receptor controls the accessbility of the TCRγ locus by Stat5 and histone acetylation. Immunity 15: 813-823, 2001.

Tokoro, Y., Shibuya, K., Osawa, M., Tahara-Hanaoka, S., Iwama, A., Kitamura, T., Nakauchi, H., and Shibuya, A. (2001) Molecular cloning and characterization of mouse tspan-3, a novel member of the tetraspanin superfamily, expressed on resting dendritic cells. Biochem. Biophys. Res. Commun. 288: 178-183.

Yamashita, Y., Kajigaya, S., Yoshida, K., Ueno, S., Ota, J., Ohmine, K., Ueda, M., Miyazato, A., Ohya, K., Kitamura, T., Ozawa, K., and Mano, H. Sak serine-threonine kinase acts as an effector of Tec tyrosine kinase. J. Biol. Chem. 276: 39012-39020, 2001.

Ueno, M., Igarashi, K., Kimura, N., Okita, K., Takizawa, M., Nobuhisa, I., Kojima, T., Kitamura, T., Morgan, S.M., Shiomura, T., Suda, T., Nakashima, K., and Taga, T. Identification of an embryonic dorsal aorta specific gene, endomucin, and its inhibitory activity on cell adhesion. Biochem. Biophys. Res. Commun. 287: 501-506.

Tulin, E.E., Onoda, N., Nakata, Y. Hasegawa, M., Nomura, H., and Kitamura, T. SF20/IL-25, a novel bone marrow stroma-derived factor that binds to mouse TSA-1 and supports lymphoid cell proliferation. J. Immunol. 167: 6338-6347, 2001.

Tulin, E.E., Onoda, N., Hasegawa, M., Nosaka, T., Nomura, H. and Kitamura, T. Genetic approach and phenotype-based complementation screening for identification of stromal cell-derived proteins involved in cell proliferation. Exp. Cell Res. in press.

Tulin, E.E., Onoda, N., Hasegawa, M., Nomura, H. and Kitamura, T. Inhibition of human endothelial cell proliferation by ShIF, a vacuolar H+-ATPAse-like protein. Oncogene in press.

Nakamura, T., Ouchida, R., Kodama, T., Kawashima, T., Watanabe, S., Kitamura, T., Morimoto, C., and Tanaka, H. Cytokine receptor common β subunit-mediated STAT5 activation confers NFκB activation through IκB-independent mechanism in a murine pro B cell line Ba/F3. J. Biol. Chem. in press.

Nosaka, T. and Kitamura, T. Pim-1 expression is sufficient to induce cytokine independence in murine hematopoietic cells, but is dispensable for BCR-ABL-mediated transformation. Exp. Hematol. in press.

Nosaka, T. Jaks and normal hematopoiesis. In The JAK-STAT pathway in hematopoiesis and disease. edited by Ward, A. C. (Landes Bioscience Publishers Eurekah Com.) in press.

Nakajima, H., Brindle, P.K., Handa, M., and Ihle, J.N. Functional interaction of STAT5 and nuclear receptor co-repressor SMRT: implications in negative regulation of STAT5-dependent transcription. EMBO J. 20: 6836-6844, 2001.

Nakajima, H. and Ihle, J.N. Granulocyte colony-stimulating factor regulates myeloid differentiation through CCAAT/enhancer-binding protein ε. Blood 98: 897-905, 2001.

Zang, H., Sato, K., Nakajima, H., McKay, C., Ney, P.A., and Ihle, J.N. The distal region and receptor tyrosines of the Epo receptor are non-essential for *in vivo* erythropoiesis. EMBO J. 20: 3156-66, 2001.

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.

Our priority is analyses of the molecular causality of the common metabolic disorders of civilization, in other word, identification of the causal gene. 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. Subarachnoid hemorrahge (SAH) due to rupture of intracranial aneurysm (IA)

Taku Yoneyama, Toshiaki Nakajima, and Ituro Inoue

Rupture of intracranial aneurysms (IA) causes subarachnoid hemorrhage, a devastating condition with high morbidity and mortality. Angiographic and autopsy studies show that IA is a common disorder with prevalence of 3-6%. 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. Although IA has a substantial genetic component, there has been little attention to the genetic determinants. We report here a genome-wide linkage study of IA in 104 Japanese affected sib-pairs in which positive evidence of linkage at chromosome 5q22-31 (MLS=2.24), 7q11 (MLS=3.22), and 14q22 (MLS=2.31) were found. The best evidence of linkage is detected at *D7S2472*, in the vicinity of the elastin gene (*ELN*), a candidate gene for IA. Fourteen distinct SNPs were identified in *ELN*, and no obvious allelic association between IA and each SNP was observed. The haplotype between the intron 20 and 23 polymorphism of *ELN* is strongly associated with IA ($P=3.81 \times 10^{-6}$), and homozygote patients are at high risk (P=0.002) , with odds ratio of 4.39. These findings suggest that a genetic locus for IA lies within or close to the *ELN* locus on chromosome 7.

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

Shingo Maeda, Toshihiro Tanaka, Kazuki Shimoonoda, Takahiro Nobukuni, 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 determinants associated with OPLL, we performed an extensive non-parametric linkage study with 126 affected sib-pairs using markers for various candidate genes by distinct analyses, SIBPAL and GENEHUNTER. 88 candidate genes were selected by comparing the genes identified by cDNA microarray analysis of systematic gene expression profiles during osteoblastic differentiation of human mesenchymal stem cells with the genes known to be involved in bone metabolism. Of the 24 genes regulated during osteoblastic differentiation, only one, the alpha B crystalline gene, showed evidence of linkage (P=0.016, NPL=1.83). Of 64 genes known to be associated with bone metabolism, 7 showed weak evidence of linkage by SIBPAL analysis (P<0.05): cadherin 13 (*CDH13*), bone morphogenetic protein 4 (*BMP4*), proteoglycan 1 (*PRG1*), transforming growth factor beta 3 (*TGFb3*), osteopontin (*OPN*), parathyroid hormone receptor 1 (*PTHR1*), and insulin-like growth factor 1 (*IGF1*). Among these genes, *BMP4* (NPL=2.23), *CDH13* (NPL=2.00), *TGFb3* (NPL=1.30), *OPN* (NPL=1.15), and *PTHR1* (NPL=1.00) showed evidence of linkage by GENEHUNTER. Only *BMP4* reached criteria of suggestive evidence of linkage. Since this gene is a well-known factor in osteogenetic function, *BMP4* should be screened in further study for the polymorphism responsible.

3. Linkage disequilibrium in the human genome for disease-gene discovery

Toshiaki Nakajima and Ituro Inoue

Linkage disequilibrium (LD) mapping for common diseases explicitly depend upon the persistence of LD. To understand the organization of LD in the human gene promises to have great impact on the strategies for identifying candidate genes. *AGT* constitutes the locus that consistently has been associated with essential hypertension (EHT). Variants associated with EHT, T235 and A(-6), vary in frequency among major ethnic groups. Sequencing of angiotensinogen gene in nonhuman primates shows that the T235 and A(-6) variants are fixed in these species. The protective M235 and G(-6) variants for EHT, that were in tight LD, are likely to have arisen during human evolution. We evaluated the organization of LD and haplotype in *AGT*. Haplotypes based on 21 SNPs showed that Caucasian and Japanese shared top four frequent haplotypes, even though the frequencies of them were quite different. In Caucasians G(-6)/M235 haplotype, protective haplotype for EHT, was very common. The difference of demographic history or natural selection might result in the increased frequency of G(-6)/M235 haplotype in Caucasian.

We compared LD parameters D′, r^2, and d^2 based on the data from 861 possible marker pairs in the *AGT*. d^2 is an LD measure for an appropriate choice for association studies. When compared between D′ and d^2, the value of d^2 varied widely in the marker pairs with $|D'|=1$. In cases with $|D'|=1$, the mean value of d^2 is 0.210 ± 0.270 and the proportion of marker pairs with $d^2<0.1$ (insufficient LD for association studies) is 0.473. These results showed that about half of cases with complete LD in the value $|D'|$ display insufficient LD ($d^2<0.1$) for association studies. Another common measure r^2 is compared with d^2. r^2 and d^2 were significantly correlated. In conclusion, an LD measure, r^2, is the better parameter than D′ in considering strategies for identifying genes for common disease.

4. Association Studies of Single Nucleotide Polymorphisms (SNPs) 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 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.

Publications

Nakajima, T., Jorde, LB., Ishigami, T., Umemura, S., Emi, M., Lalouel, J-M., Inoue, I. Nucleotide diversity and haplotype structure of the human angiotensinogen gene in two populations. *Am. J.*

Hum. Genet. in press, 2002.

Nakajima, T., Inoue, I., Cheng, T., and Lalouel, J-M. Molecular cloning and functional analysis of a factor that binds to the proximal promoter of human angiotensinogen. *J. Hum. Genet.* in press, 2002.

Onda, H., Kasuya, H., Yoneyama, T, Takakura, K., Hori, T., Takeda, J., Nakajima, T., and Inoue, I. Genome wide linkage and haplotype association studies map intracranial aneurysm to chromosome 7q11. *Am. J. Hum. Genet.* 69, 804-819, 2001.

Furushima, K., Shimo-onoda, K., Maeda, S., Nobukuni, T., Ikari, K., Koga, H., Komiya, S., Nakajima, T., Harata, S., Inoue, I. Large scale screening for candidate genes of ossification of the posterior longitudinal ligament of the spine. *J. Bone Miner. Res.* in press, 2002.

Mori, H., Ikegami, H., Kawaguchi, Y., Seino, S., Yokoi, N., Takeda, J., Inoue, I., Seino, Y., Yasuda, K., Hanafusa, T., Yamagata, K., Awata, T., Kadowaki, T., Hara, K., Yamada, N., Gotoda, T., Iwasaki, N., Iwamoto, Y., Sanke, T., Nanjo, K., Oka, Y., Matsutani, A., Maeda, E., Kasuga, M. Association of the Pro12-Ala substitution in peroxisome proliferator-activated receptor γ2 both with resistance to development of diabetes and with impairment of insulin secretion and disease severity in individuals with type 2 diabetes mellitus. *Diabetes* 50, 891-894, 2001.

The Study Group of Comprehensive Analysis of Genetic Factors in Diabetes Mellitus (Kanamori, M., Wang Y-J, Takeda, J., Inoue, I., Sanke, T., Nanjo, K., Mori, H., Kasuga, M., Hara, K., Kadowaki, T., Tanizawa, Y., Oka, Y., Iwami, Y., Ohgawara, H., Yasuda, K., Yamada, Y., Seino, Y., Yokoi, N., Yano, H., Seino, S.) S20G mutation of the amylin gene is associated with type 2 diabetes in Japanese. *Diabetologia* 44, 906-9, 2001.

Maeda, S., Ishidou, Y., Koga, H., Taketomi, E., Ikari, K., Komiya, S., Takeda, J., Sakou, T., and Inoue, I. Functional impact of human collagen α 2 (Xl) gene polymorphism in pathogenesis of ossification of the posterior longitudinal ligament of the spine. *J. Bone. Miner. Res.* 16, 948-957, 2001.

Aihara, Y., Onda, H., Teraoka, H., Yokoyama, Y., Seino, Y., Kasuya, H., Hori, T., Tomura, H., Inoue, I., Kojima, I., and Takeda, J. Assignment of SLC17A6 (alias DNPI), the gene encoding brain/pancreatic islet-type Na^+- dependent inorganic phosphate cotransporter to human chromosome 11p14.3. *Cytogenet. Cell Genet.* 92, 167-169, 2001.

Havelka, S., Vesela, M., Pavelkova, A., Halman, L., Ruzickova, S., Koga, H., Maeda, S., Inoue, I. Are diffuse idiopathic skeletal hyperostosis (DISH) and ossification of the posterior longitudinal ligament of the spine (OPLL) genetically related? *Annal. Rheum. Dis.* 60: 902-903, 2001.

Yabe, I., Sasaki, H., Yamashita, I., Tashiro, K., Tatei, A., Suzuki, Y., Kida, H., Takiyama, Y., Nishizawa, M., Hokezu, Y., Nagamatsu, K., Oda, T., Ohnishi, A., Inoue, I., and Hata, A. Predisposing chromosome of spinocerebellar ataxia type 6 (SCA6) in Japanese. *J. Med. Genet.* 38, 328-333, 2001.

Nishigori, H., Tomura, H., Kanamori, M., Yamada, S., Kikuchi, N., Sho, K., Tonooka, N., Onigata, K., Inoue, I., Kojima, I., Yamagata, K. Yang, Q., Matsuzawa, Y., Kohama, T., Miki, T., Seino, S., Kim, M., Choi, H., Moore, D., Takeda, J. Mutations in the small heterodimer partner gene are associated with mild obesity in Japan. *Proc. Natl. Acad. Sci. USA* 98, 575-580, 2001.

Takeoka, S., Unoki, M., Onouchi, Y., Doi, S., Fujiwara, H., Miyatake, A., Fujita, K., Inoue, I., Nakamura, Y., and Tamari, M. Amino-acid substitutions in the IKAP gene product significantly increase risk for bronchial asthma in children. *J. Hum. Genet.* 46, 57-63, 2001.

Maeda, S., Koga, H., Matsunaga, S., Numasawa, T., Takeda, J., Harata, S., Sakou, T., Inoue, I. Gender-specific haplotype association of collagen α 2 (Xl) gene in ossification of the posterior longitudinal ligament of the spine. *J. Hum. Genet.* 46, 1-4, 2001.

Iwasaki H, Shinohara Y, Ezura Y, Ishida R, Kodaira M, Kajita M, Nakajima T, Shiba T, Emi M. Thirteen single-nucleotide polymorphisms in the human osteopontin gene identified by sequencing of the entire gene in Japanese individuals. *J. Hum. Genet.* 46, 544-546, 2001.

Nakazawa I, Nakajima T, Ishigami T, Umemura S, Emi M. Linkage disequilibrium and haplotype analysis among eight novel single-nucleotide polymorphisms in the human tissue-type plasminogen activator (t-PA) gene. *J. Hum. Genet.* 46, 367-371, 2001.

Ota N, Nakajima T, Nakazawa I, Suzuki T, Hosoi T, Orimo H, Inoue S, Shirai Y, Emi M. A nucleotide variant in the promoter region of the interleukin-6 gene associated with decreased bone mineral density. *J. Hum. Genet.* 46, 267-272, 2001.

Nakazawa I, Nakajima T, Harada H, Ishigami T, Umemura S, Emi M. Human calcitonin receptor-like receptor for adrenomedullin: genomic structure, eight single-nucleotide polymorphisms, and haplotype analysis. *J. Hum. Genet.* 46, 132-136, 2001.

Shinohara Y, Iwasaki H, Ota N, Nakajima T, Kodaira M, Kajita M, Shiba T, Emi M. Novel single nucleotide polymorphisms of the human nuclear factor kappa-B 2 gene identified by sequencing the entire gene. *J. Hum. Genet.* 46, 50-51, 2001.

Kajita M, Iwasaki H, Ota N, Shinohara Y, Kodaira M, Nakajima T, Emi M. Novel single nucleotide polymorphisms of the human colony-stimulating factor 2 (CSF2) gene identified by sequencing the entire gene. *J. Hum. Genet.* 46, 48-49, 2001.

Iwasaki H, Ota N, Nakajima T, Shinohara Y, Kodaira M, Kajita M, Emi M. Five novel single-nucleotide polymorphisms of human interferon gamma identified by sequencing the entire gene. *J. Hum. Genet.* 46, 32-34, 2001.

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 failure, myelodysplastic syndrome and leukemia. To approach this subject, we study pathogenesis of "Fanconi anemia", a genetic disease characterized by chromosome instability and development of myeloid disorders. In addition, we have identified and are studying functions of novel molecules: (i) HSH2, an SH2 domain-containing protein which is likely to regulate cytokine signaling in hematopoietic cells, and (ii) ABT1/ABTAP, a protein complex in nucleoli and Cajal bodies, which is likely to regulate RNA transcription.

1. Mechanisms of FANCA phosphorylation

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

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 G) have been cloned. Increasing evidence indicates that the multiple FA proteins cooperate in a biochemical pathway and/or a multimer complex: FANCA is phosphorylated, forms a complex with other FA proteins including FANCC and FANCG in the cytoplasm, translocates into the nuclei, where the protein complex allows DNA damage-induced activation of FANCD2 into a mono-ubiquitinated isoform.

We previously reported that FANCA was phosphorylated in cells from normal controls, whereas the phosphorylation was defective in cells from multiple FA patients. In the present study, we have found 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. Identification of the protein kinase is expected to elucidate regulatory mechanisms which control the FA pathway.

2. Characterization of Patient-derived FANCA mutant proteins

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

FANCA, which accounts for a major population (60-70 %) of FA patients, is a hypermutable gene. More than 100 different mutations have been identified, most of which result in premature termination or large deletions (null-mutations). Group A patients

homozygous for null-mutations have a more severe clinical phenotype than those with mutations producing an altered protein. However, little has been studied on molecular basis of the genotype-phenotype correlation. Although 30 or more missense and small in-frame deletion mutants which are expected to produce an altered protein were reported, only a few have been characterized.

In the present study, twenty of the mutants were retrovirally expressed in FANCA(-) fibroblasts and extensively characterized. Some mutants (D598N, Q1128E, T1131A, F1262L, H1417D : Group I) corrected hypersensitivity to mitomycin C, a marker phenotype of FA cells, in these cells, whereas other mutants were inactive (R435C, H492R, L845P, FQ868-869del, R1055L, H1110P, F1135del, W1174del, F1239-43del, F1263del, W1302R : Group II) or partially active (L817P, P1324L, D1359Y, M1360I : Group III) in this function. Analyses of the FA pathway revealed that Group I mutants were similar to wt-FANCA in capabilities of phosphorylation, association with FANCC, localization to nuclei and induction of FANCD2 mono-ubiquitination. Pathogenicity of Group I mutants may be explained by reduced endogenous expression of these proteins. Alternatively, these mutations, especially when reported without identification of the second mutation, may be polymorphisms. By contrast, Group II and Group III mutants showed severe and mild deficiencies in the FA pathway, respectively. Activation of the FA pathway correlates with MMC sensitivity in the defined system, providing another line of evidence that this pathway plays a central role in the cellular phenotype of FA. The present results indicate that altered FANCA proteins are able to activate the FA pathway to various degrees, which may partly account for phenotypic variation of group A patients.

3. Cloning and functional analyses a novel SH2-containing adapter protein, HSH2

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

We isolated a cDNA clone encoding a novel Src homology (SH)2 domain-containing protein of 47 kD from a human cDNA library. As its transcript was predominantly expressed in hematopoietic cells, this gene was termed *HSH2* for hematopoietic SH2 protein. This protein contains several putative protein-binding motifs, SH3-binding proline-rich regions and phosphotyrosine sites, but lacks enzymatic motifs. In a yeast two-hybrid screen, we identified a cytokine-regulated tyrosine kinase c-FES and an activated Cdc42-associated tyrosine kinase ACK1 as HSH2 interactors. HSH2 bound c-FES via its C-terminal region as well as its N-terminal region including the SH2 domain, whereas it bound ACK1 via its N-terminal proline-rich region. Furthermore, these two kinases bound and tyrosine-phosphorylated HSH2 in mammalian cells. Hence, we postulate that HSH2 functions as an adapter protein involved in tyrosine kinase signaling, and possibly regulates cytokine signaling and cytoskeletal reorganization, in hematopoietic cells.

4. Identification and Functional analyses of ABT1/ABTAP, a novel protein complex in nucleoli and Cajal bodies

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

A basal transcription machinery constitutes of a number of proteins including a TATA-binding protein (TBP), TBP-associated factors (TAFs) and RNA polymerases. We recently identified a novel TBP-binding protein, ABT1, which is conserved from yeasts to mammals. Disruption of an ABT1 orthologue abolished growth/survival in *Saccharomyces cerevisiae*, suggesting that this molecule has an essential role in cell growth/survival of higher eukaryotes. An in vitro cell-free transcription assay as well as in vivo transcription assay in mammalian cells indicated that ABT1 stimulates basal transcription. However, little is known regarding regulatory mechanisms of ABT1. To elucidate the mechanism , we searched for binding proteins of ABT1 using a yeast two-hybrid screening. We isolated a cDNA of a mammalian nuclear protein, termed ABT1 associated protein (ABTAP) as an interactor of ABT1. Like ABT1, ABTAP is conserved from yeasts to mammals, and disruption of this gene abolished growth/survival of yeasts. Furthermore, the yeast orthologue of ABT1 and that of ABTAP interacted.

ABT1 localized to the nucleoli and small nuclear bodies, which were identified to be Cajal bodies based on their containing coilin and survival of motor neurons (SMN) protein. SMN is involved in genesis of snRNP and its defect causes a common infantile neuronal disease, Spinal Muscular Atrophy. When ABTAP was predominantly co-expressed with ABT1, the ABT1/ABTAP complex redistributed to small nuclear bodies without coilin or SMN, and ABT1-induced activation of Pol II basal transcription was suppressed. Increasing evidence indicates that Cajal bodies play an important role in assembly and transport/recycling of components involved in RNA transcription and processing. We postulate that the ABT1/ABTAP complex has an important regulatory role in RNA transcription and processing by dynamic translocation among nuclear structures, including nucleolus and Cajal body.

Publications

Yagasaki, H., Adachi, D., Oda, T., Garcia-Higuera, I., Tetteh, N., D'Andrea, A.D., Futaki, M., Asano, S., and Yamashita, T.: A Cytoplasmic Serine Protein Kinase Binds and May Regulate the Fanconi Anemia Protein FANCA. Blood. 98: 3650-3657, 2001

Oda, T., Muramatsu, M., Isogai, T., Masuho, Y., Asano. S., and Yamashita, T. : HSH2: A Novel SH2 Domain-containing Adapter Protein Involved in Tyrosine Kinase Signaling in Hematopoietic Cells. Biochem. Biophys. Res. Commun. 288: 1078-1086, 2001

Adachi, D., Oda,T., Yagasaki, H., Nakasato, K, Taniguchi, T., D'Andrea, A.D., Asano, S., and Yamashita, T. : Different activation of the Fanconi anemia pathway by patient-derived FANCA mutants. Blood. 98:215a, 2001

Division of Proteomics Research (ABJ Millipore)

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 gene function at the protein 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 of messenger RNA transcripts for analyzing expression profiles of genes and the use of yeast 2-hybrid screens for systematic protein interaction analysis. Proteomics probes both of these problems by direct analysis of proteins in cells or tissues. Typical studies include large-scale determination of quantitative changes in the expression levels of proteins to assess the effects of a wide variety of perturbations to cells, and comprehensive analysis of protein-protein interactions by mass identification of protein components in the functional protein complexes, membrane domains, and cellular organelles.

Besides the conventional methods for proteomics based on two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and mass spectrometry (MS), our laboratory is equipped with advanced liquid chromatography (LC)-based technologies to serve for functional proteomics.

* Multi-dimensional LC-MS/MS system that allows automatic identification of nearly a thousand protein components in functional membrane domains and organelles.

* Direct nano-flow LC-MS/MS system that allows identification of ~100 protein components within one hour, with a femtomole amount of functional complexes pull-downed from cultured cells with tagged protein as a bait.

* BIA-LC-MS/MS system for real time monitor of protein interactions by surface plasmon resonance sensor and simultaneous identification of interacting molecules by MS.

By use of these technologies as analytical platforms, we will study on the signaling networks of proteins and their spatiotemporal regulation during fundamental cellular processes such as differentiation, growth, and apoptosis of cells.