The 21st Century COE program of Tokyo University

Promotion of Genome-Based Medicine Project

研究拠点形成　ゲノム医療プロジェクト推進

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For the application of human genome data to clinics, we have been working on four projects. The four projects include, 1) prediction of sensitivity to anticancer drugs, 2) clarification of mechanisms underlying human carcinogenesis, 3) development of novel diagnostic and therapeutic strategies of human cancers, and 4) genetic diagnosis of a human disease, hereditary non-polyposis colon cancer (HNPCC). These projects are aimed to provide better diagnosis, effective treatment, and prevention of human cancer.

1. Prediction of sensitivity of gefitinib to lung cancer, and that of imatinib to CML.

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In our earlier study, we investigated expression profiles of lung adenocarcinomas that were treated with gefitinib (Iressa), which identified 12 genes that can discriminate tumors with sensitivity to the drug from those without sensitivity. Two other groups reported that genetic alteration of *EGFR*, the target of gefitinib, was associated with the efficacy. Since these studies and ours analyzed a limited number of clinical samples, sensitivity and reliability of the two prediction methods remain unresolved. Therefore, we started a prospective study to analyze both expression profile and *EGFR* mutation of tumor tissues prior to treatment with gefitinib, in collaboration with Applied Medicine, and Department of Infectious Diseases and Applied Immunology, in Research Hospital, IMSUT. An outpatient clinic for consultation of the applicants was opened in Research Hospital, IMSUT in September 2004, and department of respiratory medicine, Kawasaki Medical University joined to this project in 2005. In 2006, seven patients with lung cancer were enrolled in this study after informed consent was obtained. Tumor specimens from the seven patients were obtained by TBLB, but five were not analyzed because they did not contain enough number of cancer cells. Finally we analyzed expression profiles and genetic alteration of *EGFR* in one of the two cases, and genetic alteration alone in the other.

In another study, we investigated expression profiles of 26 chromic myeloid leukemia (CML) with high-sensitivity and those with low-sensitivity to imatinib (Glivec), and identified a

total of 79 genes differently expressed between high- and low-sensitivity groups. We developed a prediction system of the sensitivity using expression of 15 genes among the 79. For the application of the findings into clinics, we launched a prospective study to evaluate the system, and analyzed expression profiles of additional CML samples in collaboration with Applied Medicine in Research Hospital, IMSUT. An outpatient clinic for consultation of applicants was opened in June, 2004, and six patients with CML visited the clinic. After written informed consent was obtained, blood samples were taken from the patients. FISH and expression profile analyses were carried out, and calculated prediction score of the sensitivity to imatinib was reported to the patients in the clinic.

2. Comparison of gene-expression profiles between Opisthorchis viverrini- and non-Opisthorchis viverrini-associated intrahepatic cholangiocarcinoma

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Intrahepatic cholangiocarcinoma (ICC) is the second most common primary cancer in the liver, and its incidence is highest in the northeastern part of Thailand. ICCs in this region are known to be associated with infection with liver flukes, particularly *Opisthorchis viverrini* (OV), as well as nitrosamines from food. To clarify molecular mechanisms of ICC associated with or without liver flukes, we analyzed gene-expression profiles of OV-associated ICCs from 20 Thai patients, and compared their profiles with those of 20 Japanese ICCs that were not associated with OV, by means of laser-microbeam-microdissection and a cDNA microarray containing 27,648 genes. We identified 77 commonly upregulated genes and 325 commonly downregulated genes in the two ICC groups. Unsupervised hierarchical cluster analysis separated the 40 ICCs into two major branches almost completely according to the fluke status. The putative signature of OV-associated ICC exhibited elevated expression of genes involved in xenobiotic metabolism (*UGT2B11, UGT1A10, CHST4, SULT1C1*), while that of non-OV-associated ICC represented enhanced expression of genes related to growth factor signaling (*TGFBI, PGF, IGFBP1, IGFBP3*). Additional random permutation tests identified a total of 52 genes whose expression levels were significantly different between the two groups. We also identified genes associated with macroscopic type of ICCs. These data may not only contribute to clarification of common and OV-specific mechanisms underlying ICC, but also serve as a starting point for the identification of novel diagnostic markers and/or therapeutic targets for the disease.

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

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To identify novel target molecules for the diagnosis and treatment of human tumors arising in various gastrointestinal organs, we have been studying their global gene expression profiles using genome-wide cDNA microarray. Profiles of colorectal cancer (CRC), gastric cancer, hepatocarcinoma (HCC), and intrahepatic cholangiocarcinoma (ICC) have been obtained.

Using expression profiles of CRC, we selected three candidate genes for clinical application. First gene, annotated as *C10orf3*, showed elevated expression in colorectal cancer as well as in cancers arising in the stomach, lung, pancreas, and breast. This gene encoded a putative 464-amino acid protein containing a domain known as AAA (ATPases associated with a variety of cellular activities). Western blot analysis using an antibody to C10orf3 protein confirmed that the protein was over-expressed in 9 of 15 colorectal cancer tissues examined, compared to corresponding non-cancerous epithelial cells. Subsequent proteomics analysis revealed that C 10orf3 interacted with the product of tumor susceptibility gene 101 (TSG101), and that C10orf3 down-regulated TSG101 in a posttranscriptional manner. Expression of short interfering RNA caused significant decrease of *C10orf3* expression in colorectal cancer cells, and inhibited growth of the transfected cells, which was associated with an increase of apoptotic cells. The second gene was termed *TOMM34* (34kDa-Translocase of the outer mitochondrial membrane). Immunohistochemical staining revealed significant accumulation of TOMM34 protein in CRC tissues compared with their corresponding non-cancerous mucosae. Transfection of HCT116 colon cancer cells with short-interfering RNA (siRNA) specific to *TOMM34*, effectively suppressed its expression, and drastically inhibited their growth. The third gene was *FAM84A*. Northern blot analysis revealed that it was expressed in none of 22 normal tissues examined except the testis. Although immunocytochemical staining revealed localization of FAM84A protein in the sub-cellular membrane region of can-

cer cells, the staining was observed limitedly in the region lacking the attachment with neighboring cells. In addition, we found that exogenous FAM84A expression increased cell motility in NIH3T3 cells, and that phosphorylation of serine38 of FAM84A was associated with morphology of cells. These data suggest that elevated expression of C10orf3, TOMM34, and FAM84A should play an essential role in the growth or migration of cancer cells, and that suppression of their function may be a novel therapeutic strategy to human CRC.

Among the genes up-regulated in HCC compared with their non-tumor liver tissues, we focused on a novel gene termed *TIPUH1* that putatively encoded a 500-amino-acid protein containing 12 zinc-finger domains and a KRAB domain. Multiple-tissue northern blot analysis revealed testis- and placenta-specific expression of *TIPUH1* in normal tissues. Colony formation assay in soft agar showed that TIPUH1 conferred anchorage-independent growth to NIH3T3 cells, suggesting its oncogenic activity. Notably, specific siRNA to *TIPUH1* knocked down its expression in HCC cells, which resulted in their growth inhibition. We identified four TIPUH1-interacting proteins including TIF1β, a transcription-intermediary protein, and three involved in pre-mRNA processing (hnRNPU, hnRNPF, and Nucleolin), suggesting that overexpressed TIPUH1 may play a role in hepatocarcinogenesis by regulating transcription and/or RNA processing of growth control genes. These data may contribute to a better understanding of liver carcinogenesis, and the development of novel strategies for treatment of HCCs.

Among the genes whose expression was commonly elevated in ICC, we identified a novel gene termed *RASGEF1A* that encodes a putative Ras-GEF domain-containing protein. We showed that RASGEF1A protein has a guanine nucleotide exchange activity to K-RAS, H-RAS, and N-RAS proteins *in vitro*. Consistently, exogenous RASGEF1A expression increased the activity of RAS. In addition, suppression of *RASGEF1A* by small interfering RNA retarded the growth of cholangiocarcinoma cells. Interestingly, COS7 cells expressing exogenous *RASGEF1A* showed enhanced cellular motility in transwell and wound-healing assays. These data suggest that elevated expression of RASGEF1A may play an essential role for proliferation and progression of ICC, and that RASGEF1A may be a novel therapeutic target for ICC.

4. Genetic diagnosis of HNPCC

Yoichi Furukawa

Hereditary non-polyposis colorectal cancer (HNPCC) is an autosomal dominant hereditary disease accompanied by tumors arising mainly in the colon and other associated organs, such as stomach, renal pelvis, and endometrium. The frequency of HNPCC in Caucasian patients with colorectal cancer is estimated between two and five percent. However the frequency in Japanese patients with colorectal cancer remains undetermined. Therefore, Japanese Study Group for Colorectal Cancer started a collaborative project of registration of Japanese HNPCC patients and genetic analysis of mutations in *MSH2*, *MLH1*, and *MSH6*, the responsible genes for HNPCC. All patients with colorectal cancer and those who are diagnosed as HNPCC by Amsterdam's II criteria in the collaborative hospitals have been registered, and the frequency of HNPCC in registered patients with colon cancer has been determined. Collaborating to this project, we have analyzed genetic alteration in a total of 115 patients using direct sequencing and Multiplex Ligation-dependent Probe Amplification. Among the 115 cases, 8 cases were analyzed in 2006. The data will provide valuable information for the understanding of the frequency, penetrance and phenotypes of Japanese HNPCC. The results will be also used for genetic diagnosis of affected family members of the probands.

Publications

1. Ura, K., Obama, K., Satoh, S., Sakai, Y., Nakamura, Y. and Furukawa, Y. Enhanced RASGEF1A expression is involved in the growth and migration of intrahepatic cholangiocarcinoma. Clin. Cancer Res. 12: 6611-6616, 2006.
2. Jinawath, N., Chamgramol, Y., Furukawa, Y., Obama, K., Tsunoda, T., Sripa, B., Pairojkul, C. and Nakamura, Y. Comparison of gene expression profiles between opisthorchis viverrini and non-opisthorchis viverrini associated human intrahepatic cholangiocarcinoma. Hepatology 44: 1025-1038, 2006.
3. Shimokawa, T., Matsushima, S., Tsunoda, T., Tahara, H., Nakamura, Y. and Furukawa, Y. Identification of TOMM34, which shows elevated expression in the mojority of human colon cancers, as a novel drug target. Int. J. Oncol. 29: 381-386, 2006.
4. Kobayashi, T., Masaki, T., Sugiyama, M., Atomi, Y., Furukawa, Y. and Nakamura, Y. A gene encoding a family with sequence simi-

larity 84, member A (FAM84A) enhanced migration of human colon cancer cells. Int. J. Oncol. 29: 341-347, 2006.
5. Suda, T., Tsunoda, T., Uchida, N., Watanabe, T., Hasegawa, S., Satoh, S., Ohgi, S., Furukawa, Y., Nakamura, Y. and Tahara, H. Identification of secernin 1 as a novel immunotherapy target for gastric cancer using the expression profiles of cDNA microarray. Cancer Sci. 97: 411-419, 2006.
6. Silva, F.P., Hamamoto, R., Furukawa, Y. and Nakamura, Y. TIPUH1 encodes a novel KRAB zinc-finger protein highly expressed in human hepatocellular carcinomas. Oncogene 25: 5063-5070, 2006.
7. Hamamoto, R., Silva, F.P., Tsuge, M., Nishidate, T., Katagiri, T., Nakamura, Y. and Furukawa, Y. Enhanced SMYD3 expression is essential for the growth of breast cancer cells. Cancer Sci. 97: 113-118, 2006.
8. Hirata, K., Kanemitsu, S., Nakayama, Y., Nagata, N., Itoh, H., Ohnishi, H., Ishikawa, H., Furukawa, Y and HNPCC registry and genetic testing project of the Japanese Society for Cancer of the Colon and Rectum (JSCCR). A novel germline mutation of MSH 2 in a hereditary nonpolyposis colorectal cancer patient with liposarcoma. Am. J. Gastroenterol. 101: 193-196, 2006.
9. Obama, K., Kato, T., Hasegawa, S., Satoh, S., Nakamura, Y. and Furukawa, Y. Overexpression of peptidyl-prolyl isomerase-like1 is associated with the growth of colon cancer cells. Clin. Cancer Res. 12: 70-76, 2006.
10. Sakai, M., Shimokawa, T., Kobayashi, T., Matsushima, S., Yamada, Y., Nakamura, Y. and Furukawa, Y. Elevated expression of C 10orf3 (Chromosome 10 open reading frame 3) is involved in the growth of human colon tumor. Oncogene 25: 480-486, 2006.

The 21st Century Center of Excellence (COE) Program

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

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Our major goal is to dissect regulation of hematopoiesis - self-renewal of hematopoietic stem cells (HSC) and the regulation of hematopoietic cell differentiation by transcription factors-, and translate this knowledge into clinical settings to provide novel therapies for patients with hematological diseases.

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

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Immune suppressor factor (ISF) is a subunit of the vacuolar ATPase proton pump. We earlier identified a short form of ISF (ShIF) as a stroma-derived factor that supports IL-3-independent growth of a mutant subline of Ba/F3 cells. Here we report that ISF/ShIF not only supports a mutant Ba/F3, but also self-renewal and expansion of primary hematopoietic stem cells (HSC). Co-culture of murine bone marrow cells with a stromal cell line overexpressing ISF or ShIF (MS 10/ISF or MS10/ShIF) significantly enhanced their colony-forming activity and the numbers of long-term culture initiating cells (LTC-IC). Moreover, competitive repopulating activity of c -Kit^{+}Sca-1^{+}Lin^{-} HSC was significantly maintained by co-cultivation with MS10/ISF or MS10 /ShIF. These stem cell supporting activities were abolished in the proton pump mutant of ISF/ ShIF, indicating that proton transfer across cellular or endosomal membrane was critical. Gene expression analysis of ISF/ShIF-transfected cell lines revealed downregulation of secreted frizzled related protein (SFRP)-1 and tissue inhibitor of metalloproteinase-3 (TIMP-3), and the restoration of SFRP-1 and TIMP-3 expressions in MS10/ISF cells partially reversed its enhanced LTC-IC supporting activity to a normal level. These results suggest that ISF/ShIF supports HSC by modulating Wnt-activity and the extracellular matrix, and provide new insights in HSC physiology *in vivo*.

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

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

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

by stromal cells. HSC-stromal cell interaction is thought to provide unknown signals to keep HSC in immature state and makes them undergo extensive self-renewal. However, molecular mechanism of these processes is poorly understood. We are trying to address this question by following approaches. 1) Identify cell surface molecules that are expressed on hematopoietic stem cells and bone marrow stromal cells by utilizing a variety of technologies (i.e. signal sequence trap, mRNA subtraction) and analyze their function in vitro and in vivo. 2) Identify secreted proteins (i.e. cytokine, growth factor, extracellular matrix protein) from the bone marrow stroma that are potentially important for HSC regulation. From the first approach, we identified novel HSC marker, Robo4 that is specifically expressed in the primitive HSCs. From the latter approach, we identified TIMP-3, SFRPs, and Slit2 as candidate proteins, potentially regulating HSC homeostasis in the bone marrow niche. Further analysis will lead to a better understanding of stromal cell-mediated regulation of HSC self-renewal, proliferation, and differentiation.

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

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

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

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

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CCAAT/ enhancer binding protein α (C/EBPα is a member of the bZIP family of transcription factors that plays a critical role for early myeloid differentiation. C/EBPα knockout mice showed a complete differentiation block at myeloblast stage in hematopoietic system, and mature neutrophils and eosinophils are absent in the peripheral blood. Lineage specification in

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

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

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

Publications

1. Nakajima H, Shibata F, Fukuchi Y, Goto-Koshino Y, Ito M, Urano A, Nakahata T, Aburatani H, Kitamura T.: Immune suppressor factor confers stromal cell line with enhanced supporting activity for hematopoietic stem cells. Biochem Biophys Res Commun. 340:35-42, 2006.
2. Oki T, Kitaura J, Eto K, Lu Y, Maeda-Yamamoto M, Inagaki N, Nagai H, Yamanishi Y, Nakajima H, Kumagai H, Kitamura T.: Integrin alphaIIbbeta3 induces the adhesion and activation of mast cells through interaction with fibrinogen. J Immunol. 176: 52-60, 2006.
3. Nakajima H, Watanabe N, Shibata F, Kitamura T, Ikeda Y, Handa M.: N-terminal region of CCAAT/ enhancer binding protein ε is critical for cell cycle arrest, apoptosis and

functional maturation during myeloid differentiation. J Biol Chem. 281: 14494-14502, 2006.
4. Nakajima H, Shibata F, Kumagai H, Shimoda K, Kitamura T.: Tyk2 is dispensable for induction of myeloproliferative disease by mutant FLT3. Int J Hematol. 84: 54-59, 2006.
5. Fukuchi Y, Shibata F, Ito M, Goto-Koshino Y, Sotomaru Y, Ito M, Kitamura T, Nakajima H.: Comprehensive analysis of myeloid lineage conversion using mice expressing an inducible form of C/EBPα. EMBO J. 25: 3398-3410, 2006.
6. Ozaki K, Hishiya A, Hatanaka K, Nakajima H, Wang G, Hwu P, Kitamura T, Ozawa K, Leonard WJ, Nosaka T.: Overexpression of interleukin 21 induces expansion of hematopoietic progenitor cells. Int J Hematol. 84: 224-30, 2006.

Leading Project of the Regenerative Medicine

Project of Developmental Stem Cells
文部科学省　再生医療の実現化プロジェクト
幹細胞探索研究領域

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The mission of this project is to understand basic principles in stem cell biology and to explore tools applicable to stem-cell therapy. To this end, we have been determined to work on mouse hematopoietic stem cells (HSCs) as a model system for adult stem cells because HSCs are currently used in human bone marrow transplantation, and are expected more to be used in many other clinical settings. Stem cells are defined as cells capable of self-renewal and multilineage differentiation. Like many other normal cells, stem cells basically neither survive nor proliferate without extracellular stimulation. Mouse HSCs have been an excellent experimental model to study stem cell regulation because of the existence of established methods for their functional identification. We have attempted to understand which molecules directly or indirectly stimulate HSCs, how HSCs respond to such stimuli in terms of signaling, what molecular mechanisms are for HSCs to divide numerous times while maintaining self-renewal division, and finally what differentiation is all about. All these studies are devoted to the discovery of a kind of the Holy Grail in hematology and stem cell biology: a protocol for ex vivo expansion of HSCs.

1. Establishment of single-cell immunostaining procedures for mouse HSCs

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HSCs represent on the order of one in 10^4-10^5 bone marrow cells in adult mice. This scarcity of HSCs has made application of conventional biochemical analysis to HSCs almost impossible. We have developed a novel immunostaining method by which very small numbers of primary cells can be efficiently examined. The point in successful single-cell immunostaining turned out to be very simple. Cells should be kept in a liquid phase throughout the procedures so that analyzable cell numbers can be maximized, and cell shapes can remain intact. The procedures principally consist of (1) Direct sorting of cells into a droplet of buffer by flow cytometry, (2) Cell binding to the surface of a glass slide, (3) Cell fixation and permealization in a droplet of buffer, and (4) Staining of cells with antibodies in a droplet of buffer.

Using this method we are now able to study signal transduction events in HSCs. Not only the expression of proteins, but also the intracellular mobilization of membrane proteins and signal molecules can be analyzed. Moreover, signaling can be quantified by measuring fluorescent intensity using anti-phosphoprotein antibodies (SCIPhos assay). Data from signal transduction analysis by this method are seemingly correlated well with those by Western blot

analysis. Confocal laser scanning is suitable for studying intracellular localization of molecules of interest. Laser scanning cytometry is suitable for quantification of fluorescent intensities. We believe that this method will be widely used for studies of a variety of rare normal and malignant cells primarily isolated from individuals.

2. Lnk negatively regulates the probability of self-renewal in HSCs

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One of the central tasks in stem cell biology is to understand how stem cell fate i.e. self-renewal, differentiation or apoptosis is determined. We have addressed this issue using a mouse HSC model. Although *in vivo* self-renewal of HSCs has been extensively studied, its *in vitro* recapitulation remains difficult. We previously reported that HSCs undergo asymmetrical self-renewal division in culture with stem cell factor (SCF) and thrombopoietin (TPO). Since then, we have sought any condition in which HSCs can symmetrically self-renew. In this study we found that *in vitro* symmetrical self-renewal division occurs in HSCs deficient in the adaptor protein Lnk. Lnk-deficient mice have $>$ 10-fold HSCs due to increased self-renewal capacity. We found that Lnk-deficient HSCs are hypersensitive to TPO. Repopulating activity in HSCs increased after culture either with TPO or with SCF and TPO, but not with SCF alone. Paired daughter cell-experiments clearly showed that a significant portion of Lnk-deficient HSCs, as compared with wild-type HSCs, can undergo symmetrical self-renewal in the presence of SCF and TPO in their first divisions. These data suggest that Lnk acts downstream of TPO/Mpl signaling as a negative regulator for the probability of self-renewal. We further investigated TPO-mediated signal transduction pathways in HSCs. To this end, we used the single-cell immunostaining as briefly described above. We detected enhanced up-regulation in STAT5 and Akt signaling, and inversely enhanced down-regulation of p38 MAPK signaling in Lnk-deficient HSCs, as compared with wild-type HSCs. These data suggest that these combinational changes in signal transduction is associated with self-renewal in HSCs.

3. Identification of pre-HSCs using an *in vitro* HSC differentiation system from mouse ES cells

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One of the reasons why we are also interested in the development of HSCs is that we want to understand the mechanism underling a tremendous expansion of HSCs during embryonic development. We have recently shown that self-renewal capacity in adult HSCs is extensive but also limited. This may not be the case for developing HSCs in the embryo. Pre-HSCs can be defined as precursor cells of HSCs. In our hypothesis, pre-HSCs give rise to adult HSCs through embryonic and fetal HSCs. Embryonic HSCs are represented by HSCs arising in the aorta-gonad-mesonephros (AGM) region. Fetal HSCs are represented by HSCs residing in the fetal liver. Fetal HSCs should be almost comparable to adult HSCs in several aspects. However, pre-HSCs and embryonic HSCs should differ from adult HSCs, for instance, in homing, repopulating, and self-renewing capacities. We also assume that extra- and intra-cellular regulatory mechanisms are very different among these HSCs. To address these issues, we first need to isolate pre-HSCs and embryonic HSCs to be characterized.

Previously many studies have attempted to determine the anatomical origin of HSCs: the Yolk sac or the AGM region. In this study we instead studied ontogeny of HSCs in the context of differentiation pathways of HSCs from mesodermal precursors. We used an *in vitro* ES differentiation system to captivate *in vivo* development of HSCs. Kyba et al. were the first who showed that HSCs could efficiently develop *in vitro* from ES cells when HOXB4 is enforcedly expressed among them. We successfully identified target cells for HOXB4 expression needed in the embryoid body (EB) that had been formed by ES cells. Such cells were c-Kit-positive and CD41-positive (Kit^+CD41^+) cells. When HOXB4 was conditionally expressed, Kit^+CD41^+ cells acquired long-term repopulating activity in myeloablated adult mice. After HOXB4 expression, these cells tended to remain as Kit^+CD41^+ cells, but up-regulated expression levels of Bmi-1 and GATA2. Interestingly the majority of hemangioblast activity was detected in Kit^+CD41^- cells but not in Kit^+CD41^+ cells among EB cells. Moreover, most vascular endothelial precursor activity was also detected in Kit^+CD41^- cells.

From these data, we propose that Kit^+CD41^+

EB cells are pre-HSCs standing at the branching point from the hemagioblast lineage. Kit^+CD41^- EB cells possibly represent YS hematopoiesis. Further work is required to clarify whether Kit^+ $CD41^+$ pre-HSCs exist in early embryos.

Publications

Ema, H., Morita, Y., Yamazaki, S., Matsubara, A., Seita, Y., Tadokoro, Y., Takano, H., Takaki, S. and Nakauchi, H. Adult mouse hematopoietic stem cells: Purification and single-cell assays. Nat Protoc 1: 2979-2987, 2006.

Seita, J., Ema, H., Ooehara, J., Yamazaki, S., Tadokoro, Y., Yamazaki, A., Eto, K., Takaki, S., Takatsu, K., and Nakauchi, H. Lnk negatively regulates self-renewal of hematopoietic stem cells by modifying thrombopoietin-mediated signal transduction. Proc Natl Acad Sci USA *in press*, 2006.

Morita, Y., Ema, H., Yamazaki, S., and Nakauchi, H. Non-side-population hematopoietic stem cells in mouse bone marrow. Blood *108*: 2850-2856, 2006.

Furuta, C., Ema, H., Takayanagi, S-i., Ogaeri, T., Okamura, D., Masui, Y., and Nakauchi, H. Discordant developmental waves of angioblasts and hemangioblasts in the early gastrulating mouse embryo. Development *133*: 2771-2779, 2006.

Yamazaki, S., Iwama, A., Takayanagi, S-i., Morita, Y., Eto, K., Ema, H., and Nakauchi, H. Cytokine signals modulated via lipid rafts mimic niche signals and induce hibernation in hematopoietic stem cells. EMBO J *25*: 3515-3523, 2006.

Leading Project of the Regenerative Medicine

Donation Laboratories and Research Units Laboratory of Stem Cell Regulation

文部科学省　再生医療の実現化プロジェクト 幹細胞制御領域

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The major goal of our laboratory is to understand how stem cells are regulated and how we can apply this knowledge for stem-cell based regenerative medicine. Stem cells can differentiate into tissue-specific cells to repair damaged tissue, a process controlled in part by the microenvironment. Proteases, as part of the microenvironment act as processing enzymes that perform highly selective and limited cleavage of specific substrates including growth factors and their receptors, cell adhesion molecules, cytokines, chemokines, apoptotic ligands and angiogenic factors. Over the last year we studied the role of proteases during stem cell mobilization (1) and determined how a CXCR4＋ hemangiocyte promotes tissue regeneration (2).

1) Increased soluble urokinase plasminogen activator receptor (suPAR) serum levels after granulocyte-colony stimulating factor treatment do not predict successful progenitor cell mobilization in vivo.

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The soluble urokinase-type plasminogen-activator receptor (suPAR) has been implicated to play a role in granulocyte colony stimulation factor (G-CSF)-induced stem cell mobilization. We examined the correlation between suPAR serum levels and a successful progenitor cell mobilization in allogeneic donors and autologous patients. Baseline levels of full length, suPAR(I-III) and cleaved suPAR forms, suPAR(II-III) and suPAR(I) were identical in both groups. Serum levels increased significantly after G-CSF administration correlating to white blood cell counts on the day of leukapheresis in allogeneic donors. Even though CD34＋ cell mobilization was equally effective for autologous patients, here the amount of shedded suPAR after a combined chemotherapy/growth factor application was clearly reduced and did not correlate with the leukocyte count. More importantly, high suPAR levels were not predictive for CD34＋ stem cell counts in both groups, challenging the recently proposed role for uPAR as a key player in G-CSF-mediated hematopoietic progenitor mobilization.

2) Cytokine-mediated deployment of SDF-1 induces revascularization through recruitment of CXCR4＋ hemangiocytes.

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The mechanism whereby hematopoietic cytokines accelerate revascularization is unknown. We show that the magnitude of cytokine-mediated release of SDF-1 from platelets and recruitment of non-endothelial CXCR 4 + VEGFR1+ hematopoietic progenitors, "hemangiocytes", constitute the major determinant of revascularization. Soluble Kit-ligand (sKitL), thrombopoietin (TPO), and to a lesser extent EPO and GM-CSF induce the release of SDF-1 from platelets, enhancing neoangiogenesis through mobilization of CXCR4+VEGFR1+ hemangiocytes. Although revascularization of ischemic hind-limbs was diminished in GM-CSF-/-G-CSF-/- mice, there was a profound impairment in neo-angiogenesis in thrombocytopenic TPO-/-, TPO-receptor-deficient (c-Mpl-/-) mice, and sKitL-deficient MMP9-/- mice. SDF-1-mediated mobilization and incorporation of hemangiocytes into ischemic limbs were impaired in TPO-/-, c-Mpl-/-, and MMP9-/- mice. Transplantation of CXCR4+ VEGFR1+ hemangiocytes into MMP9-/- mice restored revascularization, while inhibition of CXCR4 abrogated cytokine- and VEGF-A-mediated mobilization of CXCR4+VEGFR1+ cells and suppressed neo-angiogenesis. In conclusion, hematopoietic cytokines through graded deployment of SDF-1 from platelets support mobilization and recruitment of CXCR 4 + VEGFR1+ hemangiocytes, while VEGFR1 is essential for their angio-competency for augmenting revascularization. Delivery of SDF-1 may be effective in restoring neo-angiogenesis in patients with vasculopathies.

Publications

Jin DK, Shido K, Kopp HG, Petit I, Shmelkov SV, Young LM, Hooper ATA, Amano H, Avecilla ST, Heissig B, Hattori K, Zhang F, Hicklin DJ, Wu Y, Zhu Z, Salari H, Werb Z, Hackett NR, Crystal RG, Lyden D, Rafii, S. Cytokine-mediated deployment of SDF-1 induces revascularization through recruitment of CXCR4+ hemangiocytes. Nat Med. 12: 557-97, 2006

Fietz T, Hattori K, Thiel E, Heissig B. Increased soluble urokinase plasminogen activator receptor (suPAR) serum levels after granulocyte colony-stimulating factor treatment do not predict successful progenitor cell mobilization in vivo. Blood. 107(8): 3408-9, 2006

服部浩一．生体内血管新生および組織再生に関する新仮説−骨髄細胞動員を介した血管新生制御機構．実験医学　24⑱：54−60, 2006

秋山晴代，Heissig Beate，服部浩一．造血における骨髄血管ニッチの機能解析．分子細胞治療　5⑵：18−23, 2006

Centre for Asian Infectious Diseases

IMSUT Research Center for Infectious Diseases in China

中国における感染症研究拠点

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The Institute of Medical Science, University of Tokyo (IMSUT) has established Japan-China joint laboratories for research on emerging and re-emerging infectious diseases in Asia, in collaboration with the Chinese Academy of Sciences and Chinese Academy of Agricultural Sciences. In the laboratories, Japanese and Chinese scientists conduct research on the viral pathogenicity, the genetic variation of viruses in the field, structure of viral and host proteins, and so on.

BACKGROUND

Historically speaking, China is a very important neighbor of Japan. Official diplomatic delegations were first sent from Japan during the Sui dynasty some 1400 years ago. Since late 20th century, geopolitical and economical interdependence between Japan and China has developed substantially and will deepen further in the future. China is an enormous country often symbolically referred to as the dragon. While China is developing and transforming rapidly in the coastal regions, its rural areas have been left far behind. With regard to infectious diseases, China is beset with problems ranging widely from those of a developing country to those of dense urban environments. No one can discuss emerging and re-emerging infectious diseases (ERID) without mentioning China. Severe acute respiratory syndrome (SARS) emerged in Guangdong and shocked the world in 2003. With Lake Qinghai as a reference point, avian influenza expanded westward in the Eurasian continent in 2005 and reached Africa in February 2006. The carrier rate of hepatitis viruses is very high and HIV infection is rapidly increasing.

Given these situations, academic collaboration on research in infectious diseases would be beneficial to both countries, facilitate mutual understanding, and help strengthen the stable long-term relationship between the two peoples. Establishing joint research laboratories in China is particularly important because this would allow Japanese scientists access to possible emerging pathogens and to have an opportunity to fight against possible emerging infections. Supported by a contract research fund from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) (*Japan-China Collaboration on Emerging and Re-emerging Infectious Diseases*; MEXT Project Director: Aikichi Iwamoto), IMSUT has established two joint laboratories in Beijing in

collaboration with the Institute of Biophysics and Institute of Microbiology, Chinese Academy of Sciences (IBPCAS and IMCAS, respectively); a collaborative research program with Harbin Veterinary Research Institute (HVRI), the Chinese Academy of Agricultural Science; and IMSUT's project office in Beijing.

LABORATORIES AND PROJECT OFFICE

a. Laboratory of Structural Virology and Immunology (LSVI)

LSVI has been established on the third floor of Building 1 in IBPCAS (Director; Zihe Rao). IBPCAS, established in 1958, is one of the best institutes in China in the field of structural biology. It has been the leader of structural analyses of proteins of SARS corona virus. In LSVI, Japanese and Chinese scientists have started research in the several disciplines. One is on the structure of viral and host proteins, emphasizing its relation with their biological functions and pathogenicity. Another is the immunological analysis of interplay between the pathogens and hosts. Dr. Matsuda from IMSUT serves as a principal investigator (PI) in LSVI together with two Chinese PIs. Three Chinese scientists have been recruited to work in Dr. Matsuda's laboratory.

b. Laboratory of Molecular Immunology and Molecular Microbiology (LMIMM)

LMIMM was established in one of the old buildings of IMCAS (Director; George F. Gao). IMCAS, established in 1958, has served as the *Microbiological Resources Center-China* and *China General Microbiological Culture Collection Center*. Since Professor Gao was invited from Oxford University to take the directorship in 2004, IMCAS has been strengthening its ability in research on ERID such as avian influenza, viral hepatitis and HIV/AIDS. Dr. Kitamura from IMSUT serves as a PI in LMIMM together with two Chinese PIs. Four Chinese scientists have been recruited to work in Dr. Kitamura's laboratory. IMIMM has moved to a new IMCAS building in the Olympic Park campus of Chinese Academy of Sciences in January 2007.

c. Collaborative Research Program with HVRI

HVRI (Director, Xiangang Kong) serves as a research center focused on the study of avian influenza viruses in domestic fowl and wild waterfowl in China. Japanese scientists from IMSUT led by Yoshihiro Kawaoka, together with Chinese scientists at HVRI, carry out surveillance programs and study the genetic changes in avian influenza A viruses isolated in China. In July 2006, IMSUT appointed a researcher for this project who is evaluating the replication and transmission of H5N1 avian influenza viruses in a guinea pig model at Harbin. We are currently studying the genetic variation in H5N 1 viruses and analyzing their properties. The pathogenicity of the H5N1 viruses will be studied in a macaque model in a BSL3 laboratory at HVRI.

d. IMSUT Project Office

IMSUT set up its project office in Beijing. Drs. Yoshiike and Hayashi have been responsible for establishing joint laboratories in IBPCAS and IMCAS. They are playing a very important role for maintaining and strengthening the joint research and for the close contact with the IMSUT headquarters.

IMPLEMENTATION OF COLLABORATION

The collaboration is implemented on the basis of MOUs between IMSUT and the Chinese institutes. For the joint laboratories the implementation is being controlled during the 5-year-term of the project by a steering group composed of Dean of IMSUT (Tadashi Yamamoto), MEXT Project Director (Aikichi Iwamoto), and Directors of IBPCAS (Zihe Rao) and IMCAS (George F. Gao). For the collaborative program in Harbin the implementation is being controlled by a steering committee composed of Dean of IMSUT, Director of HVRI, and some scientists involved in the program.