The 21st Century Center of Excellence (COE) program

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 are currently 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.

Yoichi Furukawa, Yusuke Nakamura[1,2], Toyomasa Katagiri[1], Yataro Daigo[2], Noriharu Sato[3], Naoyuki Takahashi[3], Tsuyoshi Fujii[4]: [1]Laboratory of Genome Technology, Human Genome Center, IMSUT, [2]Laboratory of Molecular Medicine, Human Genome Center, IMSUT, [3]Applied Medicine, Research Hospital, IMSUT, [4]Department of Infectious Diseases and Applied Immunology, Research Hospital, IMSUT

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. A total of 17 patients with lung cancer visited the clinic. Among the 17, 12 passed the criteria of our prospective study, and nine were enrolled in this study after informed consent was obtained. Department of Respiratory Medicine, Kawasaki Medical University joined to this project this year, and four patients with lung cancer in Kawasaki Medical University Hospital were enrolled into the study. Tumor specimens from a total of 13 patients were obtained by TBLB, excision of lymph node, or needle aspiration biopsies with CT. Finally we analyzed expression profiles and genetic alterations of *EGFR* in eight of the 13 cases, and the results were reported to the patients.

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 four 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. Genome-wide analysis of gene expression in human intrahepatic cholangiocarcinomas

Yoichi Furukawa, Yusuke Nakamura[1]: [1]Laboratory of Molecular Medicine, Human Genome Center, IMSUT

Intrahepatic cholangiocarcinoma (ICC) is a neoplasm arising in the liver, and its incidence is increasing in Japan as well as in western countries. Prognosis of patients with this type of tumor remains unsatisfactory, because no effective chemotherapeutic drugs are available, we have no sensitive tumor markers to detect this tumor in its early stage, and it is difficult to identify a high-risk group for the disease. To clarify molecular mechanism of the tumors and identify molecular targets for their diagnosis and treatment, we analyzed global gene-expression profiles of 25 ICCs using tumor-cell populations purified by laser-microbeam microdissection and a cDNA microarray containing 27648 genes. As a result, we identified 52 genes that were commonly up-regulated, and 421 that were down-regulated in the ICCs compared to non-cancerous biliary epithelial cells. Among the 52 up-regulated genes, we corroborated enhanced expression of P-cadherin and survivin in cancer tissues by immunohistochemical staining. Furthermore, comparison between tumors with lymph-node metastasis and those without metastasis identified 30 genes that might associate with lymph-node involvement. These data should shed light on mechanisms of ICC, and provide clues for the development of diagnostic and therapeutic strategies for this type of tumor.

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

Yoichi Furukawa, Yusuke Nakamura[1], Ryuji Hamamoto[1]: [1]Laboratory of Genome Technology, Human Genome Center, IMSUT

We previously reported that *SMYD3* expression was markedly elevated in a great majority of human colorectal (CRC) and hepatocellular carcinomas (HCC), and that SMYD3 protein functions as histone H3 lysine-4 (H3-K4)-specific di- and tri-methyltransferase. Methylation of H3 -K4 is observed frequently in transcriptionally active regions of the human genome; trimethylation of H3-K4 changes the chromatin structure, leading to transactivation of target genes. We identified HELZ as a SMYD3-interacting protein, and showed that SMYD3 forms a complex with HELZ and RNA polymerase II. We further showed that SMYD3 binds to a DNA element containing "5′-CCCTCC-3′", and that SMYD3 up-regulates *Nkx2.8* through a direct interaction with the 5′ flanking region of *Nkx2.8*. Taken together, SMYD3 regulates transcription of downstream gene(s) by altering chromatin structure through histone modification and recruitment of RNA polymerase II complex. Importantly, introduction of *SMYD3* into NIH3T3 cells enhanced their growth, and its reduced expression by short-interfering RNA (siRNA) resulted in significant growth suppression of colorectal and hepatic cancer cells. These findings suggested that SMYD3 should be a promising therapeutic target for CRC and HCC.

In addition to CRC and HCC, we have revealed that SMYD3 expression is also elevated in a great majority of breast cancer, and that its up-regulation is crucial for the growth of breast cancer cells, because silencing of SMYD3 by siRNA resulted in the inhibited growth of breast cancer cells. We have further disclosed that proto-oncogene *WNT10B* is a direct target of SMYD3. Therefore, SMYD3 should be a novel target for the treatment of breast cancer as well.

Through the investigation of mechanisms underlying elevated SMYD3 expression, we identified, in its 5′ flanking region, a region that is responsible for its transactivation. The region contained a variable number of tandem repeat (VNTR) polymorphism of a CCGCC unit. Importantly, we found significant associations between the VNTR and human cancer with elevated SMYD3. Although homozygosity for three-tandem-repeat was observed 140 of 175 (80.0%) colorectal cancers, 152 of 180 (84.4%) hepatocellular carcinomas, and 146 of 167 (87.4 %) breast cancer, the homozygosity was detected in 222 of 365 (60.8%) control healthy vol-

unteers. The data indicates that three-tandem-repeat homozygosity has increased risks of colorectal cancer ($P=9.1\times10^{-6}$, odds ratio=2.58), hepatocellular carcinoma ($P=2.3\times10^{-8}$, odds ratio=3.50) and breast cancer ($P=7.0\times10^{-10}$, odds ratio=4.48). Interestingly, this repeated sequence was proven to be a binding-site for the transcription factor E2F-1. In a reporter assay, plasmids containing three-repeats of the binding motif (corresponding to the high-risk allele) showed significantly higher reporter activity than plasmids containing two repeats (the low-risk allele). These data may imply that the common VNTR polymorphism in the *SMYD3* promoter is a susceptibility factor for some types of human cancer.

Global gene expression profiles of CRC using a cDNA microarray containing approximately 23,000 genes identified an additional three candidate genes for clinical application. Among the three genes, *PPIL1* encoded peptidyl-prolyl isomerase like 1, a cyclophilin-related protein. Colony formation assay showed a growth-promoting effect of wild type *PPIL1* on NIH3T3 and HEK293 cells. Transfection of SNUC4 and SNUC5 cells with siRNA specific to *PPIL1* reduced expression of the gene, and retarded growth of the colon cancer cells. We further identified two PPIL1-interacting proteins, SNW 1/SKIP (SKI-binding protein) and stathmin. SNW1/SKIP is involved in the regulation of transcription and mRNA splicing, while stathmin is involved in stabilization of microtubules. Therefore, elevated expression of PPIL1 may play an important role in proliferation of cancer cells through the control of SNW1/SKIP and/or stathmin.

The second candidate is a novel human gene, termed *C10orf3*, whose expression was elevated in colorectal cancers as well as in tumors arising in the stomach, lung, pancreas, and breast. The gene encodes a putative 464-amino acid protein containing a domain known as AAA (ATPases associated with a variety of cellular activities). A subsequent proteomics analysis revealed that C 10orf3 associated with the product of tumor susceptibility gene 101 (TSG101), and that C10orf3 down-regulated TSG101 in a posttranscriptional manner. Expression of C10orf3-siRNA in colorectal cancer cells caused significant decrease in *C10orf3* expression, and induced apoptosis of the cells transfected with the siRNA.

The third candidate is *SP5*, a member of the Sp transcription factor family. We uncovered that *SP5* was down-regulated after depletion of β-catenin by transduction of wild-type *APC* in SW480 colon cancer cells. Since mutation in *APC, CTNNB1, AXIN1* or *AXIN2* leads to accumulation of β-catenin and subsequent transactivation of Tcf/LEF transcription factors, we tested whether *SP5* is a direct target gene or not. An electrophoretic mobility-shift assay showed that the β-catenin/Tcf4 complex interacted with a putative Tcf-binding sequence between -285 and -279 in the 5′ flanking region of *SP5*. We also found by reporter assays that the binding element is responsible for *SP5* up-regulation. These data indicate that *SP5* is a novel direct downstream target in the Wnt signaling pathway.

Gene expression profiles of 20 intestinal-type gastric cancers identified *Immunoglobulin superfamily 11 (IGSF11)* as a candidate gene for clinical application. Its expression was elevated not only in intestinal-type gastric cancer but also in colorectal cancer and hepatocellular carcinoma. Since northern blot analysis revealed that it showed abundant expression in testis and ovary, IGSF11 is a good candidate of cancer-testis antigen (CTA). To investigate the clinical application of peptide vaccine to IGSF11, we synthesized candidate epitope peptides for IGSF 11 and tested whether the peptides elicit IGSF11 -specific CTLs. As a result, we successfully established oligo-clonal CTLs by the stimulation with IGSF11-9-207 (ALSSGLYQC). In addition, we also established additional CTLs by IGSF-9V (ALSSGLYQV), anchor-modified peptides of IGSF11-9-207. These peptides showed IGSF11-specific cytotoxicity in an HLA-A*0201-restricted fashion, suggesting that they may be applicable for cancer immunotherapy.

Among up-regulated genes in our microarray data of HCC, we identified a novel gene, termed *WDRPUH*, as a candidate for clinical application. Multiple-tissue northern blot analysis revealed its specific expression in the testis among the 16 normal tissues examined. Transfection of plasmids designed to express *WDRPUH*-specific siRNA significantly reduced its expression in HCC cells and resulted in growth suppression of the transfected cells. Interestingly, we found that WDRPUH associated directly with HSP70, proteins of the chaperonin-containing TCP-1 (CCT) complex, and BRCA2. These findings suggest that WDRPUH may modulate BRCA2, and that it may be a molecular target for the treatment of HCC.

From gene expression profiles of 25 intrahepatic cholangiocarcinomas (ICC), we focused on *PSF2* (partner of SLD five 2), a component of the GINS multiprotein complex that plays a crucial role in initiation of DNA replication. Semi-quantitative RT-PCR analysis confirmed high levels of *PSF2* expression in the ICCs, but expression of this gene was barely detectable in normal vital organs. Transfection of ETK-1 and HuH28 cholangiocarcinoma cells with *PSF2*-

specific siRNA reduced the amount of transcript and suppressed the cell growth, suggesting that PSF2 may play an important role in development of cholangiocarcinoma.

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 107 patients using direct sequencing and Multiplex Ligation-dependent Probe Amplification. Among the 107 cases, 29 cases were analyzed in 2005. 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.

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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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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 MS 10/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 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 stromal cells and important for HSC self-renewal by utilizing a variety of technologies (i.e. signal sequence trap, mRNA subtraction) and analyze their function in vitro and in vivo. 2) Identify genes that are induced in HSC by contacting with stromal cells. These genes are strong candidates that are involved in the self-renew processes evoked by stromal cell contact. We are now characterizing two novel molecules that are speculated to be important for these processes.

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 therapeutic approach to AML

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

Yumi Fukuchi[1], Fumi Shibata[1], Miyuki Ito[1], Yuko Goto-Koshino[1], Yusuke Sotomaru[8], Mamoru Ito[8], Toshio Kitamura[1], Hideaki Nakajima: [8]Central Institute for Experimental Animals

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 in-

cluding 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)

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

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 way to their application in the clinical settings.

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Promotion of Translational Research Projects

研究拠点形成　探索的プロジェクト推進

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COE Clinical Associate Seiichiro Kobayashi, M.D., D.M.Sc

特任講師　医学博士　長　村　文　孝
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Our purpose is to promote the conducts of Translational Research Projects at the Research Hospital. Reviews and advices on the study designs and protocols, and handling of problems during the conduct of clinical studies are important tasks. The role of Clinical Research Coordinator (CRC) is also necessary to perform clinical trials appropriately. Our CRCs owe the other important mission as Translational Research Coordinator. As the education for workers at the Research Hospital, we conducted the educational course on clinical studies, and 37 workers registered. We also contributed to promote and re-organize the system and procedures at the Research Hospital in terms of conducting clinical studies.

Advise/Review of clinical study protocols before the discussion at the Institutional Review Board (IRB: Chiken-Sinsa-linkai)

Fumitaka Nagamura.

One of the roles of DCTSM is to keep the quality of protocols as well as studies themselves. To perform this task, we discuss and advise on the protocols with principal investigators, and made it a rule to submit a protocol and written consent documents to DCTSM before submitting to the Institutional Review Board.

From January 2005 to December 2005, we received seven entirely protocols and numerous questions within the research hospital. All the protocols were either Phase I or Phase I/IIa studies. Pre-review of these protocols were finished within two to three weeks from the receipt. The format of pre-review is based on the style of applied in the U.S. Food and Drug Administration. Our opinions are summarized into three sections: safety issue (most concern); major problem; and minor problems/suggestions. These opinions are not obligations which posses enforcement, but those to improve clinical studies. Final decision should be made at the Institutional Review Boards. Furthermore, we performed these activities for other institutes. We received requirements from four institutes.

To assist the planning of clinical studies and writing protocols, we have disclosed "Guideline". Recently many regulations and guidelines were announced. To clear these and to match the Institute's organization, we have been engaging in the revisions of the rules of out institute and in reconstitution of the organization through Working Group.

Education on clinical studies for workers at Reseacr Hospital

Fumitaka Nagamura.

The major missions of Translational Research

Coordinator are to keep patients' right, to conduct translational research more ethically, and to perform translational research scientifically. The role of TRC is not the same as that of Clinical Research Coordinator (CRC) in terms of the aggressive intervention to keep studies ethically conducted. The problem of education for research coordinators including CRC is the new but the critical problem in Japan. To educate workers of the hospital, as well as coordinators, the division took place the educational course on clinical studies. This meeting consists of 10 sections, and participants were required to resister for preparing the course materials. Thirty-seven workers applied for this course. The content of this course consists of the basic knowledge of clinical studies, regulations, laws, coordinating skills, environment of clinical studies, and the system of the Research Hospital in terms of the conduction of Translational Research.

Trend of Drug Approval on Hematological Malignancies in the U.S. and Japan

Fumitaka Nagamura

The trends of drug approval on hematological malignancies in the U.S. and in Japan were analyzed. The purpose of this study is to clarify the problems on the drug evaluation and approval methods for fastening approval periods and evaluating efficacies/safeties more precisely, especially in cases of entirely new concepts of drugs by the comparison of two countries. Forty-six drugs were approved in the U.S., and 43 were in Japan for hematological malignancies. Twenty-seven drugs were approved in both countries. Twenty-two of 27 drugs were approved earlier in the US, and the dates of approval were earlier in the U.S. (median: 46.0 Mo, mean: 54.7 Mo). These differences have not been shortened when compared in every 10-year period. In the U.S., eight drugs were approved as "Accelerated Approval". Seven of eight "accelerated approval" drugs were approved only in the U.S. However, only one drug approved as "accelerated approval" could have shown its clinical benefit in the designated clinical trial. The ratio of non-U.S. studies was considerably low in hematological malignancies (7.0%) when compared with all oncologic drugs (23.7%). Five drugs approved only in Japan were approved in the US for diseases other than hematological malignancies, while no drug was approved in the reverse case. Accelerated approval is considered to be useful for fastening the period until approval, although the problem, that "surrogate markers" could predict the "survival and/or QOL benefit", has not been clarified, yet. The outstanding result that most of pivotal studies were not "non-U.S." study may be caused by the superiority of drug development, especially in new era of drugs for hematological malignancies and the ability to conduct the appropriate clinical trials in the U.S., or approval based on "accelerated approval". On the contrary, the expansion of the indication would be the problem in the U.S.

Activities of Translational Research Coorinator (TRC) and clinical Research Coorinator (CRC)

Hajime Kotaki, Fumitaka Nagamura, Momoyo Ohki, Miho Tabata, Kumiko Sumino.

The activities of research coordinators are important to conduct clinical studies smoothly and to manage the relationship with participants. In TR, sufficient concerns on the rights and the understandings of participants themselves should be paid compared with other clinical researches. TRCs have been organized to solve these problems, and they consist of nurse, pharmacist, psychologist, dietician and clinical laboratory technologist. DCTSM collaborates with chief of TRC, Director of Pharmacy, on the activities of TRC. Exclusive CRCs belong DCTSM and take part in clinical trials from pharmaceutical companies and medical doctor-initiative studies to maintain GCP requirements.

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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-based regenerative medicine. To this end, we have been determined to work on hematopoietic stem cells (HSCs) as a model system because HSCs have already been used in bone marrow transplantation as a prototype of stem cell therapy, and are expected to be widely used in a variety of clinical settings.

Stem cells are defined as cells capable of self-renewal and differentiation. Like most normal cells, stem cells neither survive nor proliferate without extracellular stimulation. Hematopoietic stem cells (HSCs) have been an excellent experimental model to study stem cell regulation because of established methods for their functional identification. We want to understand which molecules directly or indirectly stimulate HSCs, how HSCs respond to such stimuli in terms of signaling, what molecular mechanisms allow HSCs to divide numerous times, what molecular basis supports such division, and finally what differentiation is all about.

1. Lnk negatively regulates self-renewal of HSCs by modifying thrombopoietin-mediated signal transduction

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One of the central tasks of stem cell biology is to understand the molecular mechanisms that control stem cell self-renewal and differentiation. We have attempted to understand molecular basis underlying self-renewal in HSCs. In this study we focused on intracellular signaling events in HSCs by using mutant mice in which an adaptor protein, Lnk, is deficient (Lnk-/- mice). We have previously reported that in Lnk -/- mice, long-term marrow repopulating activity is markedly elevated due to increases in absolute number as well as self-renewal activity of HSCs. These results suggest that Lnk negatively regulates the key signaling pathways associated with self-renewal in HSCs. In search of such signals, we first evaluated direct effects of a number of cytokines on both survival and proliferation of HSCs. Serum-free culture of single WT or Lnk-/- CD34-negative, c-Kit-positive, Sca-1-positive, Lineage marker-negative ($CD34^-$KSL) cells representing highly enriched HSCs was performed in the presence of various cytokines. Lnk-/- $CD34^-$KSL cells did not respond to any cytokine which WT CD34-KSL cells did not. But, we found that Lnk-/- $CD34^-$KSL cells are hypersensitive to thrombopoietin (TPO). Importantly, in the presence of TPO, the probability of self-renewal of Lnk-/- $CD34^-$KSL cells was

greater than that of WT CD34$^-$KSL cells. Single cell phosphorylation imaging analysis that we had developed showed combinatorial changes in TPO-mediated signal transduction in Lnk-/- CD 34$^-$KSL cells as compared with WT CD34$^-$KSL cells: Activation of both JAK2/STAT and Akt signal transduction, and dephosphorylation of p -38 MAPK. Self-renewal of HSCs was suggested to be regulated by a balance in positive and negative signals. We concluded that TPO signaling pathway plays a key role in the regulation of HSC self-renewal for which Lnk acts as a negative regulator. Further work is required to determine what molecules interact with Lnk in HSC self-renewal signaling.

2. Epigenetic control of HSCs

Yuko Tadokoro[1], Masaki Okano[2], En Li[3], and Hiromitsu Nakauchi[1], and Hideo Ema: [1]Laboratory of Stem Cell Therapy, Center for Experimental Medicine, IMUT, [2]Laboratory for Mammalian Epigenetic Studies, Center for Developmental Biology, RIKEN, [3]Novartis Institutes for Biomedical Research.

Epigenetic modifications are inheritable markers in genome regions or chromatin structure, without a change in DNA sequence, for regulation of gene expression potential in developing and differentiating cells. Epigenetics has been the focus of our attention in the field of stem cell biology these days. DNA methylation and histone modification are central to epigenetic regulation. Studies of polycomb group (PcG) proteins have first shed light on epigenetic control involved in self-renewal of HSCs. Very recently it has been realized that PcG proteins interact with DNA methyltransferases for gene silencing. DNA methylation patterns are established during early embryogenesis and gametogenesis, and are maintained in somatic cells thereafter. The DNA methyltransferases Dnmt1, Dnmt3a, and Dnmt3b catalyze methylation of CpG dinucleotides in genomic DNA. Dnmt3a and Dnmt3b supposedly act as *de novo* methyltransferases while Dnmt1 acts as a 'maintenance' methyltransferase. Embryogenesis is severely impaired in Dnmt1- or Dnmt3b-deficient mice. Spermatogenesis is impaired in Dnmt3a-deficient mice. Embryonic stem (ES) cells without Dnmt3a and Dnmt3b are viable, and maintain replication potential, but progressively lose differentiation potential with repeated passage. These data imply that when stem cells undergo differentiation, Dnmt3a and Dnmt3b establish DNA methylation status in stem-cell progeny. To address this issue, we examined the role of Dnmt3a and Dnmt3b in HSCs, the best-studied adult stem cells. *De novo* DNA methyltransferases *Dnmt3a, Dnmt3b*, or both *Dnmt3a* and *Dnmt3b* were conditionally disrupted in mouse HSCs. We show that HSCs, unlike embryonic stem cells, require either Dnmt3a or Dnmt3b for self-renewal, but not for differentiation into blood cell lineages, and each separate deletion partially reduces HSC self-renewal potential. These findings establish a critical role of DNA methylation by Dnmt3a and Dnmt3b in HSC self-renewal. A simple, but important implication of this work is that DNA methylation plays a role for adult stem cell functions, in a way different from how it does for functions of ES cells and PGCs. Further studies should be devoted to clarification of whether and how epigenetic modifications can specify functions of cells at various developmental stages. In this regard, we believe that this work should stimulate epigenetic studies of stem cells as well as those of cancer stem cells.

3. Significance of non-side-population HSCs

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Most HSCs are thought to reside in so called side-population (SP) in adult mouse bone marrow (BM). However, we found that the coexistence of non-SP HSCs that do not significantly differ from SP HSCs in numbers, capacities, and cell cycle states. When costained with the Hoechst 33342 dye, CD34$^{-/low}$c-Kit$^+$Sca-1$^+$lineage marker$^-$ (CD34$^-$KSL) cell population, highly enriched in HSCs, was almost equally divided into the SP and the main population (MP) that represents non-SP cells. Competitive repopulation assays with single or 30 SP- or MP-CD34$^-$KSL cells showed similar degrees of repopulating activity and similar frequencies of repopulating cells between these populations. Secondary transplantation detected self-renewal capacity in both populations. SP analysis of BM cells from primary recipient mice showed that either SP- or MP-CD34$^-$KSL cells could give rise to both SP- and MP-KSL cells after transplantation. Cell cycle analyses revealed that SP and MP cells were equally in a quiescent state. We conclude that SP phenotype does not specify all the HSCs in mice likely because SP and MP phenotypes are reversible. This work should influence cancer studies because SP has been considered as a phenotype of cancer stem cells.

4. Development of HSCs

Chie Furuta[1], Azusa Matsubara[1], Hiroyoshi Kondo[1], Chie Ryo[1], Hiromitsu Nakauchi[1], and Hideo Ema: [1]Laboratory of Stem Cell Therapy, Center for Experimental Medicine, IMUS.

One may ask what developmental stem cells are. They are conceptual. In our hypothesis, HSCs that reside in adult bone marrow differ from embryonic and fetal HSCs in several aspects. Embryonic and fetal HSCs sequentially migrate from one organ to another, and their self-renewal potential is perhaps much greater than that of adult HSCs. Regulatory mechanisms are likely to be different among these HSCs. We want to address these issues by isolating and characterizing embryonic and fetal HSCs.

During development of embryos, hematopoiesis in the mouse begins in the blood islands of the yolk sac (YS) at E7.5. Nucleated red blood cells in blood islands produce embryonic globins. These primitive erythrocytes predominate in the circulation through E12.5, followed by eventual replacement with enucleated red blood cells that produce adult type globins. Progenitor of these definitive erythrocytes are detected initially in the YS, and later on in the paraaortic splanchnopleure (P-Sp) / aorta-gonad -mesonephros (AGM) region, the placenta, and the fetal liver. In a process presumed to be independent of these two types of erythropoiesis, HSCs arise in the P-Sp / AGM region and the YS. It is not clear how primitive and definitive hematopoietic progenitors as well as HSCs are generated in these tissues. Formation of blood cells is closely correlated with that of vascular endothelial cells in the YS. This led to the idea that both cell types arise from a common precursor, the hemangioblast. Endothelial and hematopoietic lineages share a number of expressed genes. Much work in the past has focused on the isolation and identification of hemangioblasts among cells that have in vitro differentiated from embryonic stem (ES) cells. Blast colony-forming cells (BL-CFCs) were found as candidate hemangioblasts in this model system. Recently, BL-CFCs were isolated also from the developing mouse embryo. All these studies support the common origin of vascular and hematopoietic cells. Little attention has been paid to endothelial progenitors, namely angioblasts. In an avian system, two distinct endothelial lineages have been described. One of these, derived from splanchnopleural mesoderm, is associated with hematopoiesis. The other, without hematopoietic potential, is derived from paraxial mesoderm. A similar mechanism may also operate in mammals, but no substantial evidence has been reported so far. We therefore attempted to determine the kinetics of endothelial and hematopoietic development in early gastrulating mouse embryos prior to the onset of YS hematopoiesis. We found that a significant number of angioblasts can be detected one day before hematopoietic activity is detected. Our data suggest that a distinct pathway exists in which the angioblast lineage directly differentiates from mesoderm early in development, independent of hematopoiesis. We are currently trying to physically separate angioblasts from hemangioblasts. On the other hand, we are attempting to determine which lineage HSCs are originated from.

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Leading Project of the Regenerative Medicine

Laboratory of Stem Cell Regulation

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Our research interest is to understand how we can use adult stem cells for tissue regeneration. To accomplish this goal, we are focusing on the following subjects: 1) identification and characterization of the stem cell microenvironment/niche, 2) studying the role of locoregional hematopoietic cells in the microenvironment (e.g. mast cells) during tissue regeneration, 3) studying the potential use of growth factors for tissue regeneration, 4) understanding the mechanism how proteases are involved in stem cell mobilization and stem cell-mediated tissue regeneration.

1) A role for niches in hematopoietic cell development.

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Stem cells reside in a physical niche, a particular microenvironment. The organization of cellular niches has been shown to play a key role in regulating normal stem cell differentiation, maintenance and regeneration. Hematopoietic stem cells (HSC) emerge at distinct allocation territories during ontogenesis, notably the aorto-gonadal region, the fetal liver. Adult HSC expand and differentiate exclusively in the bone marrow (BM). They can be mobilized into the blood stream. This implies that stem cells are not autonomous units of development; rather, tissue specific niches control their destiny. Interaction of HSCs with their stem cell niches is critical for adult hematopoiesis in the BM. A niche is composed of stromal cells, which either through direct cell-to-cell contact or via release of soluble factors maintain the typical features of stem cells, mainly stem cell quiescence, maintenance or expansion. HSCs are keeping the balance of the quiescence and the self-renewal in the stem cell niche, and are maintaining long-term hematopoiesis. Therefore, an understanding of cellular and chemical architecture of the stem cell niche is vital in understanding stem cell behavior. This review summarizes the recent developments in our understanding of the stem cell niche with particular focus on the HSC niche.

2) Low-dose irradiation promotes tissue revascularization through VEGF release from mast cells and MMP-9-mediated progenitor cell mobilization.

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Mast cells accumulate in tissues undergoing angiogenesis during tumor growth, wound healing, and tissue repair. Mast cells can secrete angiogenic factors such as vascular endothelial growth factor (VEGF). Ionizing irradiation has also been shown to have angiogenic potential in malignant and nonmalignant diseases. We observed that low-dose irradiation fosters mast cell -dependent vascular regeneration in a limb ischemia model. Irradiation promoted VEGF production by mast cells in a matrix metalloproteinase-9 (MMP-9)-dependent manner. Irradiation, through MMP-9 up-regulated by VEGF in stromal and endothelial cells, induced the release of Kit-ligand (KitL). Irradiation-induced VEGF promoted migration of mast cells from the bone marrow to the ischemic site. Irradiation-mediated release of KitL and VEGF was impaired in MMP-9-deficient mice, resulting in a reduced number of tissue mast cells and delayed vessel formation in the ischemic limb. Irradiation-induced vasculogenesis was abrogated in mice deficient in mast cells (steel mutant, Sl/Sl(d) mice) and in mice in which the VEGF pathway was blocked. Irradiation did not induce progenitor mobilization in Sl/Sl(d) mice. We conclude that increased recruitment and activation of mast cells following irradiation alters the ischemic microenvironment and promotes vascular regeneration in an ischemia model. These data show a novel mechanism of neovascularization and suggest that low-dose irradiation may be used for therapeutic angiogenesis to augment vasculogenesis in ischemic tissues.

3) Granulocyte-Colony Stimulating Factor promotes neovascularization by releasing vascular endothelial growth factor from neutrophils

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The granulocyte colony-stimulating factor (G-CSF) promotes angiogenesis. However, the exact mechanism is not known. We demonstrate that vascular endothelial growth factor (VEGF) was released by Gr-1+CD11b- neutrophils but not Gr-1-CD11b+ monocytes prestimulated with G-CSF in vitro and in vivo. Similarly, in vivo, concomitant with an increase in neutrophil numbers in circulation, G-CSF augmented plasma VEGF level in vivo. Local G-CSF administration into ischemic tissue increased capillary density and provided a functional vasculature and contributed to neovascularization of ischemic tissue. Blockade of the VEGF pathway abrogated G-CSF-induced angiogenesis. On the other hand, as we had shown previously, VEGF can induce endothelial progenitor cell (EPC) mobilization. Here, we show that G-CSF also augmented the number of circulating VEGF receptor-2 (VEGFR 2) EPCs as compared with untreated controls. Blocking the VEGF/VEGFR1, but to a much lesser extent, the VEGF/VEGFR2 pathway in G-CSF-treated animals delayed tissue revascularization in a hindlimb model. These data clearly show that G-CSF modulates angiogenesis by increasing myelomonocytic cells (VEGFR1+ neutrophils) and their release of VEGF. Our results indicated that administration of G-CSF into ischemic tissue provides a novel and safe therapeutic strategy to improve neovascularization.

4) 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.

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University of Tokyo Institute of Medical Science Research Center for Asian Infectious Diseases

Establishment of Japan-China Joint Laboratories on Emerging and Re-emerging Infections

アジア感染症研究拠点形成

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The Institute of Medical Science, University of Tokyo (IMSUT) is establishing Japan-China joint laboratories for studies of emerging and re-emerging infections in Asia, in collaboration with the Chinese Academy of Sciences and Chinese Academy of Agricultural Sciences. In the laboratories, Japanese scientists from IMSUT and Chinese scientists will together conduct research on the structure of viral and host proteins, viral pathogenicity, and the genetic changes of viruses in the field.

BACKGROUND

China is very important, geopolitically and economically, to Japan today and probably for much of the first half of the 21st century. Furthermore, it is in China that people have suffered from the recent and past outbreaks of new strains of human and avian influenza viruses and the SARS virus and are suffering from rapidly increasing HIV infections and the high carrier rate of hepatitis viruses. Given these situations, academic collaboration on studies of 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 grant 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 is now establishing two joint laboratories in Beijing in collaboration with the Institute of Biophysics and Institute of Microbiology, Chinese Academy of Sciences (IBCAS and IMCAS, respectively), a collaborative research program with Harbin Veterinary Research Institute (HVRI), the Chinese Academy of Agricultural Sciences, and IMSUT's project office in Beijing.

LABORATORIES AND PROJECT OFFICE

a. Laboratory of Structural Virology and Immunology (LSVI, tentatively named)

LSVI will be established in collaboration with IBCAS (Director; Rao Zihe) within one of its buildings in Beijing. IBCAS, established in 1958, is perhaps the best institute in China in the field of structural biology. Its recent contributions to science include the studies of SARS virus main protease (PNAS 100: 13190-13195, 2003) and the crystal structure analysis of SARS virus spike protein fusion core (J. Biol. Chem. 279: 49414-49419, 2004). In LSVI, Japanese and Chinese scientists will conduct research on the physical structure of viral and host proteins, particularly in relation to their biological functions and pathogenicity. IMSUT will dispatch a professor and an assistant professor to the laboratory from Japan.

b. Laboratory of Molecular Immunology and Molecular Microbiology (LMIMM, tentatively named)

LMIMM, equipped with BSL2 and BSL3 facilities, will be established in collaboration with IMCAS (Director; Gao Fu) within its new building under construction in Beijing. IMCAS, established in 1958, has been dealing with viral diseases caused by HIV, HBV, etc. and emerging and re-emerging infections in entire Asia. IMCAS has functions of *Microbiological Resources Center-China* and *China General Microbiological Culture Collection Center*, indicating that important pathogens from within and outside China are collected and stored there. In LMIMM, Japanese and Chinese scientists will conduct virological and immunological research on emerging and re-emerging viruses. IMSUT will dispatch a professor and an assistant professor to the laboratory from Japan.

c. Collaborative research program with HVRI

HVRI (Director; Xiangang Kong) serves as a research center for avian influenza virus in domestic fowl and wild ducks and geese in China. With HVRI, IMSUT is setting up a research program on avian influenza virus. Japanese scientists dispatched from IMSUT to HVRI will conduct, together with Chinese scientists, surveillance and the study of genetic changes of avian influenza virus in the field. This is one of the important steps to prepare for the coming possible pandemic of the avian influenza virus that can infect humans.

d. IMSUT research office

IMSUT is now setting up its project office in Beijing. The office will manage the personnel hired by IMSUT for the collaboration and the Japanese side budget for the laboratories. In October 2005 IMSUT appointed two people as professor for the research office, and one of them has been working in Beijing since then.

IMPLEMENTATION OF COLLABORATION

The plan for collaboration will be implemented on the basis of *the Memorandum of Understanding's* to be signed by IMSUT and the three Chinese institutes. For the joint laboratories the implementation will be controlled during 5-year-term of the project by a steering group composed of Dean of IMSUT (Tadashi Yamamoto), MEXT Project Director, and Directors of IBCAS and IMCAS. For the collaborative program in Harbin it will be controlled by a steering committee composed of Dean of IMSUT, Director of HVRI, and some scientists involved in the program.