Advanced Clinical research Center

Division of Molecular Therapy
分子療法分野

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The main theme of our research is toward the development of novel therapeutic options against intractable hematological disorders including leukemia and lymphoma. For this purpose, we are making every effort to master the mechanisms of normal and neoplastic hematopoiesis on the basis of molecular and cellular biology. We also try to develop novel therapies in the field of regenerative medicine using bone marrow-derived mesenchymal stem cells.

(1) Molecular and cellular analysis of BCR-ABL-induced leukemias
Leukemia-specific genetic rearrangements often result in chimeric transcription factors and tyrosine kinases, which appear to be the primary cause of those leukemias. We are studying the molecular and cellular aspects of BCR-ABL-induced acute and chronic leukemia as a model system.

(2) Analysis of the putative role of miRNAs in the pathogenesis of hematological malignancies:
miRNAs are a recently discovered class of small noncoding RNAs that are 18-24 nucleotides long and that downregulate target genes at a post-transcriptional level. Downregulation of specific miRNAs in certain cancers implies that some miRNAs may act as tumor suppressors. We are investigating the role of certain miRNAs in the pathogenesis of hematological malignancies as well as the possibility that miRNAs may be promising targets for therapy and diagnosis.

(3) Analysis of tumor stem cells and search for molecular targets for their elimination:
Cure of malignant tumors requires eradication of tumor stem cells. As a representative model for tumor stem cells, we are studying the identification and characterization of leukemia stem cells using cell tracking strategies and flow cytometry.

(4) Adult T-cell leukemia is a T cell malignancy which develops in HTLV-1 infected individuals after long latency period. HTLV-1 infected cells are regarded to transform through multi-step oncogenesis process. We are analyzing HTLV-1 infected cells in different stages of transformation whose phenotypes such as CD7 and CADM1 expression vary in each stage by sorting them using flow cytometer. These analyses will provide useful information regarding molecular mechanism to develop ATL.

(5) Translational research on tissue engineering:

To accomplish this goal, we are focusing on the issues including a) identification and characterization of somatic stem cells, b) search for molecules to affect the growth and differentiation of stem cells, and c) search for suitable biomaterials as the scaffold to assemble these stem cells on.

1. The *in vitro* and *in vivo* oncogenic activity of homodimeric mutant of interleukin-7 receptor α chain (IL7Rα) highlights the significance of the IL7Rα/Jak1 pathway in T-cell acute lymphoblastic leukemia.

Yokoyama K, Kobayashi S, Izawa K, Harnprasopwat R, Kotani A, Tojo A

Interleukin-7 (IL7) is essential for T cell development and homeostasis. Dysregulation of signals that control normal T-cell development has been implicated in the onset of T-cell acute lymphoblastic leukemia (T-ALL). By analogy to activating mutations in the Notch pathways, we hypothesized that any mutations in the IL7 signaling axis might also contribute to T-ALL. Direct sequencing of human IL7 receptor α chain (hIL7 RA) gene in a panel of 16 T-ALL cell lines identified two types of mutations in two different cell lines. One was an insertion mutation of 4 amino acids (LSRC) in the transmembrane region (INS, Fig. 1A) from DND-41, a $\gamma\delta^+$T-ALL cell line, and the other was a truncated, loss-of-function, mutation in the cytoplasmic region from MOLT-4. We demonstrated that hIL7RA-INS mutant spontaneously formed a homodimer and constitutively activated downstream signals including Stat family members (1, 3 and 5), Akt and Erk via Jak1, but not Jak3. Next, we investigated oncogenic activity of hIL7RA-INS in primary hematopoietic progenitor cells. To this aim, lin^- E.14 Balb/c fetal liver (FL) cells were retrovirally transduced with hIL7RA-INS in parallel with hIL7RA-wild type (WT), and then tested for their cytokine dependence *in vitro*. As expected, only hIL7RA-INS-transduced lin^-FL cells showed abrogation of cytokine dependence. hIL7RA-transduced lin^-FL cells were also transplanted into lethally irradiated syngeneic mice. Within 4-5 weeks after transplantation of lin^-FL cells transduced with hIL7RA-INS, but not with hIL7RA-WT, recipient mice developed well-tolerated myelo- and lymphoproliferative disorders, characterized by marked leukocytosis, systemic lymphadenopathy and splenomegaly (Fig. 1B). Notably, concomitant increase in hIL7 $RA^+TCR\gamma\delta^+$ T cells and decrease in B cells were observed in peripheral blood (Fig. 1C). Histological examination of bone marrow, spleen and liver specimens from diseased mice revealed moderate to severe myeloid hyperplasia, disrupted splenic architecture by disseminated mature myeloid cells and infiltration of both myeloid and mononuclear cells into hepatic parenchyma, respectively. In addition, recipient mice for hIL7RA-INS-transduced lin^-FL cells frequently manifested ruffled fur as well as mononuclear cell infiltration into salivary gland and pericardium, suggesting an autoimmune-like disorder. However, during 8 weeks of observation, these recipient mice did not develop either overt leukemia or lymphoma, indicating that additional transforming events are required for evolution to aggressive hematological malignancies. These *in vivo* findings highlighted the possibility that aberrant signals via IL7RA in hematopoietic stem/progenitor cells might preferentially stimulate myelopoiesis over lymphopoiesis, and also confirmed the essential role of IL7 RA in $\gamma\delta^+$T cell development, previously shown by IL7RA-knockout mice. Taken together, we speculated that dysregulated IL7RA signaling axis might be involved in the onset of T-ALL, especially with $\gamma\delta^+$ phenotype. Finally, the present study, together with the recent report (JEM 208: 901, 2011), emphasizes the significance of the sequential Notch-IL7RA pathways in the pathogenesis of T-ALL as well as the dominant role of the IL7RA/Jak1 axis in IL7 proliferative signal.

2. Bcr-Abl impairs T cell development from murine induced pluripotent stem cells and hematopoietic stem cells.

Bidisha C, Izawa K, Harnprasopwat R, Takahashi K, Kobayashi K, Tojo A

Chronic myeloid leukemia (CML) is a clonal myeloproliferative disorder generally believed to originate from a hematopoietic stem cell carrying the BCR-ABL fusion gene, which generally encodes 210kD and 190kD constitutively active tyrosine kinases termed as p210 and p190, respectively. In spite of the putative stem cell origin and the competence for differentiation toward mature B cells, there is a longstanding

consensus that CML never involves the T cell lineage at least in chronic phase. To gain insight into this apparent conflict, we used *in vitro* T cell differentiation model from murine pluripotent stem cells (PSCs) as well as hematopoietic stem cells (HSCs). C57BL/6 MEFs were reprogrammed using a polycistronic lentiviral Tet-On vector encoding human Oct4, Sox2 and Klf4, which were tandemly linked via porcine teschovirus-1 2A peptides, together with another lentiviral vector expressing rtTA driven by the EF-1α promoter. Almost all the vector sequences including the transgenes were deleted by adenovirus-mediated transduction of Cre recombinase after derivation of iPSCs, and only remnant 291-bp LTRs containing a single loxP site remained in the genome. A clone of MEF-iPSCs were retrovirally transduced with p190ΔccER, a ligand-controllable p190-estrogen receptor fusion protein, whose tyrosine kinase activity absolutely depends on 4-hydroxytamoxyfen (4-HT). For T cell lineage differentiation, p190ΔccER-MEF-iPSCs were recovered from a feeder-free culture supplemented with LIF and plated onto a subconfluent OP9-DL1 monolayer in the presence of Flt3 ligand and IL7 with or without 0.5 μM 4-HT. After 3 weeks of culture, iPSC-derived blood cells were collected and subjected to FACS analysis for their lineage confirmation. About 70% of lymphocyte-like cells from the 4-HT(−) culture expressed CD3, but only 20% of counterparts from the 4-HT(+) culture expressed CD3, suggesting impaied T cell development by Bcr-Abl. Next, c-Kit$^+$Sca1$^+$Lin$^-$ (KSL) bone marrow cells were prepared by FACS from 8-weeks old C57BL/6 mice treated with 5-FU. KSL cells were similarly transduced with p190 ΔccER and were subjected to the OP9-DL1 coculture system with or without 0.5 μM 4-HT. After 2 weeks of culture, 95% of lymphocytes from the 4-HT(−) culture revealed CD3$^+$TCRβ$^+$ phenotype, but only 30% of those were double positive in the presence of 4-HT. In addition, 90 % of lymphocytes from the 4-HT(−) culture progressed to the DN2 stage with c-Kit$^-$CD44$^+$ CD25$^+$ phenotype, whereas 50% of those from the 4-HT(−) culture arrested at the DN1 stage showing c-Kit$^+$CD44$^+$CD25$^-$. Since IL7 plays a central role at the stage from DN1 to DN2 of progenitor T cells, Bcr-Abl is suggested to impair T cell development possibly through interfering with the IL7 signal. The precise mechanism underlying impaired T lymphopoiesis by Bcr-Abl is under investigation.

3. BCR-ABL$^+$ leukemia-targeted therapy using miRNA-regulated vaccinia virus

Futami M, Nakamura T, Tojo A

Recent development of tyrosine kinase inhibitors (TKIs) including imatinib, nilotinib, and dasatinib has revolutionized the therapy for chronic myeloid leukemia (CML). However disease progression, mainly caused by TKI-resistant mutations in the Bcr-Abl kinase domain, still being the worrisome problem. To overcome drug resistance, anti-tumor therapy using viral infection (also known as oncolytic virotherapy) is currently being studied actively for solid tumors worldwide. Among them, JX-594, a genetically engineered vaccinia virus, has achieved an excellent clinical result against hepatocellular carcinoma in phase I/II trials. Using a vaccinia virus, we are trying to develop a CML-targeted oncolytic therapy. Vaccinia virus is a member of poxvirus family, which was widely used for the small pox prevention until mid 1970s. Although its use once became limited due to the eradication of human small pox virus in the world, the anti-tumor effect of vaccinia is making it an attractive tool again as a potential cancer therapy. Oncolytic effects are thought to be achieved by both the infection itself and the host's immune response to infected cells. Viral replication and transmission in tissue are regulated by viral thymidine kinase (TK) and viral B5R protein, respectively. Using a modification of TK and B5R gene, we are trying to make the virus more CML cell specific in order to improve the safety. Characteristics of micro RNA (miRNA) expression pattern are reported in CML patients, and down regulation of miR-203, miR-10a, miR-150, and miR-199a in CML cells correlate with disease development and progression. We designed target sequence for these miRNAs (these are complementary to guide strand of mature miRNA) and inserted the target sequence into the 3′ untranslated region of B5R gene, which is essential for viral transmission. As expected, endogenous expression of miR-203, miR-10a, miR-150, and miR-199a in normal cells inhibited the expression of miRNA-regulated B5R gene expression, whereas CML cell lines could not inhibit B5R expression due to the lack of these miRNAs. These data suggested that CML cell-specific viral transmission might be possible by regulating B5R gene via miRNA. Using quantitative RT-PCR, we validated that the expression of miR-203, miR-10a, miR-150, and miR-199a were in fact down-regulated in CML cell lines and CML patient samples compared to normal hematopoietic stem cells. Mouse tissue samples of brain, heart, lung, liver, spleen, gut, kidney, ovary, and skin showed higher expression of these miRNAs than bone marrow mononuclear cells of the same mouse, suggesting miRNA's potential protective role in normal tissues against viral adverse effect. Interestingly, not

only CML cells, but also most of all leukemic cell lines (NB4, NALM-6, Jurkat, SUP-B15) and malignant lymphoma cell line (Daudi) showed 20-100 times lower miR-203 expression than normal cells. These data suggest that miR-203-regulated B5R vaccinia virus might be a promising option for wide variety of hematological malignancies. Using mouse models, we are going to treat leukemia mice with the miRNA-regulated vaccinia virus and determine the anti-leukemia effect and safety in vivo.

4. Imatinib mesylate directly impairs class switch recombination through down-regulation of AID: its potential efficacy as an AID suppressor

Kawamata T, Kotani A, Toyoshima T, Yokoyama K, Tojo A

Activation-induced cytidine deaminase (AID) is essential for class switch recombination (CSR) and somatic hypermutation (SHM). Its deregulated expression acts as a genomic mutator that can contribute to the development of various malignancies. During treatment with imatinib mesylate (IM), patients with chronic myeloid leukemia (CML) often develop hypogammaglobulinemia, the mechanism of which has not yet been clarified. Here, we provide evidence that CSR upon B cell activation is apparently inhibited by IM through downregulation of AID. Furthermore, expression of E2A, a key transcription factor for AID induction, was markedly suppressed by IM. These results elucidate not only the underlying mechanism of IM-induced hypogammaglobulinemia but also its potential efficacy as an AID suppressor.

5. Flow cytometric analysis of CD7 and CADM1 expression clearly detect and differentiate HTLV-1 infected cells in various stages of multi-step oncogenesis of ATL development: application for diagnosis of ATL and search for molecular target of ATL therapy.

Kobayashi S, Ishigaki T, Watanabe N, Tojo A, Uchimaru K

We previously reported that leukemic T cells are specifically enriched in a unique $CD3^{dim}$ $CD7^{low}$ subpopulation of $CD4^{+}$ T cells in acute-type adult T cell leukemia. In this study we detected $CD3^{dim}CD7^{dim}$ subpopulation in peripheral blood of acute type ATL patients. This subpopulation was revealed to belong to the same clone as $CD3^{dim}CD7^{low}$ acute type ATL cells. Analysis of peripheral blood of indolent ATL showed various proportions of these $CD3^{dim}CD7^{low}$ and $CD3^{dim}CD7^{dim}$ subpopulation according to subtypes of ATL and suggested that this analysis is useful for diagnosis of subtypes of ATL. But to evaluate exactly ATL cells were to be discriminated from non-ATL cells because CD7 expression of normal CD4 T cells was down-regulated in various physiological and pathological setting, for example HIV infection. CADM1 is a tumor suppressor in various carcinomas but reported to be up-regulated in ATL cells. We integrated CADM1 to our FACS system and clearly discriminated ATL cells as $CADM1^{positive}CD^{dim}$ or $CADM1^{positive}$ $CD7^{low}$ subpopulations from non-ATL cells. Our new multi-color FACS system will be useful to diagnose indolent ATL and high risk HTLV-1 carriers for developing ATL. As it has been proposed that CADM1 over-expression accelerates the tissue infiltration of ATL cells, chemicals that inhibit CADM1signal can be good candidate for molecular target therapy for ATL and our multi-color FACS system is useful tool for screening them. We are now collaborating with Professor Y. Murakami to explore these chemicals.

6. Clinical study on bone tissue engineering

Kagami H, Agata H, Nagamura-Inoue T, Chinzei M, Tojo A.

Atrophic maxillas or mandibles are major obstacles for dental implant therapy. For example, severe periodontitis, which is a leading cause of tooth loss in the elderly, accompanied by significant bone absorption, makes dental implant therapy very difficult if not impossible to perform. Furthermore, alveolar bone regeneration is also required to improve the esthetic and prosthetic aspects of treatment outcome. Although use of dental implants is already an established clinical procedure, there are a large number of patients without adequate bone volume for placement of dental implants. For patients with severe atrophy of alveolar bone, autologous bone grafts from iliac bone, tibial bone, or mandible have been performed. However, these destructive procedures may not be feasible for all patients. Even when the amount of harvested bone is small, the procedure is inevitably accompanied by swelling and pain at the donor site. Although bioartificial bone substitutes have been frequently used, even with biological materials such as demineralized freeze-dried allografts or xenogeneic bone substitutes, the ability to induce bone regeneration is considered less efficient than native bone. Thus, the application is limited. We are carrying out a clinical study of alveolar bone tissue engineering for

dental implant therapy using bone marrow stromal cells (BMSCs), with a goal of eventual commercialization. The study has been approved by the institutional committee and by the Minister of Health, Labour and Welfare of Japan.

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Our major projects are (1) Co-ordinate control of cell division and differentiation by a crosstalk between JAK/STAT and small GTPases, (2) Molecular therapy targeting signal transduction pathways, (3) Characterization of a PIR (paired Ig receptors) family (LMIR/MAIR/CLM) and (4) Elucidation of molecular basis of leukemia, myelodysplastic syndromes, myeloproliferative disorders.

1. Co-ordinate control of cell division and cell differentiation of by the Rho family small GTPases.

Toshiyuki Kawashima, Toshihiko Oki, Yukinori Minoshima, Kohtaro Nishimura, Ying Chun Bao, Tomonori Hatori, Yasushi Nomura, Noriko Takahashi, Takaya Satoh[1], Yoshito Kaziro[2], Tetsuya Nosaka, David Williams[3] and Toshio Kitamura: [1]Kobe University, [2]Biochemistry and Cell Biology Unit, HMRO, Kyoto University Graduate School of Medicine, [3]Cincinnati Children's Hospital Medical Center, USA

In the search for key molecules that prevent murine M1 leukemic cells from undergoing IL-6-induced differentiation into macrophages, we isolated an antisense cDNA that encodes full-length mouse MgcRacGAP through functional cloning. In human HL-60 leukemic cells, overexpression of the human MgcRacGAP induced growth suppression and macrophage differentiation. Interestingly, MgcRacGAP localized to the nucleus in interphase, accumulated to the mitotic spindle in metaphase, and was condensed in the midbody during cytokinesis. These findings indicate that MgcRacGAP dynamically moves during cell cycle progression and plays critical roles in cytokinesis. Moreover, the experiment using a GAP-inactive mutant showed that the GAP activity of MgcRacGAP was required for completion of cytokinesis. We also found that MgcRacGAP is phosphorylated by Aurora B at the midbody. Intriguingly, this phosphorylation induced the Rho-GAP activity of MgcRacGAP, which was critical for completion of cytokinesis. We identified S387 as a phosphorylation site responsible for the acquirement of Rho-GAP activity during cytokinesis at the midbody. On the other hand, MgcRacGAP mainly localizes in the nucleus in the interphase. We demonstrated that MgcRacGAP directly bound transcription factors STAT3 and STAT5, and enhanced transcriptional activation of STAT proteins as a Rac GAP. Recently, we have shown that MgcRacGAP harbors functional NLS and works as a nuclear chaperon together with Rac1.

We found using an MgcRacGAP-GFP fusion protein that expression of MgcRacGAP increases in the early G1 phase in parallel with Geminin,

suggesting that MgcRacGAP may play some roles in G1 check point. In addition, our recent result has suggested that MgcRacGAP is subject to ubiquitin-dependent degradation in G0/G1 phase. In summary, our results indicate that MgcRacGAP plays distinct roles depending on the cell cycle thereby co-ordinating control of cell division and determination of cell fate, implicating multiple levels of regulation of MgcRacGAP including phosphorylation and ubiquitination in distinct biological roles in different cell cycles.

2. Molecular therapy targeting signal transduction pathways using small molecule compounds

Toshiyuki Kawashima, Akiho Tsuchiya, Yukinori Minoshima, and Toshio Kitamura:

STAT3 is frequently activated in many cancers and leukemias, and is required for transformation of NIH3T3 cells. Therefore, we have started searching for STAT3 inhibitors. We already established an efficient screening protocol for identification of STAT3 inhibitors, and identified several compounds that inhibit STAT3 activation. Through the screening of a library of small molecule compounds, we found the compounds RJSI-1 and RJSI-2 that inhibited STAT3 activation. RJSI-2 also inhibited activation of STAT1, STAT5, JAK1 and JAK2, however RJSI-2 is not a kinase inhibitor. On the other hand, RJSI-1 inhibited nuclear transport of phosphorylated STAT proteins, implicating a novel mechanism in inhibiting STAT proteins. We have also shown that these compounds are effective in a tumor-burden mouse model. In addition, we collaborate with a US biotech venture company in modification of RSJI-1 for optimization to develop anti-cancer drugs, and have developed JP 1156 which kill the tumor cells with much lower IC50.

3. Identification and characterization of a novel family of paired Ig (immunoglobulin-like) receptors LMIRs.

Yoshinori Yamanishi, Kumi Izawa, Masahiro Sugiuchi, Ayako Kaitani, Mariko Takahashi, Akie Maehara, Yutaka Enomoto, Toshihiko Oki, Fumi Shibata, Kaori Tamitsu, Si-Zhou Feng, Hideaki Nakajima[5], Jiro Kitaura, and Toshio Kitamura

We originally identified and characterized two mouse cDNAs from a mouse bone marrow-derived mast cell cDNA library. They encoded type I transmembrane proteins including a single variable immunoglobulin (Ig) motif in the extracellular domain with about 90% identity of amino acids. LMIR1 contains immunoreceptor tyrosine-based inhibition motif (ITIM) in the intracellular domain, while LMIR2 harbors a short cytoplasmic tail associating with immunoreceptor tyrosine-based activation motif (ITAM)-bearing molecules such as DAP12. In addition to LMIR1/2, related genes were identified by homology search in the close proximity on the same chromosome 11: LMIR3 is an inhibitory type receptor like LMIR1, and LMIR4, 5, 6 are activation type receptors like LMIR2. It is of note that LMIR3 has a unique property to associate with FcRγ and thereby functions as an activating receptor in concert with TLR4 stimulation. LMIRs are also called CLMs or MAIRs. Those receptors are mainly expressed in cells involved in innate immunity including mast cells, neutrophils, monocytes, and dendritic cells. In addition, some of the receptors are also expressed in some cells in colon, trachea, and lung, indicating that these receptors play important roles in innate immunity.

LMIR5 is a DAP12-coupled activating receptor predominantly expressed in myeloid cells. We have identified T cell Ig mucin 1 (TIM1) as a possible ligand for LMIR5 by retrovirus-mediated expression cloning. TIM1 interacted only with LMIR5 among the LMIR family, whereas LMIR5 interacted with TIM4 as well as TIM1. Stimulation with TIM1 or TIM4 induced LMIR5-mediated activation of mast cells. Notably, LMIR5 deficiency suppressed TIM1-Fc-induced recruitment of neutrophils in the dorsal air pouch, and LMIR5 deficiency attenuated neutrophil accumulation in a model of ischemia /reperfusion injury in the kidneys in which TIM 1 expression is up-regulated. In that model, LMIR5 deficiency resulted in ameliorated tubular necrosis and cast formation in the acute phase. Collectively, our results indicate that TIM 1 is an endogenous ligand for LMIR5 and that the TIM1-LMIR5 interaction plays a physiological role in immune regulation by myeloid cells.

We have established gene-disrupted mice lacking LMIR3, LMIR4 or LMIR5. Investigation to elucidate the biological roles of LMIRs on immune cells is now underway. In addition, we have identified ligands for LMIR3 which we are now characterizing.

4. Molecular basis of acute leukemia, myelodysplastic syndromes (MDS), MDS overt leukemia, and myeloproliferative disorder (MPD).

Naoko Watanabe, Yukiko Komeno, Naoko Kato, Yutaka Enomoto, Toshihiko Oki, Ko-

ichiro Yuji, Hideaki Nakajima[4], Yuka Harada[5], Hironori Harada[5], Toshiya Inaba[6], Tetsuya Nosaka, Jiro Kitaura, and Toshio Kitamura: [4]Keio University School of Medicine [5]Department of Hematology/Oncology and [6]Department of Molecular Oncology, Research Institute for Radiation Biology and Medicine, Hiroshima University

To elucidate the molecular mechanisms of leukemia, MDS, and MPD, we established mouse model using bone marrow transplant (BMT); we transduced mouse bone marrow cells with genes of leukemogenic mutations derived from patients including MLL-fusions and BCR-Abl, and mutant forms of AML1 and C/EBPa using retroviruses. The bone marrow cells transduced with these mutant genes derived from patients were transplanted to irradiated mice. Using this mouse BMT model, we have shown several interesting things; 1) Combination of class I (MLL-Sept6) and class II mutations lead to development of acute leukemia; 2) A class II mutation (AML1 mutations) induced MDS-like disease, and some of the mice progressed to acute leukemia with additional mutations such as overexpression of Evi1; 3) Combination of BCR-Abl and Hes1 expression induced CML blast crisis (BC) like disease. In fact, overexpression of Hes1 was demonstrated in 8 of 20 patients with CML-BC but not in patients with CML-chronic phase; 4) Two classes of C/EBPa mutations (N-terminal and C-terminal mutations) collaborate with each other in inducing acute leukemia in mouse BMT models, probably working as class I and class II mutations.

We also establish bone marrow-derived immature cell lines transduced with MLL fusions and Hes1 overexpression. While the differentiation of these cell lines is blocked probably by MLL-fusions and Hes1 overexpression, they still remain dependent on IL-3. In the mouse BMT model, we are beginning to understand that leukemogenesis (acute leukemia as well as MDS/AML and CML-BC) require multiple mutations; mutations that block differentiation, and mutations that block apoptosis or induce factor-independent proliferation. Based on the mouse BMT model, we assume that there are the second hit mutations in addition to mutations such as MLL-fusions and AML-1 in patients' leukemic cells. To identify such mutations, we use retrovirus-mediated expression cloning method. To this end, we make cDNA libraries of patients' leukemic cells, and will isolate cDNAs that give rise to the autonomous growth of the cytokine-dependent cell lines established as stated above. In this way, we isolated ITD-Flt3 (constitutively activated Flt3 mutant found in 30 % of patients with acute myeloid leukemia) and RasGRP4 that activate Ras pathways. We are now testing the effects of RasGRP4 in mouse BMT models.

Recent progress using high-speed sequencing has identified mutations in genes encoding epigenetic factors. Experiments are now under way to clarify the molecular mechanisms by which mutations of epigenetic factors including TET2, EZH2 and ASXL1 induce hemopietic malignancies using mouse BMT models and transgenic mice.

5. The function of TSC-22 as a tumor suppressor.

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Transforming growth factor-beta (TGF-beta)-stimulated clone-22 (TSC-22) was originally isolated as a TGF-beta-inducible gene. We identified TSC-22 as a potential leukemia suppressor. Two types of FMS-like tyrosine kinase-3 (Flt3) mutations are frequently found in acute myeloid leukemia: Flt3-ITD harboring an internal tandem duplication in the juxtamembrane domain associated with poor prognosis and Flt3-TKD harboring a point mutation in the kinase domain. Comparison of gene expression profiles between Flt3-ITD- and Flt3-TKD-transduced Ba/F3 cells revealed that constitutive activation of Flt3 by Flt3-TKD, but not Flt3-ITD, upregulated the expression of TSC-22. Forced expression of TSC-22 suppressed the growth and accelerated the differentiation of several leukemia cell lines into monocytes, in particular, in combination with differentiation-inducing reagents. On the other hand, a dominant-negative form of TSC-22 accelerated the growth of Flt3-TKD-transduced 32 Dcl.3 cells. Collectively, these results suggest that TSC-22 is a possible target of leukemia therapy.

We have characterized the function of TSC-22 using TSC-22-deficient mice and soft agar colony formation of NIH3T3 cells, and have demonstrated that TSC-22 inhibited transformation both in vitro and in vivo, indicating that TSC-22 is a tumor suppressor.

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The long-term goal of our division is medical sciences on infectious diseases in two directions, from clinic to bench and from bench to clinic. Our current main subject is immunopathogenesis of HIV-1 infection. We are focusing on how cellular immune responses fight against HIV-1 and how immune system is disrupted and develop AIDS after long-lasting exposure to HIV-1. We are also developing detection system for drug-resistant HIV-1. We work together with the staffs in the Department of Infectious Diseases and Applied Immunology in the IMSUT hospital and apply the research results to the people living with HIV-1/AIDS.

1. Analysis of T cell dysfunction during chronic HIV infection

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Chronic HIV-1 infection is characterized by immune cell dysfunctions driven by chronic immune activation. Plasma HIV-1 viral load (VL) is closely correlated with disease progression and the level of immune activation. However, the mechanism by which the persistent presence of HIV-1 damages immune cells is still not fully understood. To evaluate how HIV-1 affects disruption of T cell-mediated immune responses during chronic HIV-1 infection we determined the functional profiles of T cells from subjects with chronic HIV-1 infection. We measured the capacity of peripheral blood mononuclear cells (PBMCs) to produce 25 specific cytokines in response to nonspecific T cell stimulation, and found that the capacity to produce Th-1-related cytokines (MIP-1α, MIP-1β, RANTES, IFN-γ, and MIG), sIL-2R, and IL-17, but not Th-2-related cytokines, was inversely cor- related with plasma VL. The capacities to produce these cytokines were interrelated; notably, IL-17 production had a strong direct correlation with production of MIP-1α, MIP-1β, RANTES, and IFN-γ. In both CD4＋ and CD8＋ T cells, dysfunctional production of cytokines was associated with T cell activation (CD38expression) and exhaustion (PD-1 and/or CTLA-4 expression) status of memory subsets. Although the capacity to produce these cytokines was recovered soon after multiple log 10 reduction of plasma viral levels by antiretroviral therapy, memory CD8＋ T cells remained activated and exhausted after prolonged virus suppression. We also found IL-2 production at early time point after T cell stimulation was significant lower in high VL subjects than low VL subjects. Our data suggest that HIV-1 levels directly affect the ability of memory T cells to produce specifically Th1- and Th17-related cytokines during chronic HIV-1 infec-

tion.

2. Impact of Maraviroc intensification on HIV-1 specific CD8+ T cell response in recently infected HIV-1 patients initiating treatment with Raltegravir plus tenofovir/emtricitabine.

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Maraviroc (MVC) is an antiretroviral drug which binds to CCR5, co-receptor of HIV infection, and blocks HIV entry. Some clinical studies reported that treatment with MVC has resulted in larger increases in CD4+ T cell counts than other HIV drugs. We have also observed faster CD4 increase in subjects with standard triple therapy with MVC intensification compared to subjects without MVC intensification in our phase III clinical trial. As CD4+ T cells are important to maintain functional CD8+ T cells, the apparently more robust CD4 T cell recovery in Maraviroc intensified subjects compared to the standard triple therapy treated individuals might be associated with broader, stronger, and more functional CTL responses. We assessed magnitude, breadth, and polyfunctionality of HIV-specific CD8+ T cells in both groups, and found broader HIV-specific response tend to remain longer in MVC intensified subjects than control subjects.

3. Replication capacities of chimeric NL4-3 encoding *gag-protease* from modern HIV-1 isolates are significantly reduced compared to those derived from isolates in the early days of epidemic in Japan

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Human Immunodeficiency Virus type I (HIV-1) evolve rapidly and adapt to various host selection pressures. As a result, HIV-1 in modern days possibly have distinct property from isolates in early days of the epidemic. So far, few studies have demonstrated alternation in viral replication capacity since the discovery of HIV-1. In the present study, we compared Gag-protease associated HIV replication capacity between early and modern days of epidemic in Japan.

Total 167 antiretrovirals-naïve asymptomatic HIV positive Japanese were enrolled; all of them were infected with cladeB viruses and visited our clinic from 1994 to 2009; the majority of them were MSM. Chimeric NL4-3 encoding *gag-protease* derived from plasma collected at near first visit to the clinic were generated; relative replication capacity of the chimeric viruses to wild-type NL4-3 were measured by infecting them to LTR-driven GFP-reporter T-cell line. Multivariate analyses were performed to examine correlation between viral replication capacity (VRC), CD4+T cell counts, plasma virus loads (pVL) and the year of patient's first visit.

There were no significant differences in CD4+T cell count and pVL between the patients in the early days and those in the modern days of HIV epidemic. We found that VRC of chimeric viruses were inversely correlated with the year of patient's first visit ($r=-0.01$, $p=0.003$); the significance remained even after adjusting with CD4+T cell count and pVL ($r=-0.01$, $p=0.005$). In a different statistical procedure, modern viruses (2005-2009) displayed significantly reduced VRC as compared to viruses in the early days (1994-1999) (relative to wild-type: 1.14 vs 1.30, $p=0.01$). These findings indicate that cladeB HIV-1 circulating among Japanese population have been attenuated as the epidemic matures; and modern HIV isolates in Japan have reduced replication capacity as compared to old viruses. It will be warranted to investigate the mechanisms of this alternation in viral replication capacity.

4. Induction and maintenance of neutralizing antibodies after single dose of non-adjuvanted split 2009 pandemic H1N1 influenza A vaccine in HIV-1 positive population

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Several studies have examined immunnogenicity of 2009 pandemic H1N1 influenza A (pdmH1N1) vaccine in HIV+populations using hemagglutinin inhibition test; but the results are

inconsistent and none of them analyzed kinetics of the antibody responses. In the present study, we investigated neutralizing antibody (NT) responses to pdmH1N1 in HIV+population over 4 months after vaccination.

103 HIV+ adult outpatients of the IMSUT hospital (median CD4+T cell count: 451 cells/mm^3, 81% on cART) and 17 age-matched healthy controls (HC) were enrolled, and received single dose of pdmH1N1 (A/California/7/2009) vaccine in 2009/2010 season. Serum samples were collected at baseline and 2 month post-vaccination from all of them; and additional samples were obtained, if available, at one month and 4 month post-vaccination for kinetics study. NT titers to pdmH1N1 were determined by microneutralizing antibody assay.

There were no difference in NT titers at baseline between HIV+subjects and HC. At 2 month post-vaccination, 49.5% (51/103) of patients and 58.8% (10/17) of HC showed positive responses (defined as $\geq$4-fold increase), however there were no significant difference between them ($p=0.60$). In HIV+subjects, following factors were not different between responders and non-responders (age, current and nadir CD4+T cell count, plasma virus loads, time from HIV diagnosis, with or without cART). Nevertheless, in the kinetics study of 9 patients and 10 HC, while the time to peak NT titer did not differ between the groups (both at 1 month post-vaccination), we observed significantly greater decline (from 1 month to 4 month post-vaccination) in NT titer in HIV+subjects than those in HC (75% vs 50%, $p=0.036$). Despite neutralizing antibody response rates to pandemic H1N1 vaccine in HIV+population appeared comparable to those in healthy controls and there were no clear association between CD4+T cell count and NT responses, the capability of maintaining vaccine-induced NT responses might be impaired in HIV-infected populations.

5. HIV-1 Drug Resistance test using a sequence specific oligonucleotide probes and Luminex assay system

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HIV-1 Drug resistance (DR) a major obstacle to the long-term efficacy of antiretroviral therapy. Although PCR-based viral sequencing is the standard of genotypic HIV-1 DR testing, the development of new classes of antiretroviral drugs (ARVs), such as integrase inhibitors and entry inhibitors, requires that wider gene segments be sequenced in the countries where these new ARVs are available. In some developing countries, expanded access to treatment has increased the need for testing for resistance to the subset of ARVs that are affordable in those countries. Considering the wide usage of NRTIs and NNRTIs, a new simplified system to detect the DR mutations in the RT coding region is warranted. We developed a strategy that combines a PCR amplification- sequence-specific oligonucleotide probes protocol and Luminex technology to detect 6 mutations in HIV-1 RT gene: M41L, K65R, K70R, K103N, M184V and T215Y/F in single tube. The system appears capable of detecting DR mutation if they comprise at least 10-20% of the population. We used the assay to determine the presence or absence of the 6 RT mutations in plasmas from 74 clade B HIV-1-infected patients, and compared the results to those obtained by sequencing. The assay system specificity would be 95.5%, 100%, 90.0%, 71.4%, 81.0%, and 95.7%, and the sensitivity 78.8%, 91.5%, 95.3%, 80.6%, 88.5%, and 93.8% for M41L, K65R, K70R, K103N, M184V and T215 Y/F, respectively. Our data suggest that PCR-SSOP-Luminex assay can be used effectively to detect clinically significant DR mutations in HIV-1.

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Our division has been conducting basic research projects related to the cancer and transplantation immunology. The reagents, modalities, and concepts developed in this division have been clinically applied as translational research projects by the clinicians of Department of Surgery in our research hospital. We believe that bidirectional information exchange between the bench and the bed side would be one of the most important requirements for the successful development of novel and effective therapies.

Development of innovative cancer therapy using immunologic approaches

I. Induction of memory Th1 cell responses with IL-23 produced by human mature dendritic cells stimulated with OK-432 combined with PGE2

Marimo Sato-Matsushita, Akira Kanamoto, and Hideaki Tahara

The OK-432 (Picibanil) is a biological response modifier derived from the weakly virulent Su strain of Streptococcus pyogenes and available in GMP quality. It has been reported that OK-432 could induce the monocyte-derived human DCs to become mature to produce significant amounts of IL-12 without interfering the presentation of pulsed epitope-peptide. Previously in 2004, we reported that the migratory capacity of human monocyte derived OK-432-stimulated dendritic cells can be promoted with the co-administration of prostaglandin E2 (PGE2) (OK-P-DCs). Thus, OK-P-DCs appeared to be useful for clinical DC therapy. In the current study, we found that the immature DCs stimulated by OK-432 with or without PGE2 could secrete high amount of bioactive IL-12 and IL-23. Recently identified cytokine IL-23, which is composed of the IL-23 p19 subunit and the IL-12/23 p40 subunit, has been shown to preferentially act on Th1 effector or memory CD4+ T cells and induce their IFN-g production and proliferation. We examined the biological and immunological effect of IL-23 produced by OK-P-DCs. The results of such experiments suggest that OK-P-DCs induce memory type Th1 immune responses against tumor cells in effector phase through IL-12 and IL-23 secretion. These results indicated that OK-P-DCs might efficiently induce memory Th1 cell responses with IL-12 related cytokines.

II. Induction of systemic and therapeutic antitumor immunity using dendritic cells genetically modified to express interleukin-23

Marimo Sato-Matsushita, Akira Kanamoto, and Hideaki Tahara

We have reported that the systemic administration of IL-23 induces potent antitumor immunity primarily mediated with the Th1-type response. In this study, we investigated whether

bone marrow- derived dendritic cells (BM-DCs) adenovirally transduced with genes encoding murine IL-23 have therapeutic benefits for antitumor immunotherapy. We made RGD fiber-mutant adenovirus (Ad) vectors encoding IL-23 or EGFP. The MCA205 fibrosarcoma was intradermally inoculated to C57BL/6 on day 8, and the mice were intratumorally injected with BM-DCs transduced with Ax3CAmIL23/RGD (Ad-IL-23-DCs). The tumors of mice treated with AD-IL-23-DCs resulted in significant growth suppression when compared to that with BM-DCs transduced Ad-EGFP-F/RGD. Ad-IL-23-DCs treatment induced MCA-205-specific and potent CTL responses. In addition, The significant induction of IFN-γ and IL-17 and decrease of Tregs in TIL were strongly suggested in the mice injected with Ad-IL-23-DCs. This strategy designed to deliver genetically modified DCs to tumor sites is associated with systemic and therapeutic antitumor immunity and could be an alternative approach to those using delivery of DCs loaded with defined tumor antigens.

III. Early activation and interferon-γ production of tumor-infiltrating mature CD27high natural killer cells

Yoshihiro Hayakawa, Marimo Sato-Matsushita and Hideaki Tahara

Natural killer (NK) cells are known to be critically involved in the control of tumors through their direct cytotoxic function, but have also been proposed as an initial source of interferon (IFN)-γ that primes subsequent adaptive tumor-specific immune responses. Although mounting evidence supports the importance of NK cells in antitumor immune responses, the immunological characteristics of NK cells infiltrating the tumor microenvironment and the mechanisms that regulate this process remain unclear. In the present study, we found that NK cells infiltrate early developing MCA205 tumors, and further showed that mature CD27 (high) NK cells were the predominant subpopulation of NK cells accumulating in the tumor microenvironment. The tumor-infiltrating NK cells displayed an activated cell surface phenotype and provided an early source of IFN-γ. Importantly, we also found that host IFN-γ was critical for NK cell infiltration into the local tumor site and that the tumor-infiltrating NK cells mainly suppressed tumor growth via the IFN-γ pathway. This work implicates the importance of IFN-γ as a positive regulatory factor for NK cell recruitment into the tumor microenvironment and an effective antitumor immune effector response.

IV. Evidences for cancer immune-escalation process and the role of inflammatory cytokines

Yoshihiro Hayakawa, Marimo Sato-Matsushita and Hideaki Tahara

Although an inflammatory microenvironment is an essential component of tumors, the precise mechanism remains to be clarified by which type of immune response can positively or negatively control cancer progression to metastatic phenotypes. In this study, we aim to demonstrate the immune response can be involved in cancer malignant progression of cancer, particularly providing inflammatory tumor microenvironment. To examine the regulatory mechanism of host inflammatory responses in cancer malignant progression, we employ an unique in vivo mouse model in which low tumorigenic original cancer cells acquire highly malignant and metastatic phenotypes after exposure to host inflammatory microenvironment induced by inflammation initiator. By using this model, we investigated the roles of inflammatory mediators, specifically IL-17 and IFN-γ in the process of inflammation-associated cancer malignant progression termed as cancer immune-escalation process. We found that IL-17 was critical component for cancer immune-escalation process, and further currently investigating the role of those two cytokines in regulating the initial tumor growth and acquisition of metastatic ability upon in vivo inflammation-associated tumor progression.

V. Tumor-associated macrophages regulate tumorigenicity and anticancer drug responses of cancer stem/initiating cells.

Masahisa Jinushi, Akinori Takaoka, Hideaki Tahara

Recent evidence has unveiled the critical role of tumor cells with stem cell activities in tumorigenicity and drug resistance, but how tumor microenvironments regulate cancer stem/initiating cells (CSCs) remains unknown. We clarified the role of tumor-associated macrophages (TAMs) and their downstream factor milk-fat globule-epidermal growth factor-VIII (MFG-E8) in the regulation of CSC activities. Bone marrow chimeric systems and adoptive cell transfers elucidated the importance of MFG-E8 from TAMs in conferring to CSCs with the ability to promote tumorigenicity and anticancer drug resistance. MFG-E8 mainly activates signal transducer and activator of transcription-3 (Stat3) and Sonic Hedgehog pathways in CSCs and further ampli-

fies their anticancer drug resistance in cooperation with IL-6. Thus, the pharmacological targeting of key factors derived from tumor-associated inflammation provides a unique strategy to eradicate therapy-resistant tumors by manipulating CSC activities.

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Our long term goal is to define the molecular and structural basis for the mechanisms of the immune abnormalities observed in various immune-mediated disorders such as autoimmue disease as well as to cure patients suffering from the above immune-mediated disorders. To accomplish this goal, we have focused on defining the structure and function of cell surface and intracellular molecules expressed in human T cells and other cells and on understanding how the immune regulatory system works in normal and disease conditions. Moreover, we will establish the translational research to cure such diseases. Our study will provide new insights into understanding the precise molecular mechanisms that underlie immune abnormalities found in various autoimmune diseases as well as other immune-mediated disorders and will lead to the development of new rational therapy for the manipulation of the abnormalities found in such diseases.

I. β1 integrins and Cas-L/NEDD9

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β1 integrins play crucial roles in a variety of cell processes such as adhesion, migration, proliferation, and differentiation of lymphocytes. Previously we showed that co-immobilized anti-β1 integrin mAbs or its ligand with a submitogenic dose of anti-CD3 mAb induced a marked increase of IL-2 secretion and proliferative response of T cells, indicating that β1 integrins are costimulatory molecules of T cells. Pp105 was first described in our laboratory as a protein predominantly tyrosine phosphorylated by the ligation of β1 integrins in H9 T cells. By cDNA cloning, we demonstrated that pp105 was a homologue of p130Cas (Crk-associated substrate)/BCAR1 (Breast Cancer Antiestrogen Resistance 1), and designated as Cas-L (Cas lymphocyte type). It has been shown that Cas-L, HEF1 (human enhancer of filamentation), and NEDD9 (neural precursor cell expressed, developmentally down-regulated 9) are identical gene products. We found that transfection of Cas-L cDNA into Jurkat T cells restored β1 integrin-mediated costimulation and cell migration, indicating that Cas-L plays a key role in the β1 integrin-mediated T cell functions.

Our present projects aim at investigating the biological significance of Cas-L *in vitro* and *in vivo.* Our approach may shed a light on the clinical relevance of Cas-L-mediated signaling pathways in inflammatory diseases and malignancies.

a. NEDD9/HEF1/Cas-L is a potential prognostic biomarker for human lung cancer.

In substantial population of non-small cell lung cancer (NSCLC), expression and activation of EGFR has been reported, and is regarded as a novel molecular target. A growing body of evidence has shown the signaling crosstalk between EGFR and integrins in cellular migration and invasion. NEDD9/HEF1/Cas-L is an integrin signaling adaptor protein composed of multiple domains serving as substrate for a variety of tyrosine kinases. In the present study, we aimed at elucidating a role of Cas-L in the signaling crosstalk between EGFR and integrins.

Using NSCLC cell lines, we performed immunoblotting and cellular migration/invasion assay in vitro. Next, we analyzed metastasis assays in vivo by the use of xenograft transplantation model. Finally, we retrospectively evaluated clinical samples and records of NSCLC patients.

We demonstrated that tyrosine phosphorylation of Cas-L was reduced by the inhibition of EGFR in NSCLC cell lines. Overexpression of constitutively-active EGFR caused tyrosine phosphorylation of Cas-L in the absence of integrin stimulation. By gene transfer and gene knockdown, we showed that Cas-L plays a pivotal role in cell migration and invasion of those cells in vitro. Furthermore, overexpression of Cas-L promoted lung metastasis of a NSCLC cell line in NOG mice. Finally, univariate and multivariate Cox model analysis of NSCLC clinical specimens revealed a strong correlation between Cas-L expression and recurrence-free survival as well as overall survival.

Our data thus suggest that Cas-L is a promising biomarker for the prognosis of NSCLC, and its expression can promote NSCLC metastasis.

b. Analysis on collagen-induced arthritis of Cas-L/Nedd9 knockout mice.

Rheumatoid arthritis (RA) is an autoimmune polyarthritis that is accompanied by the infiltration of T cells into articular joints, proliferation of synovium, degradation of articular cartilage, and osteolytic changes in the affected joints. In the affected joints of RA patients, there have been reported an elevated expression of beta 1 integrins (VLAs) and their ligands such as VCAM-1. Previously, we found that the levels of Cas-L are markedly upregulated in various tissues of HTLV-I tax transgenic mice, a murine model of RA. In particular, large amount of Cas-L positive lymphocytes and leukocytes migrating into the inflamed joints, suggesting a role of Cas-L in pathophysiology of RA. To further evaluate the pathophysiological role of Cas-L in the development of RA, we employed collagen-induced arthritis model using Cas-L knockout mice on a C57BL6 background.

We compared three groups of mice, Cas-L $-/-$ (homo), Cas-L $-/+$ (hetero), and littermate controls (wt) on the incidence and severity of CIA. Although the incidence of arthritis was unaltered, wt and hetero mice showed higher severity of arthritis compared to homo mice, which was evaluated by scoring and histology. X-ray analysis suggest that the joint space narrowing and bone destruction of hind limb is more apparent in wild type mice than that of Cas-L $-/+$ and Cas-L $-/-$ mice. The serum levels of inflammatory cytokines, TNF-α, IL-17, and IL-6 were higher in wt and hetero mice, whereas that of anti-inflammatory cytokine, IL-10 was higher in homo mice. We are now performing microarray analysis of mRNA obtained from the affected joints of those mice. Finally, we hope that our study might suggest a pathophysiological role of Cas-L in vulnerability to rheumatoid arthritis in mouse models.

c. Protective role of Crk-associated substrate lymphocyte against Citrobacter rodentium infection.

Crk-associated substrate lymphocyte type (Cas-L) is an integrin signaling adaptor protein composed of multiple domains serving as substrate for a variety of tyrosine kinases. Cas-L functions in diverse biological processes such as cell attachment, migration and invasion, apoptosis, and cell cycle progression. The role of Cas-L in immune responses in vivo and host defense mechanisms against bacterial infection remains to be elucidated.

To elucidate the function of Cas-L in host defence, we employed Cas-L-lacking mice (Cas-L $-/-$). Citrobacter rodentium (C. rodentium) (strain DBS100) were cultured overnight at 37°C in Luria broth with agitation. Cultures were harvested by centrifugation and concentrated to 10-fold. Mice were orally inoculated by gavage with 200 μl of the bacterial suspension containing approximately $3\times10^8\sim4.5\times10^9$ CFU bacteria which was assessed by plating on MacConkey agar. The general conditions, form of stool, and body weight were monitored every day. After 7 and 10 days, mice were euthanized and distal colon, spleen, and liver were examined pathologically. Simultaneously, we determined CFU of the homogenate of these organs by serial dilution with PBS and spreading on MacConkey agar to examine the colonization of the bacteria in these organs. Serum samples were collected and subjected to cytokine measurement by Luminex.

The weight loss of Cas-L $-/-$ mice was greater than that of wild-type mice. The numbers of bacterial colonization of colon and

spleen were 10-fold larger in Cas-L −/− mice compared to the wild-type mice. The serum levels of IFN-γ, IL-6, IL-1β, TNF-α, IL-12/23p40 were elevated in the case of Cas-L −/− mice 7 days after infection.

Cas-L −/− mice may be more susceptible to bacterial infection than the wild-type mice, suggesting a pivotal role of Cas-L in the protection against bacterial infection.

II. Structural basis for CD26 mediated T cell costimulation and function in normal and disease conditions.

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CD26 is a 110-kDa cell surface glycoprotein that posseses dipeptidyl peptidase IV (DPPIV) (EC. 3.4.14.5) activity in its extracellular domain and a primary marker of activated T cells. In the resting state, CD26 is preferentially expressed on a subset of CD4 memory T cells where they account for the majority of IL-2 secretory capabilities and help for B cell Ig production and are the primary responders to recall antigen such as tetanus toxoid. CD26 is also capable of providing a potent costimulatory or "second" signal which can augment other activation pathways leading to proliferation, cytokine production and effector functions. The mechanism of costimulation remains unclear since the cytoplasmic domain consists of only 6 amino acid and lacks a phosphorylation site, leading to the conclusion that CD26 interacts with other cell surface molecules. We have already shown that CD26 may interact with CD45RO which modulates TcR/CD3 activity through its intracellular tyrosine phosphatase domain. Recently, we have detected another CD26 binding protein, the mannose-6-phosphate/insulin-like growth factor II receptor (M6P/IGFIIR) as being critical for this interaction for CD26 mediated T cell costimulation in addition to adenosine deaminase (ADA). More recently, we have shown that CD26 localizes into lipid rafts, and targeting of CD26 to rafts is necessary for signaling events through CD26. Importantly, aggregation of CD26 by anti-CD26 mAb crosslinking also causes coaggregation of CD45 into rafts. In addition, we have demonstrated that recombinant soluble CD26 (sCD26) has an enhancing effect on T cell proliferation in the presence of the recall antigen, tetanus toxoid. This enhancement resulted in an increase in the surface expression of the costimulatory molecule CD86 on monocytes following sCD26 binding to Caveolin-1 expressed on monocytes. Moreover, we showed that CD26 plays a role in Tumor growth and Invasion.

Currently we are focusing on the molecular and structural and structural basis for CD26-mediated T cell activation signaling and are searching for its ligand directly involved in CD 26-mediated T cell costimulation. Furthermore we are focusing on the translational research of utilization of anti-CD26 mAb, Caveolin-1 fusion protein and recombinant soluble CD26 for treatment of malignant tumors, immune-mediated disorders and immune deficiency diseases. The phase I/II clinical trial utilizing humanized CD 26 antibody for the treatment of malignant mesothelioma has already been started at Gustave Roussy Institute and Cochin Hospital in Paris. We hope that phase I/II clinical trial will start in Japan soon.

a. A fine strategy for immunetolerance induction therapy using blockade of CD26-mediated T cell costimulation with soluble Caveolin-1-Ig fusion protein

Recently, we have demonstrated that caveolin -1 is a binding protein of CD26, and that CD26 on activated memory T-cells interacts with caveolin-1 on tetanus toxoid (TT)-loaded monocytes. Moreover, following CD26-caveolin-1 interaction on TT-loaded monocytes, caveolin-1 is phosphorylated, with linkage to NF- B activation, followed by upregulation of CD86. To examine the binding of caveolin-1 to CD26 in T-cells more precisely, we made soluble Fc fusion proteins containing the N-terminal domain of caveolin-1 (Cav-Ig), and found that ligation of CD26 by solid-phased Cav-Ig induces T-cell proliferation in the presence of TCR/CD3 costimulation.

Presentation of antigen to the T cell receptor (TCR) without costimulation results in a state of antigen-specific unresponsiveness on rechallenge, known as anergy in vitro and tolerance in vivo. However, blockade of costimulation via the CD40 or CD28 pathways, which effectively leads to a state of tolerance of naïve donor-specific T cells in allogeneic transplantation, may not be effective against donor-specific memory T cells elicited either by exposure to donor antigens or viral pathogens. Since CD26 is preferentially expressed on a specific population of lymphocytes, the subset of CD4+CD45 RO+memory T cells, and is unregulated follow-

ing T cell activation, blockade of CD26-mediated T cell costimulation may lead to effective T cell anergy in response to recall or allogeneic antigens. In this year, we therefore evaluated the effect of in vitro treatment of recombinant, soluble Cav-Ig as a surrogate ligand for CD26 binding in an effort to block CD26-related functions on CD4+lymphocytes, hypothesizing that this approach could prevent efficient T cell activation during autologous and allogeneic immune responses. Consistent with previous work involving antibody blocking studies, blockade of CD26 costimulation by Cav-Ig renders CD4+T cells unresponsive not only to TT-pulsed autologous APC, but also to allogeneic APC. These results suggest that blockade of CD26-mediated costimulation provides immune tolerance to an allogeneic response, such as that found in transplantation. In this regard, therapeutic use of Cav-Ig in immune disorders may be tested in vitro and in vivo.

b. A novel mechanism of human CD8+ T cell function via CD26 molecule

CD26 is originally found as a T cell activation antigen, and contains dipeptidyl peptidase IV (DPPIV) enzymatic activity in its extracellular domain. Our laboratory has vigorously examined and found that CD26 molecule is functioned in T cell costimulation on human CD4+ T cells. Indeed, CD26 is preferentially expressed on human CD4+ memory T cells, and CD4+ CD45RO+CD26+ T cells exhibit maximal response to recall antigens. Human T-helper 1 (T_H 1) cells display a higher expression of CD26, and are much more sensitive to CD26-mediated costimulation than human T_H2 cells. In contrast, the role of CD26 in human CD8+ T cells still remains to be elucidated, while CD8+ T cells, as well as CD4+ T cells, express CD26 molecules. In this year, we have shown that the effector function of CD26-mediated costimulation of human CD8+ T cells. In comparison with CD28-medaited costimulation, which is one of established T cell costimulation, CD26-mediated costimulation in human CD8+ T cells showed delayed proliferation than that of CD28 stimulation, but finally expanded to a similar extent. The secretion of inflammatory cytokines, TNF-α and IFN-γ, was strongly induced after CD26-mediated costimulation. In contrast, the secretion of IL-2 and IL-5 was significantly less as compared with CD28-mediated costimulation. Finally, we have shown that the expression of Granzyme B, one of the major effector molecules in the cytotoxic activity of CD8+ T cells, was markedly enhanced by CD26-mediated costimulation in dose dependent manner of anti-CD26 monoclonal antibody stimulation. Moreover, with CD26-mediated costimulation, CD8+ T cells were observed to kill target cells in a Granzyme B-dependent manner. Taken together, Our data strongly suggest that CD26 plays a very important role in CD8+ T cell dependent defense against viruses and tumor cells, and also involves in pathology such as autoimmune diseases and graft-versus-host disease.

c. CD26-based molecular target therapy for graft-versus-host disease in hematopoietic stem cell transplantation

Graft-versus-host disease (GVHD) remains a major cause of morbidity and mortality in allogeneic hematopoietic stem cell transplantation (alloHSCT). In GVHD, mature donor T cells that accompany the stem cell graft attack recipient tissues, especially the skin, liver, gastrointestinal tract, and lung. Therefore, all patients undergoing alloHSCT receive GVHD prophylaxis to impair T cell function; however, treatment to prevent GVHD can be deleterious since mature donor T cells play a critical role in mediating reconstitution of the adaptive immune system. Recipients of alloHSCT are thus at great risk for infections, particularly when prolonged immunosuppression is required for treatment of GVHD. Although the role of CD26/DPPIV in GVHD needs to be studied in more detail, treatment with a murine antibody against human CD26 was reported to have an effect in patients with steroid-resistant acute GVHD following alloHSCT (Bacigalupo A., et al., Acta Haematol 1985:73:185, de Meester, et al., Immunobiology 1993:188:145). To examine the efficacy of CD26-targeting therapy in GVHD more profoundly, we established mouse GVHD model using human peripheral blood lymphocytes (huPBL) (xenograft GVHD mouse model; x-GVHD). After NOD/Cg-Prkdcscidil2rgtm1Sug/Jic mice were injected with appropriate numbers of huPBL, mice show symptoms of GVHD such as loss of weight, loss of hair, deterioration of activity, and thinning of ear pads. Histopathological examination revealed that CD3+CD8+CD26+ human lymphocytes were infiltrated in the skin, intestinal mucosa, salivary gland, lung and liver of the x-GVHD mice. In this mouse model, humanized anti-CD26 monoclonal antibody (mAb) was injected two weeks later of onset of x-GVHD, and the symptoms of GVHD were improved after ten injections of humanized anti-CD26 mAb. Moreover, x-GVHD was observed to be suppressed when humanized anti-CD26 mAb was prophylactically administered. Taken together, it may be possible that the full therapeutic potential of alloSCT will be realized by

approaches that aim to minimize GVHD by targeting CD26-mediated T cell regulation.

d. In vitro effects of CD26/DPPIV-specific inhibitor, sitaglipitn on immune and inflammatory cell responses

In addition to being a marker of T-cell activation, CD26/DPPIV is also associated with T-cell signal transduction processes as a costimulatory molecule, and the enzymatic activity of CD26/DPPIV appears to play an important role in enhancing cellular responses to external stimuli. Inhibition of DPP-4 is a novel oral treatment for type 2 diabetes. DPPIV inhibition increases insulin secretion and reduces glucagon secretion by preventing the inactivation of glucagon-like peptide-1 (GLP-1), thereby lowering glucose levels. As described above, since DPPIV activity is reported to be involved in human T-cell function, DPPIV inhibitor treatment is necessary for analyzing adverse effects on various parameters of immune function. In this regard, other investigators recently reported to compare T-cell activation in diabetic patients treated with and without sitagliptin. It is observed that sitagliptin treatment had no apparent influence on T-cell activation in vitro, suggesting that defective CD 26-dependent T-cell activation cannot explain the increased rate of infection in diabetic patients treated with DPPIV inhibitors. In this study, however, CD4+ lymphocytes isolated from the donors were stimulated with phytohemagglutinin (PHA). Since PHA is a very strong mitogen to lymphocytes, and therefore, this assay system might not detect more subtle immune defects involved in CD26-mediated T-cell costimulation responding to recall antigens. The primary objective is to determine whether the specific DPP-4 inhibitor sitagliptin has effects on immune function of CD4+ T-cells and endothelial cells in inflammatory circumstance. Specifically, we have determined whether sitagliptin has alterations in the magnitude or type of memory response of human CD4+ T-cells. For this purpose, human peripheral CD4+ lymphocyte is stimulated with anti-CD3 plus anti-CD26 or anti-CD28 mAbs as we developed previously, and various cytokine production is measured using ELISA. Moreover, CD4+ T-cells are isolated from TT-immunized healthy adult donors. T-cell proliferation and cytokine production are examined It is obeserved that sitagliptin decreased CD26-mediated T-cell proliferation and Th1/Th2/inhibiyory cytokine production. Moreover, only higher dose of Sitagliptin decreased CD28-mediated T-cell proliferation and IFN-γ/IL-5/IL-10. We next exmained an effect of sitagliptin on memory response of CD4+ T-cells. For this purpose, we conducted recall antigen, tetanus toxoid-driven T-cell stimulation system. In this systme, sitagliptin, as well as P32/98, decreased tetanus toxoid-mediated T-cell memory responses. Taken together, our present data indicate that DPPIV specific inhibiton affect on T-cell immune responses, and may lead to abrogation of T-cell immunity by immbalances of T-cell responses. These researches will reveal an important issue in DPPIV inhibition and infection/autoimmunity, may lead to provide new drugs on immunity or diabetes mellitus.

e. Clinical application of CD26/DPPIV and malignant mesothelioma

Malignant pleural mesothelioma (MPM) is an aggressive malignancy arising from the mesothelial cells lining the pleura. It is generally associated with a history of asbestos exposure and has a very poor prognosis. In fact, the median survival is less than 12 months, with most patients dying within 10-17 months of their first symptoms. Once rare, the incident of MPM has increased in industrialized nations as a result of past wide spread exposure to asbestos. The incident is predicted to increase further in the next decades, especially in developing countries where asbestos has not yet been prohibited. Due to the lack of efficacy of conventional treatments, novel therapeutic strategies are urgently needed to improve outcomes. The identification of potential biomarkers would be helpful in managing three clinical aspects of MPM: early diagnosis, prognosis and response to therapy. However, there are presently no established indicators that have clinical significance for MPM. Given the fact that MPM remains a rare disease with several subtypes, the lack of accessibility to uniform tumor populations renders the search for biomarkers very difficult. Recently, we showed that CD26 is preferentially expressed on malignant mesothelioma cells but not on normal mesothelial cells, and suggested that membranous expression of CD26 indicates an importance in treatment of patients with MPM . More importantly, humanized anti-CD26 antibody inhibited growth of malignant mesothelioma cells and induced long term survival of tumor-transplanted SCID mice. Although we recently showed that cells from certain CD26-positive mesothelioma cell lines appeared to include the cancer stem cell characteristics for malignant mesothelioma in addition to CD24 and CD9-positive cells, the role of CD26 for MPM in the clinical setting as a prognostic and therapeutic biomarker has not yet been clarified. We showed that the CD26 molecule is expressed on the cell membrane of the epithelial and biphasic,

but not the sarcomatoid, type of mesothelioma. Importantly, treatment outcome prediction study showed that CD26 membrane expression on MPM was closely correlated with disease responsiveness to chemotherapy. Meanwhile, our *in vitro* studies showed that mesothelioma cells expressing high level of CD26 displayed high proliferative activity, and microarray analysis of CD26 knockdown and CD26-transfected mesothelioma cells showed that CD26 expression was closely linked to expression of genes contributing to cell proliferation, cell cycle regulation, drug- induced apoptotic action, and chemotherapy resistance. These data further argue for the potential clinical significance of CD26 in MPM. In conclusion, our present work suggests that CD26 is an important biomarker for predicting sensitivity to chemotherapy, supported by our *in vitro* studies and microarray analyses. Moreover, since membranous CD26 expression can potentially predict tumor sensitivity to chemotherapy, knowledge of membranous CD26 expression in MPM may affect clinical care of patients and treatment decision.

III. Therapeutically targetting transcription factors

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We are interested in the mechanism of eukaryotic gene expression and development of novel therapy and/or drugs that target transcriptional machineries. For this purpose, our recent work is mainly focused on conditional regulation of transcription factors including the glucocorticoid receptor. Our recent achievement has been now applied in clinical settings in the Research Hospital.

Glucocorticoid hormones are effective in controlling inflammation and immunity, but underlying mechanisms are largely unknown. It has been shown that both positive and negative regulation of gene expression is necessary for this process. The genes whose activity is negatively modulated in the anti-inflammatory process code for several cytokines, adhesion molecules. Most of them do not carry a classical binding site for regulation by the GR, but have instead regulatory sequences for transcription factors such as AP-1 or NF-κB. Considering various severe side effects of glucocorticoids, it may be pharmacologically important to dissociate these negative regulatory function of the GR from induction of genes for metabolic enzymes, expression of which have been shown to be positively regulated by the GR. We propose that a certain class of compounds (surprisingly, some of them are non-steridal chemicals) may dissociate transactivation and transrepression function of the GR and offer opportunities for the design of such compounds that could function more effectively as antiinflammatory drugs. In this line, we are developing novel therapeutic strategy. On the other hand, we have developed an efficient system to screen out the target genes of GR in glucocorticoid-responsive tissues, and are working with clarification of tissue-specific effects of glucocorticoids.

a. Development of Dissociating Ligand for the GR

The GR function could be differencially regulated by ligands. We have recently shown that not only synthetic glucocorticoids but also certain bile acids could differentially modulate GR function. Moreover, the effects of those compounds are indicated to be ascrived to the ligand binding domain of the receptor. In this line, we are going to isolate the dissociating ligand that preferencially promotes transrepression function of the GR. Recently we have demonstrated that certain ligands can modulate interdomain communication of the GR, which will eventually contribute to isolation of novel category of ligands.

On the other hand, receptor specificity is another important aspect of novel GR regulator. In this line, we have shown that cortivazol is extremely specific for GR and does not bind to MR. We are studying the molecular basis for this receptor specificity of the ligand using cortivazol as a model. Our recent microarray study demonstrated that GR and MR have differential role in homeostatic regulation in non-classical corticosteroid target tissues including the heart. Notably, collaboration with Professor Miyano's laboratory greatly contributed to development of this program.

b. Molecular biology of small nuclear RNA binding protein HEXIM1

Expression of HEXIM1 is induced by treatment of vascular smooth muscle cells with a differentiation inducer hexamethylane bisacetamide. It is shown that HEXIM1 binds 7SK snRNA and inhibits P-TEFb-mediated transcriptional elongation process. Physiological significance of HEXIM1 is studied using newly generated transgenic mice. HEXIM1 Tg mice show cardiac resistance to elevation in pulmonary arterial pressure with less hypertrophic response, suggesting that HEXIM1 might be a therapeutic candidate of pulmonary hypertension in connective tissue

diseases.

c. Clarification of tissue-specific effects of glucocorticoids

We performed target gene identification and clarification of their biological significance in cardiac muscles and skeletal muscles.

1. Cardiac muscles. We found that the expression of genes that encode 2 key enzymes in a common pathway of prostaglandin biosynthesis were upregulated by glucocorticoids via the GR in cardiomyocytes: phospholipase A2 group IVA (*Pla2g4a*; encoding cytosolic calcium-dependent phospholipase A2 [cPLA2]), which belongs to the class of cPLA2s that preferentially cleave arachidonic acid from membrane phospholipids; and prostaglandin-endoperoxide synthase 2 (*Ptgs2*; encoding COX2), which converts arachidonic acid into PGH2. Importantly, ALD did not have similar stimulatory effects on these genes. The induction of *Pla2g4a* and *Ptgs2* by GR is specific for cardiomyocytes, since GR has been shown to transrepress the activation of these proinflammatory genes in most cells. Therefore, we sought to investigate the major types of prostanoids produced in cardiomyocytes after exposure to glucocorticoids and to clarify the roles of these products in cardiac physiology. Among the genes for PGH2 isomerases, expression of *Ptgds*, which encodes lipocalin-type prostaglandin D synthase (L-PGDS), was selectively upregulated by a GR-specific ligand. Consistent with this result, PGD2 was the most prominently induced prostaglandin by GR-specific ligand stimulation of cultured cardiomyocytes and in vivo hearts. Using isolated Langendorff-perfused hearts and cultured cardiomyocytes, we demonstrated that the activation of L-PGDS-mediated production of PGD2 was crucial for the cardioprotection against ischemia /reperfusion conferred by glucocorticoid-GR signaling. Our results suggest a novel interaction between glucocorticoid-GR signaling and the arachidonic acid cascade-mediated cardiomyocyte survival pathway. Recently, we have characterized the cardiac receptor for PGD2 and clinical application of this study is now ongoing in collaboration with the Department of Cardiology, Keio University School of Medicine.
2. Skeletal muscle. Muscle comprises~40% of body mass and contributes not only to the structure and movement of the body but also to nutrient storage and supply. In adult mammals, skeletal muscle hypertrophy/atrophy is characterized by an increase/decrease in the size (as opposed to the number) of individual myofibers, respectively. The control of muscle mass is believed to be determined by a dynamic balance between anabolic and catabolic processes. Mammalian target of rapamycin (mTOR) is a crucial component of the anabolic machinery for protein synthesis. Prototypically, insulin/IGF-1 activates mTOR via the PI3K-Akt pathway. Protein degradation in skeletal muscle cells is essentially mediated by the activity of two conserved pathways: the ubiquitin-proteasomal pathway and the autophagic/lysosomal pathway. The ubiquitin-proteasomal pathway is responsible for the turnover of the majority of soluble and myofibrillar muscle proteins. The activity of this pathway is markedly increased in atrophying muscle due to the transcriptional activation of a set of E3 ligase-encoding genes, e.g., atrogin-1 and MuRF1. Autophagy also plays an important role in the degradation of skeletal muscle, and is indicated to be a consequence of an ordered transcriptional program involving a battery of genes, e.g., LC3 and Bnip3. These positive and negative pathways are balanced in a highly coordinated manner for the determination of myofiber size and total muscle volume; however, distortion of this balance with a relative increase in degradation results in the generalized decrease of myofiber size and muscle atrophy (Hoffman and Nader, Nat. Med. 2004). Pioneering studies demonstrated that muscle atrophy is a result of active processes that are transcriptionally controlled through the expression of a particular gene set; the forkhead box O (FoxO) transcription factors are common components of a number of atrophy models and act as critical liaison molecules for protein degradation and autophagy via the transcriptional regulation of, for example, atrogin-1, MuRF1, LC3, and Bnip3 (Mammucari et al., 2007; Sandri et al., 2004; Stitt et al., 2004; Zhao et al., 2007). In clear contrast, it is evident that each disease has proper signaling pathways to FoxOs and that other components of the cellular machinery often participate in the progression of atrophy. Therefore, for the development of therapies against muscle atrophy, it should be addressed how the transcriptional program triggered by a particular atrophy pathway is orchestrated and how the balance of muscle protein synthesis and degradation is distorted in each disease.

Typically, glucocorticoid-induced muscle atrophy is characterized by fast-twitch type II glycolytic muscle fiber loss with reduced or no impact on type I fibers. The mechanism of such fi-

ber specificity is yet unknown. Previous reports suggested that the glucocorticoid-GR system has anti-anabolic and catabolic effects and promotes degradation via the induction of a set of genes including atrogin-1, MuRF1, and myostatin. Although the involvement of FoxO transcription factors is reported in the gene regulation of atrogin-1 and MuRF1 under the presence of excess glucocorticoids, the biochemical role of GR in the transcriptional regulation of muscle tissue has not yet been determined. Therefore, we investigated how GR-mediated gene expression coordinately modulates anti-anabolic and catabolic actions to understand the functional coupling of metabolism and volume regulation in muscle.

We identified REDD1 and KLF15 genes as direct targets of GR. REDD1 is known to be induced by various stressors, including glucocorticoid, and to inhibit mTOR activity via the sequestration of 14-3-3 and the increase of TSC1/2 activity. We clearly identified the functional GRE via the promoter analysis of REDD1 gene. On the other hand, KLF15 is a recently discovered transcription factor that is involved in several metabolic processes in skeletal muscle; e.g., KLF15 transcriptionally upregulates the gene expression of branched-chain aminotransferase 2 (BCAT2), a mitochondrial enzyme catalyzing the first reaction in the catabolism of branched-chain amino acids (BCAA) to accelerate BCAA degradation and alanine production in skeletal muscle. Moreover, phenotypic analysis of cardiac-specific KLF15 knockout mice revealed marked left ventricular hypertrophy, indicating the negative regulatory role of KLF15 on muscle mass. We here demonstrated that KLF15 participates in muscle catabolism via the transcriptional regulation of atrogin-1 and MuRF1. Moreover, KLF15 affects mTOR through BCAA degradation and negatively modulates myofiber size. mTOR activation inhibits GR-mediated transcription by suppressing GR recruitment onto target genes, strongly suggesting a mutually exclusive crosstalk between mTOR and GR. Pharmacological activation of mTOR with BCAA attenuated GR-mediated gene expression, leading to the substantial restoration of muscle in glucocorticoid-treated rats. We, therefore, indicate the critical importance of the interaction of GR and mTOR in the regulation of metabolism-volume coupling in skeletal muscle. Given this, we have just started the clinical trial in IMSUT hospital to verify our scenario in glucocorticoid-treated patients.

IV. Cancer Stem Cells

Hiroto Yamazaki, Motohiko Naito, Toshihiro Okamoto and Chikao Morimoto

Characterization of cancer stem cell properties in CD24 and CD26-positive human malignant mesothelioma cells

Malignant mesothelioma (MM) is an asbestos-related malignancy characterized by rapid growth and poor prognosis. In our previous study, we have demonstrated that several cancer stem cell (CSC) markers correlated with CSC properties in MM cells. Among these markers, we focused on two markers, CD24, the common CSC marker, and CD26, the additional CSC marker. We conducted further analysis of CSC properties in CD24 and CD26-positve cells of the MM cell lines. We established RNAi-knockdown cells and found that these markers significantly correlated with chemoresistance, proliferation, and invasion potentials *in vitro.* Interestingly, Meso-1 cells express both CD24 and CD26, but each CSC property correlated with the expression of alternative marker. In addition, downstream signals of these markers were explored by microarray analysis and their expressions were correlated with several cancer-related genes. Furthermore, phosphorylation of ERK by EGF stimulation was significantly affected depending on the expression of CD26, but not CD24. These results suggest that CD24 and CD26 share and regulate CSC potentials of MM and could be promising targets for CSC-oriented therapy.

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Advanced Clinical Research Center

Division of Clinical Genome Research
臨床ゲノム腫瘍学分野

Professor	Yoichi Furukawa M.D., Ph.D.	教　授	医学博士	古　川　洋　一
Associate Professor	Tsuneo Ikenoue M.D., Ph.D.	准教授	医学博士	池　上　恒　雄
Assistant Professor	Kiyoshi Yamaguchi Ph.D.	助　教	薬学博士	山　口　貴世志

We have been working on the following four projects, 1) identification of novel therapeutic strategies of human cancer, 2) functional analysis of molecules associated with human cancer, 3) establishment and investigation of mouse models of human cancer, and 4) development of novel diagnostic strategies for hereditary tumors. These projects are aimed to develop strategies for better diagnosis, effective treatment, and prevention of human cancer.

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

Kiyoshi Yamaguchi, Rui Yamaguchi[1], Seiya Imoto[2], Satoru Miyano[1,2], Yusuke Nakamura[3], and Yoichi Furukawa: [1]Laboratory of Sequence Analysis, [2]Laboratory of DNA Information Analysis, [3]Laboratory of Molecular Medicine, Human Genome Center, IMSUT

In our earlier study aimed to clarify molecular mechanisms underlying colorectal cancer and to discover target molecules for therapy, we analyzed gene expression profiles of colorectal cancer using cDNA microarray. As a result, we found that MRG-binding protein gene (*MRGBP*) was up-regulated in the majority of colorectal tumors. Immunohistochemical staining of adenomas and carcinomas in the colon and rectum showed that high levels of MRGBP expression were observed more frequently in carcinomas (45%) than adenomas (5%), linking its role to malignant properties of colorectal tumors. Importantly, silencing MRGBP by siRNA inhibited cellular DNA synthesis, resulting in suppression of cell proliferation. These data suggest that MRGBP contributes to colorectal carcinogenesis through rendering advantages in proliferation to cancer cells.

We also focused on defective in sister chromatid cohesion 1 homolog (*DCC1*) gene that was overexpressed in the majority of colorectal tumors. Promoter analysis clarified that the overexpression of DCC1 was resulted in part from its promoter transactivation by E2F1. The elevated expression of DCC1 rendered advantages in cell proliferation, whereas DCC1 depletion reduced cell viability. In addition, colorectal cancer cells expressing exogenous DCC1 induced BCL2 expression and acquired resistance against apoptotic stimuli. These results may provide a novel explanation of drug resistance.

These findings may contribute to the better understanding of colorectal carcinogenesis, and open a new avenue to the development of novel therapeutic and/or diagnostic approaches to this type of tumor.

2. Functional analysis of SMYD3 in the development of zebrafish

Tomoaki Fujii, Kiyoshi Yamaguchi, Sumiko Watanabe[1], Yoichi Furukawa: [1]Division of Molecular Developmental Biology, IMSUT

In our earlier studies, we found that SMYD3 has methyltransferase activity to histone H3 lysine 4, and that its up-regulation is involved in the tumorigenesis of human colon, liver, and breast. To clarify the function of Smyd3 *in vivo*, we studied its expression pattern and its role in zebrafish. Recently we revealed that zebrafish embryos express two forms of *smyd3* from early developmental stages. To clarify the role(s) of Smyd3 in the development, we suppressed the translation of Smyd3 in the embryos using smyd3-specific morpholino oligonucleotides. Zebrafish embryos injected with the morpholinos, termed Smyd3 morphants, showed pericardial edema and curved trunk, suggesting that Smyd3 plays a crucial role in the development of heart and skeletal muscle. To elucidate the mechanism of cardiac and skeletal muscle defects, we further investigated the expression of cardiac and skeletal muscle genes. Although in situ hybridization demonstrated that expression of four cardiac transcription factors including *gata4, scl, nkx2.5*, and *hand2*, was almost normal, expression of cardiac chamber-specific markers including *cmlc2, amhc*, and *vmhc* was deregulated at late developmental stages. These results implied that Smyd3 does not play a role in the cardiac specification, but does in the morphogenesis of the heart. Regarding the trunk defect, expression of terminal differentiation markers of skeletal muscle including *mck, mylz2*, and *smyhc 1* was normal in the morphants. Although expression of two myogenic regulatory factors, *myod and myog*, was markedly decreased in the trunk of controls at late stages, their expression persisted in the morphants. These results suggested that Smyd3 is not involved in cell specification or differentiation, but is involved in maturation of differentiated myogenic cells.

3. Establishment and investigation of novel mouse models of gastrointestinal, pancreas, and liver cancer

Tsuneo Ikenoue, Yoshinari Asaoka[1], Hideaki Ijichi[1], and Yoichi Furukawa: [1]Department of Gastroenterology, Graduate School of Medicine, University of Tokyo

Genetically engineered mice are useful tools for studying human diseases, including cancer. In this project, we have already succeeded in the establishment of a mouse model of pancreatic ductal adenocarcinoma (PDAC) using pancreas-specific deletion of *TGFβ type II receptor* and *K-ras* activation. In addition, we recently succeeded in the development of a novel mouse model displaying aggressive intrahepatic cholangiocarcinoma (ICC). This model was generated by the liver-specific deletion of *Pten* and *K-ras* activation, because it has been reported that PI3K/AKT and RAS signaling pathways are frequently involved in human ICC. Now we are also developing other mouse models of PDAC and ICC. Intense investigation of these models should provide better understanding of PDAC and ICC and facilitate the development of new therapies to these tumors.

4. Genetic diagnosis using next generation sequencer

Masao Nagasaki[1], Satoru Miyano[2], Kiyoshi Yamaguchi, Tsuneo Ikenoue, and Yoichi Furukawa: [1]Functional Genomics, [2]Laboratory of DNA Information Analysis, Human Genome Center, IMSUT

We started a project, the determination of germ-line mutations in patients suspected for familial colorectal cancer using next generation sequencer. This project was approved by the institutional review board in 2011. One patient who was diagnosed as familial polyposis of the colon but was not identified any pathogenic mutations in *APC* by the conventional PCR-direct sequence method, was enrolled in this study. This project is aimed to detect mutations that are undetectable by Sanger sequence method, and to identify novel genetic alterations associated with familial tumor.

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Advanced Clinical Research Center

Division of Innovative Cancer Therapy
先端がん治療分野

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Associate Professor	Yasushi Ino, M.D., Ph.D.	准教授　医学博士	稲　生　　靖

The major research topic of our laboratory is to develop oncolytic virus therapy for various malignant tumors. Oncolytic viruses are designed so that they can infect, replicate selectively within, and destroy tumor cells. G47Δ, a recombinant, triple-mutated oncolytic herpes simplex virus type 1 (HSV-1), exhibits potent anti-tumor efficacy while maintaining safety. A G47Δ clinical trial at IMSUT Hospital for glioblastoma is underway.

Creation of novel recombinant oncolytic HSV-1

The use of genetically-engineered oncolytic viruses is a novel therapeutic strategy for cancer. Various kinds of virus have been studied worldwide as oncolytic viruses, but genetically engineered HSV-1 is particularly useful because of following favorable characteristics: (1) It shows little toxicity to normal tissues, and there exist theoretical backgrounds for tumor cell selectivity. (2) The viral genome is stable. (3) It can efficiently infect wide range of tumor types and exhibits a potent oncolytic activity. (4) Cell-to-cell spread is minimally affected by circulating antiviral antibodies. (5) Inflammatory reactions to the virus are generally mild and repeated administrations are possible. (6) There are antiviral drugs available to terminate viral replication when undesired events occur. (7) Antitumor immune responses are elicited in the course of oncolytic activities by the virus. (8) The large size of HSV-1 genome (～152kb) allows insertion of large or multiple foreign genes.

Conventional homologous recombination techniques had required time-consuming processes to create new recombinant oncolytic HSV-1. We have established an innovative recombinant HSV-1 construction system using bacterial artificial chromosome and two sets of recombinases (Cre/loxP and FLP/FRT). This system allows a rapid generation of multiple new recombinant HSV-1 with desired sequences inserted into a specific locus.

Application of oclolytic HSV-1 for malignant glioma is a major study interest in our laboratory. In addition, in vitro and in vivo tumor models of other cancers, including renal cancer, prostate cancer, bladder cancer and malignant mesothelioma, have also been used for testing efficacy and safety.

Studies using glioma-derived cancer stem cells

There exists a small population of tumor-initiating, stem-like cells within the tumor. Because cancer stem-like cells (CSC) are reported to be resistant to current therapies and responsible for recurrence, a novel approach that can eliminate CSCs is needed to cure the disease. We currently use glioma-derived CSCs to study new therapeutic approaches including oncolytic virus therapy using genetically engineered HSV-1. G47Δ has been shown to kill CSCs efficiently. In order to target CSCs, we have created novel

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Advanced Clinical Research Center,

Division of Advanced Medicine Promotion
先端医療開発推進分野

Professor Fumitaka Nagamura, M.D., D.M.Sc

教 授 医学博士 長 村 文 孝

Division of Advanced Medicine Promotion was established in December 2011. Our mission is to assist the development of translational researches. For this purpose, it is critical to discover the new "seeds" and to eradicate many blockades during development. In this sense, our role is the translation from the results of basic science at our Institute to the conduct of clinical trials at the Research Hospital. In Research Hospital, we work together with staffs of Department of Clinical Trial Safety Management. Concurrently, for the reduction of blockades during translational researches, we engage in research on Regulatory Science.

1. Studies on Blockades of Conducting Clinical Trials in Japan.

Fumitaka Nagamura

Dissolution of blockades of conduct clinical trials, especially for translational research, is the critical problem in Japan. The performance of conducting clinical trial, so to speak that of clinical development, is far inferior to that of basic science. There are many differences in the circumstance for conducting clinical trials between in Japan and in the U.S. In Japan, investigator-initiated Investigational New Drug Application (Ishi-Syudou Chiken) can be used as the basis for approval, however, Sponsor-Investigator (= Research, Investigator-initiated) Investigational New Drug Application in the U.S. cannot be used as that purpose. The aim of our research is to reveal the existence of blockages and to seek the solution, and apply that for the development of translational research.

2. Assistance of Clinical Trials/TRs at Research Hospital

Kazufumi Matsumoto, Kumiko Sumino, Noriko Fujiwara, Minako Kohno, Makiko Tajima, Fumitaka Nagamura

In Research Hospital, we work together with staffs of Department of Clinical Trial Safety Management. The assistance of Translational (Clinical) Research Coordinators is indispensable for the conduct of clinical trials, especially for TR. The activities of Coordinators are results of the collaboration between Division of Advanced Medicine Promotion and Department of Clinical Trial Safety Management.

3. The Development of the Scholastic Program for the Graduate Students of Nurses in the Area of Translational Research.

Kazufumi Matsumoto, Makiko Tajima, Kumiko Sumino, Noriko Fujiwara, Fumitaka Nagamura.

Translational Research (TR) is the early phase of clinical trials, which applied the developments of basic researches for patients with incurable and/or life-threatening diseases. High-educated nurses are indispensable for the conducts of TRs in terms of the protection of participants in TRs and the conducts of scientifically appropriate TRs. We developed the scho-

lastic program for the graduate students of nurses in the area of TR. We planed and implemented the two-weeks program to foster the expert research nurse aimed at the graduate students. It consists of the lectures on the feature points of TR (e.g. ethical considerations of TR, and the role of research nurse), role-plays of Institutional Review Board and obtaining Informed Consent, case conference, and the experience of the actual operations. We evaluated the reports and the questionnaires from the students to explore the degree of their understandings and satisfactions for this program. These reports and questionnaires were analyzed in accordance with the qualitative method. Six students participated in the program and we evaluated the reports and the questionnaires. Students could understand the role of research nurse and the necessary ability and organization to play this role appropriately. They were satisfied with the content and the quality of lectures and role-plays, however, the experiences of the actual operations did not meet their demands due to the less acquisition of the practical expertise. Generally, our program meets the demands of the students, however, the improvement of the content on the experience of the actual operations is the next issue.

4. Education and training for ethics board members: Is e-learning the solution?

Makiko Tajima, Fumitaka Nagamura.

The Japanese Ministry of Health, Labour and Welfare recently revised the Ethical Guidelines for Clinical Studies. The revised guidelines require education and training for members of a research ethics committee (REC). Some training programs on ethics are offered in an e-learning format for added convenience to learners. E-learning, also called online learning, web-based learning, or distance learning, has potential to be an effective training tool. We conducted a literature and internet search to assess the feasibility of e-learning in REC member training. E-learning is suitable for studying bioethical principles as well as laws and regulations relevant to clinical research. It is also useful to share criteria for protocol review and to update information on science and technology. E-learning is especially effective when the same training courses are offered repeatedly and when members are unable to assemble at the same location and same time due to geographical and temporal limitations. E-learning materials are shared among REC members, faculty, staff, and students, and may be open to the public. Disadvantages of e-learning include a high dropout rate due to lack of social interaction and the requirement for additional human and financial resources. To overcome these disadvantages, conventional methods such as lectures, workshops, and on-the-job training (OJT) should be incorporated into the training program. E-learning that includes the use of learning networks, such as online discussion forums, may provide results similar to those of workshops and OJT.

E-learning can be a valuable tool for REC member training. Combining different types of training media, or blended learning, is recommended to conduct effective training.

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