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 malignant disorders including leukemia, lymphoma and various cancers. For this purpose, we are making every effort to master the mechanisms of normal and neoplastic stem cells on the basis of molecular and cellular biology as well as medical informatics. We also try to develop novel therapies in the field of regenerative medicine using bone marrow-derived mesenchymal stromal 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) Study 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) Investigation of cancer stem cells and search for molecular targets for their elimination:
We are focusing on cancer, stem cells, and cancer stem cells. We aim to elucidate molecular mechanisms how growth factor signaling regulates tumorigenesis and maintenance of stem cells and cancer stem cells. Moreover, by taking not only molecular biology but also new bioinformatics approaches, we aim to identify novel cancer biomarkers and molecular targets for cancer therapy. Our ultimate goal is to translate them into clinic.

(4) Clinical study of clonal evolution of HTLV-1-infected T cells into leukemia:
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) basic and clinical studies on bone tissue engineerings.

1. Signals via homodimeric mutant of interleukin-7 receptor α chain (IL7Rα) highlight the significance of the IL7Rα/Janus kinase 1 pathway in T-cell acute lymphoblastic leukemia cells.

Yokoyama K, Kobayashi S, Izawa K, Kotani A, Harashima A, Tojo A

Interleukin-7 (IL7) is a requisite and potent growth and survival factor for early T-cell development. Signaling pathways that control normal T-cell development have been implicated in the leukemogenesis of T-cell acute lymphoblastic leukemia (T-ALL). By analogy to activating mutations in the Notch pathway, we hypothesized that T-ALL might result from mutations of interleukin-7 receptor chain (IL7R) gene. Direct sequencing of IL7R gene in a panel of 16 T-ALL cell lines identified two types of mutations in two different cell lines. One was an insertional mutation in the transmembrane region (INS), and the other was a cytoplasmic deletional loss-of-function mutation. We demonstrated that INS spontaneously formed a homodimer and activated IL-7R signaling pathway via Janus kinase (Jak)1, but not Jak3. These findings might expand the significance of the sequential Notch-IL7 pathway in the pathogenesis of T-ALL and highlight the dominant role of the IL7R/Jak1 axis in IL7 growth signaling.

2. Bcr-Abl impairs T cell development from murine 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 hematopoietic stem cells (HSCs). 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 co-culture 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. Anti-multiple myeloma therapy using miRNA-regulated vaccinia virus

Futami M, Nakamura T, Tojo A

Vaccinia virus is a member of poxvirus family, which was widely used for the small pox prevention until mid 1970s. Although its use 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 candidate for potential cancer therapy. Oncolytic effects are thought to be achieved by both the infection itself and the host's immune response to infected cells. Although active oncolytic potential is required for better outcome, infection and transmission of virus has to be inhibited in normal tissues for safety in clinical use. Micro RNAs (miRNAs) are 19-25 nucleotide small RNAs that negatively regulate gene expression. Interestingly, expression patterns of miRNAs are dif-

ferent from normal tissues in most cancer and leukemia cells. To improve the tumor specificity of the virus, we are trying to regulate viral transmission by utilizing endogenous micro RNA (miRNA) expression pattern. Vaccinia B5R gene is critical for viral transmission from infected cells. By inserting target sequence of an miRNA (which is expressed in normal cells but not in tumor cells) into the 3' untranslated region of B5R gene, we tried to regulate vaccinia virus to grow only in tumor cells. To determine which of the disease would be the optimal target for vaccinia therapy, first, I infected different cell lines derived from acute myelogenous leukemia, chronic myeloid leukemia, acute lymphoblastic leukemia, malignant lymphoma, adult T-cell leukemia/lymphoma, and multiple myeloma *in vitro*. Very interestingly, multiple myeloma cells showed exceptionally high susceptibility (10-100 times higher than others) to vaccinia virus, which led us to focus on multiple myeloma. Using quantitative RT-PCR of miRNAs, I found that miR-10a expression was inhibited in multiple myeloma cells compared to normal counterpart. Mouse tissue samples of the brain, heart, lung, liver, spleen, gut, kidney, ovary, and skin showed higher expression of miR-10a than bone marrow mononuclear cells of the mouse, suggesting that miR-10a would protect normal tissues from viral infection while the virus would infect and kill multiple myeloma cells. Using *in vitro* infection and *in vivo* mouse multiple myeloma transplant models, we are going to determine the efficacy and safety of miR-10a regulated vaccinia virus.

4. Imatinib mesylate directly impairs class switch recombination through down-regulation of AID.

Kawamata T, Toyoshima T, Yokoyama K, Oyaizu N, Ando K, Tojo A, Kotani A

Activation-induced cytidine deaminase (AID) is essential for class switch recombination and somatic hypermutation. 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 often develop hypogammaglobulinemia, the mechanism of which has not yet been clarified. Here, we provide evidence that class switch recombination on B-cell activation is apparently inhibited by IM through down-regulation 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. The CADM1 vs CD7 plot in multicolor flow cytometry reflects multistep oncogenesis in HTLV-1 infection.

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

CADM1 (cell adhesion molecule 1), initially identified as a tumor suppressor gene in lung cancer, was recently reported to express highly and ectopically in primary adult T-cell leukemia-lymphoma (ATL) cells. In this study, we show that CADM1 vs CD7 plot in $CD4^+$ cells in flow cytometry well reflects disease progression in human T-cell leukemia virus type I (HTLV-I) infection. In the plot, three subpopulations, namely P ($CADM1^{negative(neg)}$ $CD7^{positive(pos)}$), D ($CADM1^{pos}CD7^{dim}$), and N ($CADM1^{pos}$ $CD7^{neg}$), were consistently observed. HTLV-I infected cells were efficiently enriched in $CADM1^+$ subpopulations (D and N). Normal controls showed P-dominant pattern. As disease progresses from asymptomatic carriers (AC) with low proviral load (PVL) to ACs with high PVL and indolent ATL, the D and N subpopulations gradually increased. As disease further progresses to acute-type ATL, expansion of the N subpopulation was observed. Therefore proportion of the three subpopulations is considered to well reflect disease stage. Clonality analysis revealed that the putative high-risk ACs with increased D and N subpopulations had clonally expanded cells in these subpopulations. Intriguingly, gene expression microarray analysis of each subpopulation with fluorescence activated cell soring revealed that these ACs and indolent ATL cases showed similar gene expression pattern in subpopolations containing clonal cells. These findings suggest that the CADM1 vs CD7 plot is a powerful tool not only in clinical evaluation of disease status but also for elucidation of multistep ontogenesis in HTLV-I infection.

6. Analysis of the role of H3K36 demethylase Fbxl10 in normal hematopoiesis

Izawa K, Ozawa M, Yoshida N, Tojo A

Histone methylation influences mammalian development and cell differentiation via regulating transcription, chromatin structure and epigenetic inheritance. F-box and leucine-rich repeat protein 10 (Fbxl10) is a histone H3 lysine 36 (H3K36) demethylase and implicated in cell cycle regulation, cell death, senescence, and tumorigenesis. As for hematopoiesis, Fbxl10 is highly expressed in primitive hematopoietic cells and down-regulated during maturation, and forced overexpression of Fbxl10 in $CD34^-$KSL cells results in maintenance of self-renewing HSCs. It is also highly expressed in various types of leukemia cells. To gain insight into the

physiological role of Fbxl10 in hematopoiesis, we performed comparative analysis of peripheral blood (PB) cells and bone marrow progenitor (lineage$^-$/c-kit$^+$/Sca1$^+$: KSL) cells between Fbxl10$^{+/+}$ (WT), Fbxl10$^{+/-}$ (h-KO) and Fbxl10$^{-/-}$ (n-KO) mice. There was a considerable dispersion in the number of white blood cells and their differential counts from individual KO mice, but no difference in red blood cell counts. The number of KSL cell-derived colonies in the methylcellulose culture was slightly reduced in KO mice, compared with WT mice. FACS analysis showed that the KSL cell ratio per BM nucleated cells was lowest in n-KO mice. Furthermore, it was shown that the KSL cell ratio in BM from 23-weeks old mice was lower than that from 13-weeks old mice. These results suggests that Fbxl10 is dispensable for the hematopoietic development, but is significantly involved in the maintenance of HSCs.

7. Establishment of murine iPSC-derived hematopoietic progenitor cell lines which can yield mature blood cells.

Izawa K, Yamaguchi K, Furukawa Y, Yamamoto M, Tojo A

Hematopoietic stem/progenitor cells (HS/PCs) constitute a quite minor part of bone marrow (BM) nucleated cells and cannot be expanded *in vitro* with sustained hematopoietic ability for a long time. It is also difficult to efficiently direct pluripotent stem cells including ESCs and iPSCs toward the stage of HSCs. GATA2 is an essential transcription factor for hematopoietic development and is expressed in HS/PCs. We generated iPSCs (GGKI-iPSCs) from GFP+ BM cells from heterozygous GATA2-GFP knock-in mice in which GFP cDNA was inserted into exon 2 of the GATA2 gene. Colony forming assays confirmed that HPCs exist in a GFP+ fraction of BM cells in those mice, suggesting that HS/PCs derived from GGKI-iPSCs may be identified according to GFP expression. First, GGKI-iPSCs were induced toward hematopoietic differentiation over an OP9 cell monolayer with a cocktail of cytokines. Interestingly, after two months of culture, we found that GFP+ cells were continuously proliferating. They increased in number by 100-fold per week and were sustained for at least 3 months without loss of their properties. Next, GFP+ cells were FACS-sorted according to its expression level. In a methylcellulose assay, the number of colonies from GFP^{low} cells is 100 times higher than that from GFP^{high} cells. The microarray and RT-PCR analysis suggested that GFP^{low} fraction includes myeloid signatures, while GFP^{high} fraction has early erythroid signatures. Furthermore, transplantation experiments revealed that CD11b+ mature myeloid cells in PB could be detected in mice transplanted with GFP^{low}, but not GFP^{high}, cells. As a result, we succeeded in establishment of murine iPSC-derived hematopoietic progenitor cell lines which can yield mature blood cells.

8. Novel molecular mechanisms of acquired resistance to gefitinib in non-small lung cancer (NSCLC)

Nakata A, Yoshida R, Yamaguchi R, Tamada Y, Fujita A, Adi F Gazdar, Shimamura T, Imoto S, Higuchi T, Miyano S, Gotoh N.

Epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs), such as gefitinib or erlotinib, were remarkably effective in some patients with lung cancer harboring EGFR kinase domain mutations. Although these patients were initially sensitive to EGFR-TKIs, they eventually have acquired resistance to EGFR-TKIs within several years. The several molecular mechanisms of acquired resistance to EGFR-TKI have already been reported. It has revealed that 50% of patients who have acquired resistance to EGFR-TKIs have secondary mutation in EGFR (T790M) and 20% of them have the amplification of the MET oncogene. However, some NSCLC patients still suffer from gefitinib-resistance caused by unknown mechanisms. To explore novel molecular mechanisms for gefitinib-resistance, we established the gefitinib-resistant PC9 M2 cells that were spontaneously derived from gefitinib-sensitive PC9 cells cultured in the presence of a low amount of gefitinib for a long time. In PC9 M2 cells, we found neither the mutation of EGFR T 790M nor amplification of MET oncogene and thus the novel mechanisms may underlie the acquired resistance to gefitinib in these cells. We analyzed gene expression profiles of PC9 and PC9M2 cells with or without stimulation with EGF in the presence or absence of gefitinib in a detailed time course by using DNA microarray. We found that Wnt signaling-related genes were upregulated in PC9M2 cells compared with PC9 cells. Furthermore, phosphorylation of GSK3α and the accumulation of β-catenin were increased in PC9M2 cells. We next investigated the effect of β-catenin knockdown in PC9M2 cells by using siRNA for β-*catenin*. We found suppression of β-catenin expression partially restored the sensitivity to EGFR-TKIs in PC9M2 cells. Thus our findings suggest that enhanced Wnt/β-catenin signaling is associated with acquired resistance to EGFR-TKIs. Targeting Wnt/β-catenin pathway may be useful for overcoming the acquired resistance to EGFR-TKIs.

9. Development of a novel diagnostic method based on the gene signature to predict high-risk patients of early stage lung cancer

Nakata A, Miyano S, Tojo A, Gotoh N.

Non-small cell lung cancer (NSCLC) is the commonest and the most fatal histological subtype of lung cancer. About 10-30% of stage IA patients die due to recurrence after surgery of curative intent. Therefore, the identification of prognostic biomarkers for stage IA lung adenocarcinoma with poor prognosis is of great importance to select patients who will be benefited by adjuvant therapy. We indentified 139 genes as the EGFR tyrosine kinase-influenced key genes. We used them as expression signatures to train a risk scoring model that classifies patients in high- or low-risk (the risk of death or recurrence in 5 years). This model was trained by using a data set composed of 253 North American patients with lung adenocarcinomas. Then, the predictive ability of the risk scoring model was examined in two independent cohorts composed of North American and Japanese patients. The model enabled the statistically significant identification of high-risk stage IA lung adenocarcinoma in both cohorts, with hazard ratios (HRs) for death of 7.16 ($P = 0.029$) for North American and of 10.98 ($P = 0.008$) for Japanese. The set of 139 genes includes many ones that have already been reported in the literature to be involved in biological aspects of cancer phenotypes such as ADAM family genes (ADAM10, ADAM19), matrix metalloprotease (MMP) family genes, NMU and Ube2c, but are yet unknown to be involved in EGF signaling. These results strongly re-emphasizes that EGF signaling status underlies aggressive phenotype of cancer cells, and also suggests the first set of genes that are useful for the identification of high-risk stage IA lung adenocarcinoma patients. We are now examining prognostic ability of each of these genes by using many frozen lung adenocarcinoma tissues by qRT-PCR. We aim at developing a novel diagnostic method based on qRT-PCR measurement to predict high-risk patients of early stage lung adenocarcinoma after surgery. These predicted high-risk patients should undergo adjuvant chemotherapy or radiotherapy that may improve prognosis.

10. A PI3K-dependent gene expression program that regulates mammosphere formation of breast cancer cells.

Hinohara K, Shimamura T, Fukuda H, Tominaga K, Nida A, Yamaguchi R, Imoto S, Tsuji E, Tada K, Kanauchi H, Ogawa T, Miyano S, Tojo A, Gotoh N.

Breast cancer stem cells (BCSCs) are responsible for mammary tumorigenesis. To develop more effective cancer therapies, it would be reasonable to target molecules that have a critical role in the maintenance of BCSCs. However, our understanding of the cellular and molecular mechanisms underlying BCSC properties is limited. We found that heregulin (HRG), a ligand for ErbB3, induced mammosphere formation of BCSCs through a phosphatidyl inositol 3-kinase (PI3K) pathway. To investigate transcriptome dynamics during ErbB/PI3K activation, we performed time series microarray analyses under condition of HRG treatment and/or PI3K inhibition, and obtained an ErbB/PI3K gene signature that includes 635 genes. To examine whether the ErbB/PI3K gene signature is associated with BCSC-related traits, we analyzed the association of the signature with histological grade of breast cancer, because BCSCs are enriched in high-grade breast tumors when compared to low-grade breast tumors. Of note, the ErbB/PI3K gene signature was highly enriched in high-grade breast tumors, raising the possibility that the ErbB/PI3K activity correlates with stemness potential. We found that insulin-like growth factor 2 (IGF2), included in the ErbB/PI3K gene signature, increased mammosphere formation of breast cancer cells. These findings suggest that ErbB/PI3K signaling maintains BCSCs through production of growth factors such as IGF2 by autocrine/paracrine mechanisms.

11. Amphiregulin/EGFR pathway contributes to mammoshpere formation in human breast cancer

Fukuda H, Hinohara K, Shimamura T, Watanabe T, Miyano S, Tojo A, Gotoh N.

We found the expression of Amphiregulin (AR), a ligand for EGFR, was induced via HRG/PI3K/NF-κB pathway. It was previously reported that AR is expressed in breast cancer tissues at increased levels than in normal tissues and that AR is involved in resistance to chemo- and hormone therapeutic agents. However, the role of AR/EGFR pathway in regulating properties of BCSCs remains largely unknown. To investigate the effect of AR/EGFR pathway, we examined mammosphere formation in four breast cancer cell lines treated with AR or EGF. AR induced mammosphere formation in all four cell lines more frequently than those of EGF. AR induced sustained activation of Akt or Erk, whereas EGF induced transient activation of Akt or Erk in breast cancer cells. We found EGF but not AR stimulation led to rapid downregulation of EGFR. These results suggest that the increase levels of mammosphere formation by AR is due to slower kinetics of EGFR downregulation by AR stimulation than by EGF stimulation. In addition, AR-induced mammosphere formation was reduced by treatment with inhibitors for PI3K or MEK. Thus AR/EGFR signaling appears to maintain mammosphere formation through strong activation of PI 3K or MEK/Erk pathway.

12. A Potential Role of FRS2β, a Feedback Inhibitor for ErbB, during Mammary Tumorigenesis

Machida Y, Iejima D, Mizutani A, Sakamoto R, Yoshida N, Gotoh N.

FRS2β has phosphotyrosine binding domein and binds to ErbB2, a member of tyrosine kinase family. We have previously shown that FRS2β binds to activated ERK and inhibits ErbB2-induced cell growth by the activation of ErbB2. FRS2β acts as a feedback inhibitor for ErbB2 signal. FRS2β is expressed in only restricted areas, including small number of cells in mammary epithelial cells. We generated mutant FRS2β mice by gene targeting, and crossed them with MMTV-ErbB2 mice in which overexpression of ErbB2 induces breast cancer. Although cells expressing FRS2β in breast tissues showed reduced proliferation and disruption of FRS2β should remove the feedback inhibition, tumor growth in FRS 2β(−/−) mice was greatly reduced and eventually, all FRS2β(+/+) mice died faster than FRS2β(−/−) mice. Immunohistocheminal analysis revealed that α-smooth muscle actin positive cancer associated fibroblast (CAF) were abundant in mammary tumor of FRS2β(+/+) mice, compared with FRS2β(−/−) mice. Thus presence of CAF might affect tumor growth. Recently, accumulating evidence suggest that cancer tissues are derived from cancer stem cells and that they are transformed normal stem/progenitors. The EGF-induced mammosphere forming ability of lactational breast cells were decreased in FRS2β(−/−) mice compared with those of FRS2 β(+/+) mice, suggesting that stem/progenitor cells in breast tissues are decreased in FRS2β(−/−) mice. From these results, FRS2β plays an inhibitory role in the onset of mammary tumorigenesis but a stimulatory role in tumor growth. The complex role of FRS2β in mammary tumorigenesis suggests its important roles for cancer stem cells.

13. 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 and currently in progress.

Publications

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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, Kohtaro Nishimura, Toshihiko Oki, Yukinori Minoshima, Ying Chun Bao, Tomonori Hatori, Yasushi Nomura, Noriko Takahashi, Takaya Satoh[1], and Toshio Kitamura: [1]Osaka City University.

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 anticancer drugs, and have developed JP1156 which kill the tumor cells with much lower IC50. Now we have demonstrated JP1156 is effective in inhibiting growth of various tumor cell lines in mouse tumor-burden models.

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[2], Jiro Kitaura, and Toshio Kitamura: [2]Keio University School of Medicine

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 LMIR4-8 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 TIM1 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 TIM1 is an endogenous ligand for LMIR5 and that the TIM1-LMIR5 interaction plays a physiological role in immune regulation by myeloid cells.

We have recently identified ceramides as ligands for LMIR3, and demonstrated that LMIR3 plays critical roles in inhibiting allergic response caused by mast cells using LMIR3 knockout mice. Our results suggest that ceramides present in the skin attenuate the activation of mast cells when they are activated by IgE and antigens.

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, Koichiro Yuji, Hideaki Nakajima[2], Yuka Harada[3], Hironori Harada[3], Toshiya Inaba[4], Tetsuya Nosaka[5], Jiro Kitaura, and Toshio Kitamura: [3]Department of Hematology/Oncology and [4]Department of Molecular Oncology, Research Institute for Radiation Biology and Medicine, Hiroshima University, and [5]Mie University School of Medicine.

To elucidate the molecular mechanisms of leukemia, MDS, and MPD, we established mouse model using bone marrow transplantation (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 hemopoietic malignancies using mouse BMT models and transgenic mice.

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

Masaki Nakakmura, Jiro Kitaura, Yang Lu, Yutaka Enomoto, Toshihiko Oki, Yukiko Komeno, Katsutoshi Ozaki, Mari Kiyono, Hidetoshi Kumagai, [2]Hideaki Nakajima, Tetsuya Nosaka, Hiroyuki Aburatani[6], and Toshio Kitamura: [6]Research Center for Advanced Science and Technology, The University of Tokyo

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 32Dcl.3 cells. Collectively, these results suggest that TSC-22 is a possible tumor suppressor.

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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Our overall goal 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 to HIV-1 and how immune system is disrupted and develops AIDS. We are also working on viral pathogenesis in HIV-infected patients. 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. Structural insights into major/early and minor/late escape mutations at a CTL epitope in HIV-1 infection

Akihisa Shimizu, Ai Kawana-Tachikawa, Atsushi Yamagata[1,2], Chung Yong Han, Dayong Zhu, Hitomi Nakamura[3], Michiko Koga, Tomohiko Koibuchi[4], Jonathan Carlson[5], Eric Martin[6], Chanson J. Brumme[7], Shi Yi[8], George F. Gao[8], Zabrina L. Brumme[6,7], Shuya Fukai[1,2], Aikichi Iwamoto: [1]Structural Biology Laboratory, Life Science Division, Synchrotron Radiation Research Organization and Institute of Molecular and Cellular Biosciences, The University of Tokyo, [2]Department of Medical Genome Sciences, Graduate School of Frontier Sciences, The University of Tokyo, [3]Department of Infectious Diseases Control, International Research Center for Infectious Diseases, The University of Tokyo, [4]Department of Infectious Diseases and Applied Immunology, Hospital, The Institute of Medical Science, The University of Tokyo, [5]Microsoft Research, Los Angeles, CA, USA [6]Faculty of Health Sciences, Simon Fraser University, Burnaby, BC, Canada. [7]British Columbia Centre for Excellence in HIV/AIDS, Vancouver, BC, Canada. [8]CAS Key Laboratory for Pathogenic Microbiology and Immunology, Institute of Microbiology, Chinese Academy of Science, Beijing, China.

Viral proteins are processed to peptides (p), bound to the polymorphic major histocompatibility complex (HLA in human) class I molecules and expressed on the cell surface as pHLA complexes. Cytotoxic T lymphocytes (CTL) recognize pHLA via T cell receptors (TCR) and exert strong immune pressure. Single amino acid mutants of HIV-1 which circumvent processing, binding or CTL recognition may appear, selected in vivo and deteriorate the immune system. Here we show the crystal structures of the three types of the TCR interactions with the HIV-1 peptide (Nef134-10) from the wild type or a major mutant in complex with HLA-A* 2402. We also show the crystal structure of TCR interactions with the peptide from the wild type and a minor mutant. Our results demonstrate that CTL with functional TCR which contribute to eliminate the wild type are coexisting with a processing mutant. Our results may indicate that drug development targeting a modification in virus-specific protein processing may be a new armament for improving the cellular immune responses against

HIV-1.

2. Impact of an Amino Acid Change within Overlapping CTL Epitopes in HIV-1 Infection

Chungyong Han, Akihisa Shimizu, Atsushi Yamagata, Shuya Fukai, Zabrina L. Brumme, Shi Yi, George F. Gao, Dayong Zhu, Noriaki Hosoya[1], Hitomi Nakamura, Michiko Koga, Tomohiko Koibuchi, Ai Kawana-Tachikawa, and Aikichi Iwamoto: [1]Department of Infectious Diseases Control, International Research Center for Infectious Diseases, The University of Tokyo

In HIV-1 infection, amino acid substitutions around CTL epitopes allow the virus to evade the CTL responses. As numerous CTL epitopes have been reported across the HIV-1 proteome and some of which overlap with other epitopes, an amino acid substitution in an epitope by CTL selection pressure may affect the CTL recognition of another epitope. Nef126-10 (NYTPGPGIRY) and Nef134-10 (RYPLTFGWCF), overlapping by 2 amino acids, are restricted by HLA-A*2402, and the substitution of F for Y at Nef135 frequently occurs in HLA-A*2402-positive individuals. Although both of the epitopes were frequently recognized (50.0% and 80.4%, respectively, in 46 individuals), the Nef126-10-specific response was observed only in the individuals with Nef135F substitution. To clarify the mechanism, we examined the epitope-specific recognition by CTL clones to these epitopes, either the wild types (Nef 126-10(wt) and Nef134-10(wt)) or Nef135F mutants (Nef126-10(F) and Nef134-10(F)). The endogenously derived Nef126-10(F) and Nef134-10(wt) were well recognized by the epitope-specific CTLs and, on the contrary, the recognition of Nef126-10(wt) and Nef 134-10(F) was considerably diminished. Additionally, in the longitudinal analysis of the Nef126-10-specific response and the appearance of Nef135F mutant in one patient, the substitution was followed by the emergence of the Nef126-10-specific CTL response. These data suggest the Nef135 substitution alters the CTL recognition of Nef126-10 and Nef134-10 epitopes. In conclusion, this study demonstrates a mechanism, which generates *de novo* CTL epitope after one amino acid change that might result from the CTL pressure to the overlapping epitope. This novel "epitope switching" mechanism that occurs in host immunity might help the understanding of cellular immunity and CTL vaccine development.

3. Anti-APOBEC3G activity of HIV-1 Vif protein from elite controllers is attenuated compared to those from untreated chronic progressors or those from individuals with acute infection

Tadashi Kikuchi, Yukie Iwabu[1], Ai Kawana-Tachikawa, Michiko Koga, Noriaki Hosoya, Shigeru Nomura, Zabrina L. Brumme, Heiko Jessen[2], Florencia Pereyra[3], Alicja Trocha[3], Bruce D. Walker[3], Aikichi Iwamoto[1], Kenzo Tokunaga[1], Toshiyuki Miura[4]: [1]National Institute of Infectious Diseases, Tokyo, Japan [2]Jessen Praxis, Berilin, Germany, [3]MIT and Harvard, Ragon Institute of MGH, Charlestown, Boston, USA, [4]ViiV Healthcare

HIV-1-infected individuals who control viremia to below the limit of detection without antiviral therapy have been termed elite controllers (EC). Functional attenuation of some HIV-1 proteins has been reported in EC . However, little is known about role of the HIV-1 accessory protein Vif function in EC, which enhances HIV-1 infectivity through APOBEC3G degradation. In this study, the anti-APOBEC3G function of Vif was compared between EC, chronic progressors (CP) and individuals with acute infection (AI).

Forty-nine EC, 49 CP and 44 AI were studied. vif genes were amplified by nested RT-PCR using concentrated plasma. To compare anti-APOBEC3G activity of Vif proteins among those groups, VSV-G-pseudotyped viruses were generated by co-transfecting 293T cells with expression plasmids encoding patient-derived Vif, APOBEC3G, VSV-G, together with a vif/env-deficient HIV-1 proviral DNA clone carrying a luciferase reporter gene. VSV-G-pseudotyped viruses were normalized for p24 antigen and used to infect 293T cells and luciferase activity was measured at 48 h postinfection.

Anti-APOBEC3G activity of Vif from EC was significantly reduced compared to those from CP or AI. These results remained significant after excluding individuals expressing protective HLA alleles B*27 and/or B*57. No significant difference was observed between CP and AI. There were no common polymorphisms (away from consensus B) that could explain reduced anti-APOBEC3G activity of Vif derived from EC.

In conclusion, Anti-APOBEC3G activity of Vif proteins derived from EC was reduced. This reduced activity was independent of presence or absence of known protective HLA alleles. Common Vif mutations in EC unlikely explain the observed reduction; rather it might be attributable to unique mutations to each EC Vif protein.

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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 involved in related clinical trials 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. The practical application for cancer immunotherapy through the investigation chronic inflammation in IL-23/Th17 axis

Marimo Sato-Matsushita and Hideaki Tahara

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, the mice were injected intratumorally 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 T-regs 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. The evaluation of chronic inflammation in Ad-IL-23-DCs treatment using immunological analyses and immunohistochemical methods is currently on going.

II. Analysis of immunotherapy markers in oncology

Marimo Sato - Matsushita, Hideaki Tahara, Francesco M Marincola[#] ([#]Disease and Immunogenetics Section, Department of Transfusion Medicine, Clinical Center, Associate Director National Institute of Health)

We focused on novel cutting-edge strategies suitable for high-throughput screening of clinical samples for the identification. Such biomarkers will be more likely identified by paired comparison of pre- and post-treatment samples, and selection and validation of biomarkers relevant to disease outcome and/or serve as surrogate equivalents to clinical outcome. The critical factor in identification of predictive markers for treatment is the availability of samples from patients homogenously treated within the treatment arm from clinical trials. Thus, collection of tissue samples needs to be mandated in

each clinical trial. Paired pre-treatment and post-treatment samples collected at various time points need to be considered for these studies to identify optimal collection/measurement time points. Currently available high-throughput genomic/epigenetic/proteomic approaches for profiling of small amount of tissues should facilitate progress in this area. In the current study, we evaluated molecular profile of peripheral cells from healthy donors, chronically viraemic HCV-treatment-naïve patients and patients who spontaneously achieved virus eradication by whole genome gene expression analysis (GeneChip® Human Gene 1.0 ST Array - Affymetrix).

III. Development of cancer immunotherapy using the blockade of MFG-E8

Marimo Sato-Matsushita, Hideaki Tahara

The secreted protein, milk fat globule epidermal growth factor-8 (MFG-E8), stimulates disease progression through coordinated vβ3 integrin signaling in tumor and host cells. MFG-E8 enhances tumor cell survival, invasion, and angiogenesis, and contributes to local immune suppression. We have shown that systemic MFG-E8 blockade cooperates with cytotoxic chemotherapy, molecularly targeted therapy, and radiation therapy to induce destruction of various types of established mouse tumors. The combination treatments evoke extensive tumor cell apoptosis that is coupled to efficient dendritic cell cross-presentation of dying tumor cells. Our previous findings suggest that systemic MFG-E8 blockade might intensify the antitumor activities of existing therapeutic regimens through coordinated cell-autonomous and immune-mediated mechanisms also in human. In order to apply these finding to treat cancer patients, we are currently developing the antibodies specific to the human MFG-E8 and extend our study on human samples.

IV. IL-17-producing NK1.1$^-$ CD27$^-$ γδT cells promote tumor malignant progression by inducing inflammatory microenvironment.

Yoshitaka Kimura[#], Marimo Sato-Matsushita, Hideaki Tahara and Yoshihiro Hayakawa[##] ([#]The University of Tokyo, [##]Institute natural Medicine, University of Toyama)

Inflammatory microenvironment is an essential component of tumors and important for carcinogenesis and metastasis of tumor cells, however, the precise details of inflammatory immune responses to promote tumor malignant progression are still unclear. To characterize such tumor-promoting inflammatory immune responses, we employ a unique in vivo model in which low tumorigenic cell line QR-32 acquires high malignant phenotype after exposure to host inflammatory responses induced by an inflammation initiator. By using this model, we investigated the role of inflammatory cytokines IL-17 and IFNγ in tumor malignant progression process. We demonstrated that IL-17 and IFNγplayed positive and negative roles, respectively, in the malignant progression of tumor cells and IL-17 played a predominant role in this process. Adoptive transfer of inflammatory cells from wild-type mice into IL-17-deficient mice recovered in vivo progression of QR-32 cells and the exact source of IL-17 within such inflammatory cells was determined as NK1.1$^-$CD27$^-$γδT cells. Furthermore, CD11b$^+$ Ly-6G$^+$ neutrophils infiltrated into the inflammatory site primed by IL-17-producing NK1.1$^-$ CD27$^-$γδT cells in the presence of QR-32 and IL-17 played an important role for maintaining such tumor-associated inflammatory microenvironment. Collectively, our data clearly implicate that the inflammatory tumor microenvironment triggered by IL-17-producing NK1.1$^-$CD27$^-$γδT cells is important for tumor malignant progression. We are now further characterizing γδT cells in the inflammatory microenvironment promoting tumor malignant progression and exploring the components for downstream inflammatory immune responses triggered by IL-17.

V. Toll-like receptor 3 signaling converts tumor-supporting myeloid cells to tumoricidal effectors

Hiroaki Shime, Hideaki Tahara, Tsukasa Seya

Smoldering inflammation often increases the risk of progression for malignant tumors and simultaneously matures myeloid dendritic cells (mDCs) for cell-mediated immunity. PolyI:C, a dsRNA analog, is reported to induce inflammation and potent antitumor immune responses via the Toll-like receptor 3/Toll-IL-1 receptor domain- containing adaptor molecule 1 (TICAM-1) and melanoma differentiation-associated protein 5/IFN-β promoter stimulator 1 (IPS-1) pathways in mDCs to drive activation of natural killer cells and cytotoxic T lymphocytes. Here, we found that i.p. or s.c. injection of polyI:C to Lewis lung carcinoma tumor-implant mice resulted in tumor regression by converting tumor-supporting macrophages (Mfs) to tumor suppressors. F4/80+/Gr1- macrophages (Mfs) infiltrating the tumor respond to polyI:C to rapidly produce inflammatory cytokines and thereafter accelerate M1 polarization. TNF-α was increased within 1 h in both tumor and serum upon polyI:C injection into tumorbearing mice, followed by tumor hemorrhagic necrosis and growth suppression. These tumor responses were abolished in TNF-α−/− mice. Furthermore, F4/80+ Mfs in tumors extracted from polyI:

Cinjected mice sustained Lewis lung carcinoma cytotoxic activity, and this activity was partly abrogated by anti-TNF-α Ab. Genes for supporting M1 polarization were subsequently up-regulated in the tumor-infiltrating Mfs. These responses were completely abrogated in TICAM-1−/− mice, and unaffected in myeloid differentiation factor 88−/− and IPS-1−/− mice. Thus, the TICAM-1 pathway is not only important to mature mDCs for cross-priming and natural killer cell activation in the induction of tumor immunity, but also critically engaged in tumor suppression by converting tumor-supporting Mfs to those with tumoricidal properties.

VI. Development of robotized cell culture system

Shigeyuki Wakitani[#], Marimo Sato-Matsushita, Hideaki Tahara ([#]Department of Health and Sports,Mukogawa Women's University)

In collaboration with Kawasaki Heavy Industries, Inc., we are developing robotized cell culture system which could be applied to a variety of procedures including virus production as a funded project by NEDO.

VII. Treatment of malignan pleural mesothelioma using replication-defective recombinant adenoviral vector expressing the suppressor of cytokine signaling 3 (SOCS3). (Manufacture of the viral vector for preclinical studies in non-human primates)

Tetsuji Naka[#], Hiroyuki Mizuguchi[##], Takafumi Nakamura[###], Hisako Katano[####], Hideaki Tahara ([#]Laboratory for Immune Signal, National Institute of Biomedical Innovation, Osaka, Japan [##]Laboratory of Biochemistry and Molecular Biology, Graduate School of Pharmaceutical Sciences, Osaka University, [###]Tottori University, [####]University of Tokyo)

In collaboration with the research team, we have prepared the replication-defective recombinant adenoviral vector expressing the suppressor of cytokine signaling 3 (SOCS3) for treatment of malignant pleural mesothelioma. We have supported the vector production using Vector Facility in IMSUT utilizing the master and working cell banks of 293 cells, which we established previously. The purified final products are to be used for preclinical study in monkey.

Publications

1. Nakamura Y, He X, Kato H, Wakitani S, Kobayashi T, Watanabe S, Iida A, Tahara H, Warman ML, Watanapokasin R, Postlethwait JH. SOX9 Is Upstream of MicroRNA-140 in Cartilage. Appl Biochem Biotechnol 166(1): 64-71. 2012 (Epub 2011 Nov 4.)
2. Shime H, Matsumoto M, Oshiumi H, Tanaka S, Nakane A, Iwakura Y, Tahara H, Inoue N, Seya T. TLR3/TICAM-1 signaling converts tumor-supporting myeloid cells to tumoricidal Effectors. Proc Natl Acad Sci U S A. 109(6): 2066-71. 2012.

Advanced Clinical Research Center

Division of Clinical Immunology
免疫病態分野

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Our long term goal is to define the molecular and structural basis for the mechanisms of the immune abnormalities underlying various immune-mediated disorders as well as to cure patients with these 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. (With the fulfillment of Professor Morimoto's tenure at University of Tokyo, the division was closed in March 31, 2012.)

I. β1 integrins and Cas-L/NEDD9

Satoshi Iwata, Tomonori Katayose, Yoshiko Kichikawa, Shunsuke Kondo, Hiromi Ichikawa, Akiko Souta-Kuribara, Osamu Hosono, Hiroshi Kawasaki, Hirotoshi Tanaka, and Chikao Morimoto.

The 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. The pp105 was first described in our laboratory as a protein predominantly tyrosine phosphorylated by the ligation of β1 integrins in H 9 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. Impact of the integrin signaling adaptor protein NEDD9 on prognosis and metastatic behavior of human lung cancer.

In a substantial population of non-small cell lung cancer (NSCLC), expression and activation of EGF receptor (EGFR) have 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 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 NEDD9 in the signaling crosstalk between EGFR and integrins. Using NSCLC cell lines, we conducted 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 patients with NSCLCs. We showed that tyrosine phosphorylation of NEDD9 was reduced by the inhibition of EGFR in NSCLC cell lines. Overexpression of constitutively active EGFR caused tyrosine phosphorylation of NEDD9 in the absence of integrin stimulation. By gene transfer and gene knockdown, we showed that NEDD9 plays a pivotal role in cell migration and invasion of those cells in vitro. Furthermore, overexpression of NEDD9 promoted lung metastasis of an NSCLC cell line in NOD/Shi-scid, IL-2Rγ (null) mice (NOG) mice. Finally, univariate and multivariate Cox model analysis of NSCLC clinical specimens revealed a strong correlation between NEDD9 expression and recurrence-free survival as well as overall survival. Our data thus suggest that NEDD9 is a promising biomarker for the prognosis of NSCLCs and its expression can promote NSCLC metastasis.

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

Kei Ohnuma, Takaaki Ooki, Wakae Fujimaki, Ryo Hatano, Junpei Yamamoto, Kaoru Komoriya, Satoshi Iwata, Osamu Hosono, Hiroshi Kawasaki, Hirotoshi Tanaka and Chikao Morimoto (in collaboration with Nam H Dang, University of Florida, USA, Taketo Yamada, Keio University, Takumi Kishimoto, Okayama Rousai Hospital, Kouki Inai, Hiroshima University).

CD26 is a 110-kDa cell surface glycoprotein that possesses 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 acids 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 successfully isolated another CD26-binding protein, the mannose-6-phosphate/insulin-like growth factor II receptor (M6P/IGFIIR) as being critical for this interaction for CD 26 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 CD26 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. In pursuing surrogate prognostic markers in patients with malignant mesothelioma along with CD26, we also elucidated that CD9 is an independent favorable prognostic marker.

A. CD26-mediated co-stimulation in human CD8 (+) T cells provokes effector function via pro-inflammatory cytokine production.

CD26 is an activation marker of human CD4(+) T cells, and is associated with T-cell signal transduction processes as a co-stimulatory molecule. We have previously demonstrated that high CD26 cell surface expression on CD4(+) T cells is correlated with the production of T helper type 1 cytokines,

whereas CD26(+) T helper cells stimulate antibody synthesis in B cells. Although the cellular and molecular mechanisms involved in CD26-mediated CD 4(+) T-cell activation have been extensively evaluated by our group and others, the role of CD26 in CD8(+) T cells has not been clearly elucidated. In the present study, we examine the effector function of CD8(+) T cells via CD26-mediated co-stimulation in comparison with CD28-mediated co-stimulation. We found that CD26(high) CD8(+) T cells belong to the early effector memory T-cell subset, and that CD26-mediated co-stimulation of CD8(+) T cells exerts a cytotoxic effect preferentially via granzyme B, tumor necrosis factor-α, interferon-γ and Fas ligand. The effector function associated with CD26-mediated co-stimulation is enhanced compared with that obtained through CD28-mediated co-stimulation, suggesting that the CD26 co-stimulation pathway in CD8(+) T cells is distinct from the CD28 co-stimulation pathway. Targeting CD26 in CD8(+) T cells therefore has the potential to be useful in studies of immune responses to new vaccine candidates as well as innovative therapy for immune-mediated diseases.

B. CD26 overexpression is associated with prolonged survival and enhanced chemosensitivity in malignant pleural mesothelioma.

Malignant pleural mesothelioma (MPM) is an aggressive and therapy-resistant neoplasm arising from the pleural mesothelial cells, without established indicators to predict responsiveness to chemotherapy.

Our study involving 79 MPM patients showed that 73.4% of MPM expressed CD26 on cell membrane. The majority of epithelioid and biphasic types of MPM expressed CD26 on the cell membrane, whereas the sarcomatoid type showed a lack of CD26 surface expression. Although the sarcomatoid type was associated with poor prognosis ($P< 0.0001$), no significant relationship between CD26 expression and survival was observed. On the contrary, there was a trend for an association between response rate to chemotherapy and CD26 expression ($P=0.053$), with a higher level of CD26 expression more likely to be linked to better response to chemotherapy. Moreover, CD26 expression was a significant factor associated with improved survival in patients who received chemotherapy [median survival time (MST), 18.6 vs. 10.7 months, $P= 0.0083$]. Furthermore, CD26 expression was significantly associated with better prognosis in patients receiving non-pemetrexed-containing regimens (MST, 14.2 vs. 7.4 months, $P=0.0042$), whereas there was no significant association between CD26 expression and survival time for patients receiving pemetrexed-containing regimens. Our in vitro and microarray studies showed that mesothelioma cells expressing high CD26 displayed high proliferative activity, and CD26 expression was closely linked to cell-cycle regulation, apoptosis, and chemotherapy resistance. Our results strongly suggest that CD26 is a clinically significant biomarker for predicting response to chemotherapy for MPM.

C. CD9 expression as a favorable prognostic marker for patients with malignant mesothelioma.

CD9 is involved in cell growth, adhesion and motility and its expression is reported to be of prognostic significance in various types of human malignancies. We found increased cell migration in the mesothelioma cell lines MSTO-211H and TUM1 following in vitro shRNA-mediated knockdown of CD9 expression. We investigated CD9 expression in 112 malignant pleural mesotheliomas. CD9 expression was observed in 62 of 71 epithelioid, 13 of 20 biphasic and only 1 of 21 sarcomatoid mesotheliomas. Among the epithelioid mesotheliomas (EMs), CD9 expression was observed in all of the 33 cases with a differentiated type (EM-D) and in 29 of the 38 cases with a less-differentiated type (EM-LD). Patients with CD9 expression showed higher 1- and 2-year survival rates (63 and 25%) compared to the patients without CD9 expression (39 and 11%). Univariate analysis revealed that patients with CD9 expression demonstrated a more favorable survival ($P=0.0025$) along with other clinicopathological factors, including age younger than 60 years, IMIG stage I-II, epithelioid histology, EM-D and patients who underwent extrapleural pneumonectomy or received chemotherapy. Multivariate analysis identified CD9 expression as an independent prognostic factor with a hazard ratio (HR) of 1.99 in the analysis of all mesotheliomas ($P=0.0261$) and an HR of 2.60 in the analysis of EMs ($P= 0.0376$). CD9 expression is an independent favorable prognostic marker of malignant mesothelioma.

III. Cancer Stem Cells

Hiroto Yamazaki, Motohiko Naito, Toshihiro Okamoto and Chikao Morimoto

Recent studies suggest that tumors contain cancer stem cells (CSCs) and their stem cell characteristics are thought to confer therapy-resistance. However, whether malignant mesothelioma (MM) cell has any stem cell characteristics is not known. To understand the molecular basis of MM, we first performed serial transplantation of surgical samples into NOD/SCID mice and established new cell lines. Next, we performed marker analysis of the MM cell lines and found that many of them contain SP cells and expressed several putative CSC markers such as CD9, CD24, and CD26. Interestingly, ex-

pression of CD26 closely correlated with that of CD 24 in some cases. Sorting and culture assay revealed that SP and CD24(+) cells proliferated by asymmetric cell division-like manner. In addition, CD9(+) and CD24(+) cells have higher potential to generate spheroid colony than negative cells in the stem cell medium. Moreover, these marker-positive cells have clear tendency to generate larger tumors in mouse transplantation assay. Taken together, our data suggest that SP, CD9, CD24, and CD26 are CSC markers of MM and could be used as novel therapeutic targets. We conducted research to further characterize these molecules, especially CD24 and CD26.

A. 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: CD24, the common CSC marker, and CD26, the additional CSC marker. We further analyzed the CSC properties of CD24 and CD26-positve MM cells. We established RNAi-knockdown cells and found that these markers were significantly correlated with chemoresistance, proliferation, and invasion potentials in vitro. Interestingly, while Meso-1 cells expressed both CD24 and CD26, the presence of each of these two markers was correlated with different CSC property. In addition, downstream signaling of these markers was explored by microarray analysis, which revealed that their expressions were correlated with several cancer-related genes. Furthermore, phosphorylation of ERK by EGF stimulation was significantly affected by the expression of CD26, but not CD24. These results suggest that CD24 and CD26 differentially regulate the CSC potentials of MM and could be promising targets for CSC-oriented therapy.

IV. Therapeutically targetting transcription factors

Hirotoshi Tanaka, Noritada Yoshikawa, Noriaki Shimizu, Takako Maruyama, Chikao Morimoto

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 is now been applied in clinical settings in IMSUT 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-steroidal 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 anti-inflammatory 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.

(i) Development of Dissociating Ligand for the GR

The GR function could be differentially 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 ascribed to the ligand binding domain of the receptor. In this line, we are going to isolate the dissociating ligand that preferentially 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.

(ii) 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 hexamethylene bisacetamide. It is shown that HEXIM1 binds 7SK snRNA and inhibits P-TEFb-mediated transcriptional elongation process. Right ventricular hypertrophy (RVH) and right ventricular (RV) contractile dysfunction are major determinants of prognosis in pulmonary arterial hypertension (PAH) and PAH remains a severe disease. Recently, direct interruption of left ventricular hypertrophy has been suggested to decrease the risk of left-sided heart failure. HEXIM1 is a negative regulator of P-TEFb, which activates RNA polymerase II (RNAPII)-dependent transcription and whose activation is strongly associated with left ventricular hypertrophy. We hypothesized that during the progression of PAH, increased P-TEFb activity might also play a role in RVH, and that HEXIM1 might have a preventive role against such process. We revealed that, in the mouse heart, HEXIM1 is highly expressed in the early postnatal period and its expression is gradually decreased, and that prostaglandin I_2, a therapeutic drug for PAH, increases HEXIM1 levels in cardiomyocytes. These results suggest that HEXIM1 might possess negative effect on cardiomyocyte growth and take part in cardiomyocyte regulation in RV. Using adenovirus-mediated gene delivery to cultured rat cardiomyocytes, we revealed that overexpression of HEXIM1 prevents endothelin-1-induced phosphorylation of RNAPII, cardiomyocyte hypertrophy, and mRNA expression of hypertrophic genes, whereas a HEXIM1 mutant lacking central basic region, which diminishes P-TEFb-suppressing activity, could not. Moreover, we created cardiomyocyte-specific HEXIM1 transgenic mice and revealed that HEXIM1 ameliorates RVH and prevents RV dilatation in hypoxia-induced PAH model. Taken together, these findings indicate that cardiomyocyte-specific overexpression of HEXIM1 inhibits progression to RVH under chronic hypoxia, most possibly via inhibition of P-TEFb-mediated enlargement of cardiomyocytes. We conclude that P-TEFb/HEXIM1-dependent transcriptional regulation may play a pathophysiological role in RVH and be a novel therapeutic target for mitigating RVH in PAH.

iii) 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 fiber 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.

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Advanced Clinical Research Center

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

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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 carcinogenesis, 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 cancer

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

We previously analyzed gene expression profiles of primary colorectal cancer to identify novel molecular targets for the treatment. The profiles identified a set of genes whose expression levels were frequently elevated in the tumors compared with their matched non-cancerous colonic mucosa. Among the genes we focused the proto-oncogene defective in sister chromatid cohesion 1 homologue (*DSCC1*), and have been interrogating the molecular basis for deviant expression of DSCC1 in colorectal cancer and its ability to promote survival of cancer cells. Although *DSCC1* is located at chromosomal region 8q, which is one of the most frequently amplified chromosomal regions in colorectal tumors, copy number gain or amplification was not involved in *DSCC1* up-regulation. Subsequently, *in silico* analysis and reporter gene assay revealed a potential role for the E2F family of DNA -binding proteins in controlling DSCC1 expression. Gain- and loss-of-function experiments demonstrated that DSCC1 is involved in the viability of cancer cells in response to genotoxic stimuli. In addition, we performed gene expression profile analysis with DSCC1-siRNA, and identified a set of genes regulated by DSCC1. Subsequent analysis uncovered that DSCC1 induces the expression of anti-apoptosis gene *BCL2*, suggesting its vital role in the DSCC1-related resistance to genotoxic insults. These data suggested that DSCC1 may serve as a biomarker for chemo-resistance, and that its suppression may be a useful option for the treatment of colorectal cancer.

We also focused on SET and MYND domain containing 3 (SMYD3) as a therapeutic target for colorectal cancer. Accumulating evidence suggests that SMYD3 catalyzes methylation of histone lysines, with implications for human carcinogenesis. However, the mechanism by which SMYD3 promotes cancer progression remains largely unknown. Since SMYD3 both methylates histone tails and interacts with RNA polymerase II, we hypothesized that SMYD3 might regulate downstream genes through simultaneous modulation of chromatin structure and recruitment of transcription factors. To clarify

its role in gene expression, we combined microarray expression profiling with ChIP-chip analyses, using SMYD3-specific siRNAs and a SMYD3 antibody, respectively. The analyses revealed a set of genes whose expression is regulated by SMYD3. These data should be helpful for expanding our understanding of human carcinogenesis involving overexpression of SMYD3.

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 vivo*

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

SMYD3, SET and MYND domain containing protein 3 is a histone methyltransferase whose expression levels are enhanced in human colon, liver, and breast cancer. We have revealed that zebrafish Smyd3 plays a crucial role in morphogenesis of heart and skeletal muscle. We additionally investigated its role in the development and differentiation of hematopoietic cells because these cells as well as heart and skeletal muscle cells are derived from mesoderm. We knocked down Smyd3 in zebrafish embryos using morpholino oligonucleotides, and generated Smyd3 morphants. Expression of various differentiation markers of myeloid and lymphoid cells has been analyzed in the morphants. The markers include *hbbe1* (erythrocytes), *mpx* (macrophages), *plastin* (granulocytes), *rag1* (lymphocytes), *pu.1* (myeloid progenitors), *ikaros* (lymphoid progenitors), and *cmyb* (definitive stem cells). In addition, we have been analyzing phenotype of Smyd3 knockout mice. These studies will clarify the physiological function of Smyd3 in fish and mammals.

3. Establishment and investigation of novel mouse models of human cancer

Tsuneo Ikenoue, Tomoaki Fujii, Mitsuho Imai[1], Hideaki Ijichi[1], and Yoichi Furukawa: [1]Department of Gastroenterology, Graduate School of Medicine, University of Tokyo

Genetically engineered mice (GEM) are useful tools for studying human diseases, including cancer. In this project, we succeeded in the establishment of mouse models of pancreatic and liver cancer. In addition, we recently generated a novel mouse strain carrying a conditional knockin allele of the *Fbxw7* gene, which is frequently mutated in human colon, liver, and hematopoietic malignancies. Using this strain, we are trying to establish new mouse models of these tumors. Intense investigation of these models should provide better understanding of these malignancies and facilitate the development of new therapies to them.

4. Genetic diagnosis using next generation sequencer

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

We have started two projects using next generation sequencer (NGS); 1) the determination of germline mutations in patients suspicious for hereditary colorectal cancer, and 2) whole genome analysis of hematopoietic malignancies and colorectal tumors. For these two projects, we have established a highly secure system in collaboration with Human Genome Center. In this system, CPUs and storage in the supercomputer are theoretically separated, and desk-top computers accessible to the system were prepared in a secured room. Analytical pipelines are under construction. These projects are aimed to return the data of personal genome and/or cancer genome to patients in IMSUT Hospital, and apply them to their diagnosis and treatment.

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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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Assistant Professor	Motokazu Ito, M.D., Ph.D.	助　教	医学博士	伊　藤　元　一

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 oncolytic HSV-1 that utilize tumor/tissue-specific promoters.

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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 clinical trials, especially for translational researches. For this purpose, it is critical to discover the new "seeds" and to eradicate many blockades until the utilization. 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. At 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 conduction clinical trials, especially for translational research, is the critical problem in Japan. 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

Noriko Fujiwara, 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. Scholastic Program for the Graduate Students of Nurses in the Area of Translational Research.

Noriko Fujiwara, Makiko Tajima, Fumitaka Nagamura

TR is the early phase of clinical trials, which applied the developments of basic researches for patients with incurable and/or life-threatening diseases. Highly 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 scholastic program for the graduate students of nurses in the area of TR. We planed and implemented the one-week 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. 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. Stressor Scale for Clinical Research Coordinators: development and psychometric testing.

Matsumoto K, Sumino K, Fukahori H, Kamibeppu K, NagamuraF

Job stress is viewed as a situation where working conditions interact with individual worker characteristics and result in disruption of psychological or physiological homeostasis. Clinical research coordinators, also known as research nurses, are professionals who play a central role in clinical trials. They face various problems associated with their responsibilities; however, few studies have reported on their stress. To manage their stress, it is necessary to identify the sources of stress (i.e. stressors). The 56-item preliminary instrument was developed based on literature review and expert discussions. A total of 589 clinical research coordinators in 186 hospitals in Japan were surveyed in 2011. Statistical analyses on construct and concurrent validity, internal consistency, and test-retest reliability were performed. A six-factor solution with 23 items was selected using exploratory factor analysis: 'quantitative workload', 'conflict with investigators', 'ambiguity of work', 'conflict with other clinical research coordinators and with supervisors', 'demands from an affiliate other than the hospital', and 'difficulty in caring for trial participants'. Confirmatory factor analysis affirmed construct validity, with a demonstrated acceptable fit between the factor structure and the observed data. All factors had significant correlations with burnout and psychological distress, which indicated acceptable concurrent validity. Cronbach's alpha coefficients ranged from 0.73-0.82. Intra-class correlation coefficients indicated almost satisfactory test- retest reliability. Our new instrument has acceptable validity and reliability for evaluating job stressors for clinical research coordinators.

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Mae H, Ooi J, Takahashi S, Kato S, Kawakita T, Ebihara Y, K Tsuji, Nagamura F, Echizen H, Tojo A. Acute kidney injury after myeloablative cord blood transplantation in adults: the efficacy of strict monitoring of vancomycin serum trough concentrations. Transpl Infect Dis 0:1-6, 2012

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長村文孝　FDAにおける治験・承認審査の要点と傾向　稀少疾患／難病の診断・治療と製品開発　技術情報協会　255−260，2012

長村文孝　国内外のがんワクチンの開発動向と臨床からの期待　ワクチンの市場動向と開発・製造実務集　技術情報協会　673−689，2012

長村文孝　国内副作用情報報告からFDAへの英文副作用報告書を作成する　世界の薬事規制対応・承認申請　成功のコツ　技術情報協会　印刷中