*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 hematological malignancies:*
*Tumor-specific genetic alterations often result in transcriptional dysregulation and activation of signal transduction pathways as well as defective tumor suppressors, which appear to be the primary cause of those tumors. We are studying the molecular and cellular aspects of hematological malignancies as a model system. Furthermore, we performed clinical sequencing in tight collaboration with Human Genome Center and Health Intelligence Center to establish a platform for precision medicine.*

*(2) Development of anti-cancer therapy using recombinant vaccinia virus:*
*Oncolytic virotherapy is an emerging type of cancer therapy in which a native or genetically modified virus selectively infects and replicates in the tumor and destroys tumor cells. The anti-tumor effects of oncolytic virus alone were generally insufficient in pre-clinical and clinical trials. Using genetic engineering, we loaded oncolytic viruses with foreign transgenes to increase the potency of the therapeutic effect.*

*(3) Development of a novel cell therapy using the genome editing with CRISPR/Cas9:*
*Cell therapy using mesenchymal stem cells and chimeric antigen receptor expressing-T cells (CAR-T cells) are promising therapeutic options for refractory diseases. While cell therapies are remarkably effective, very expensive cost hampers them to be applied for regular clinical use. We used CRISPR/Cas9 for the gene editing to generate a universal cell therapy.*

*(4) 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.*

*(5) 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.*

## 1. Preclinical study of recombinant vaccinia viruses in mouse immunocompetent syngeneic tumor models

**Chit UW, Futami M, Tojo A: Division of Molecular Therapy**

Vaccinia virus (VV) is a useful tool for the oncolytic virotherapy. We previously reported that miRNA and thymidine kinase (TK)-doubly regulated vaccinia virus (MDVV) selectively infected human multiple myeloma (MM) cells and prolonged survival of severe combined immunodeficiency (SCID) mice bearing MM tumor. In the current study, we observed whether the MDVV is similarly effective in an immunocompetent setting. A C57BL/6 (B6) mouse-derived lung cancer 3LL cells were transplanted subcutaneously into B6 mice, and infectivity of MDVV *in vivo* was examined. MDVV was successfully delivered to subcutaneous 3LL tumors by IV injection, and significant tumor regression and prolongation of survival were observed (21.8 days vs. 29.4 days, respectively, P= 0.0094). Although the tumor regressed during the first week, it began to increase in size again after MDVV disappeared from the body, leading to death in all mice. The limited retention of MDVV was thought to be the main cause of suboptimal anti-tumor effect in the B6/3LL model. To enhance the effectiveness, we tried to produce the anti-tumor immunity using artificial immunoadjuvant cells that stimulate natural killer T (NKT) cells, NK cells, and cytotoxic T cells. These cells co-expressed the CD1d molecule and tumor antigen/GM-CSF fusion. The immunoadjuvant cells were generated by transducing 3LL tumor cells to express CD1d and ovalbumin (OVA)/GM-CSF fusion. B6/3LL subcutaneous tumor mice were injected IV with the immunoadjuvant cells, and significant tumor size reduction and prolongation of survival was observed.

## 2. Development of a novel cell therapy using the genome editing with CRISPR/Cas9

**Meshitsuka S, Ikeda M, Futami M, Tojo A: Division of Molecular Therapy**

Cell therapies using mesenchymal stem cells (MSCs) are effective for the treatment of graft versus host disease (GvHD) following allogeneic stem cell transplantation. However, human leukocyte antigen (HLA) alleles usually mismatch between patients and donors, and transplanted MSCs are eventually rejected by the host immunity. The knockout (KO) of HLA gene would unlock the HLA restriction and facilitate the development of universal cell therapy. For a longer retention of transplanted MSCs in the recipient, a genome editing to knockout HLA molecule was performed. As HLA class I molecules are expressed on the cell surface together with β-2 microglobulin (B2M), knockout (KO) of B2 M leads to loss of expression of HLA. Using the electroporation, MSCs were transfected with Cas9 protein and a short guide RNA (sgRNA) targeting B2M. Successful KO of B2M and HLA class I was confirmed on day 7. We confirmed that B2M$-/-$ MSCs retains the immunosuppressive effect as strong as parental MSCs using the mixed lymphocyte reaction (MLR) in the presence of MSCs. Although loss of HLA would protect MSCs from cytotoxic T lymphocytes (CTLs), loss of HLA deprives a protective effect of HLA through the binding to inhibitory receptors on the natural killer (NK) cells. To avoid from both CTLs and NK cells, HLA-G, an almost invariant non-classical HLA, was fused with B2M, and the B2M/HLA-G fusion was successfully transduced into MSCs using a lentiviral vector. We are also attempting to insert the B2M/HLA-G fusion into MSCs with a genome editing method using CRISPR/Cas9. To establish a sophisticated method by which efficient and safe gene KO and/or knock-in (KI) are carried out, we examined several methods using CRISPR/Cas9. While a high efficiency of KO could be achieved by the transfection of either a CRISPR/Cas9-expressing plasmid (pX330) or a mixture of sgRNA and Cas9 protein, the efficiency of KI was very low using a conventional electroporation of sgRNA, Cas9 protein, and donor DNA (in the form of plasmid). The limiting factor in KI seemed to be the cytotoxicity due to the large DNA

size that was transfected as a DNA donor. We compared several different types of DNA donors, including plasmids with homology arms (HA) on both sides of the inserted gene, plasmids without HA on both sides, the linear double strand DNAs, and the single strand DNAs. We found that transfection of sgRNA/Cas9 with a plasmid that have sgRNA recognition sites on both sides of transgene (with no HAs) showed a relatively low cytotoxicity and a good KI efficiency (homology-independent transgene insertion). Using the method that we have confirmed, we will put forward our genome editing experiments for the development of a new cell therapy.

### 3. Proportion of $CD4^+CADM1^+$ population predicts disease progression in HTLV-1 asymptomatic carrier and indolent ATL

**Kobayashi S[1], Makiyama J[2], Watanabe E[3], Ishigaki T[4,5], Kawamata T[2], Tojo A[1,2], and Uchimaru K[6]: [1]Division of Molecular Therapy, [2]Department of Hematology/Oncology, IMSUT Hospital, [3]IMSUT clinical flow cytometry laboratory, [4]Department of Laboratory Medicine, IMSUT Hospital, [5]Division of Stem Cell Therapy, [6]Laboratory of Tumor Cell Biology, Department of Computational Biology and Medical Sciences, Graduate School of Frontier Sciences**

We previously reported that human T-cell leukemia virus type I (HTLV-1) asymptomatic carriers (AC) and indolent adult T-cell leukemia-lymphoma (iATL) could be divided into four groups based on the cell adhesion molecule 1 (CADM1) versus CD7 plot in flow cytometry. However, the clinical outcome in long-term follow-up has not been fully elucidated. Hence, we evaluated HTLV-1 AC and iATL cases according to the $CADM1^+$ (%) to reveal which cases were at high risk of disease progression. Peripheral blood samples of AC (n=48) and iATL (n=26) cases were analyzed by flow cytometry to classify according to the $CADM1^+$ (%) and their clinical courses were followed. There were 20 (AC 20) in G1 ($CADM1^+$<=10%), 17 (AC 17) in G2 (10% <$CADM1^+$<=25%), 19 (AC 9, smoldering type ATL 9, chronic type ATL 1) in G3 (25%< $CADM1^+$<=50 %), and 18 (AC 2, smoldering type ATL 6, chronic type ATL 10) cases in G4 (50%<$CADM1^+$). In G1 and G2, apparent clinical disease progression was not observed up to 8 years' follow-up (median follow-up time 3.8 years). In G3, two cases received systemic chemotherapy due to disease progression. In G4, the probability of systemic therapy-free survival at 5 years was 42.1%. Moreover, all but one AC cases, who progressed into ATL defined at 5% or more abnormal lymphocytes were in G3 and G4 initially. We could stratify HTLV-1 AC and iATL cases according to the $CADM1^+$ (%). It was suggested that the proportion of the $CD4^+CADM1^+$ population could predict clinical disease progression in HTLV-1 AC and iATL.

### 4. Cell lineage-level targeted sequencing to identify acute myeloid leukemia with myelodysplasia-related changes.

**Yokoyama K[1,2], Yokoyama N[3,6], Nakamura S[2], Ogawa M[2], Takei T[2], Shimizu E[4], Yamaguchi R[4], Imoto S[5], Miyano S[4], Tojo A[1,2]: [1]Department of Hematology/Oncology, IMSUT Hospital, [2]Division of Molecular Therapy, [3]Department of Applied Genomics, IMSUT Hospital, [4]Laboratory of Genome Database, [5]Division of Health Medical Data Science, [6]Division of Clinical Genome Research**

Acute myeloid leukemia (AML) is a clonal myeloid neoplasm that typically arises de novo; however, some cases evolve from a preleukemic state, such as myelodysplastic syndrome (MDS). Such secondary AMLs and those with typical MDS-related clinical features are known as AMLs with myelodysplasia-related changes (AML-MRC). Because patients with AML-MRC have poor prognosis, more accurate diagnostic approaches are required. In this study, we performed targeted sequencing of 54 genes in 3 cell populations (granulocyte, blast, and T-cell fractions) using samples from 13 patients with MDS, 16 patients with clinically diagnosed AML-MRC, 4 patients with suspected AML-MRC but clinically diagnosed as AML not otherwise specified (AML-NOS), and 11 patients with de novo AML. We found that overlapping mutations, defined as those shared at least by the blast and granulocyte fractions, were significantly enriched in patients with MDS and AML-MRC, including those with suspected AML-MRC, indicating a substantial history of clonal hematopoiesis. In contrast, blast-specific nonoverlapping mutations were significantly enriched in patients with de novo AML. Furthermore, the presence of overlapping mutations, excluding *DNMT3A, TET2,* and *ASXL1,* effectively segregated patients with MDS and AML-MRC or suspected AML-MRC from patients with de novo AML. Additionally, the presence of ≥3 mutations in the blast fraction was useful for distinguishing patients with AML-MRC from those with MDS. In conclusion, our approach is useful for classifying clinically diagnosable AML-MRC and identifying clinically diagnosed AML-NOS as latent AML-MRC. Additional prospective studies are needed to confirm the utility of this approach.

### 5. Personalized circulating tumor DNA dynamically predicts response and/or relapse in hematological malignancies.

**Nakamura S[1], Yokoyama K[1,2], Yusa N[3], Ogawa M[1], Takei T[1], Kobayashi A[1], Ito M[1], Shimizu E[4], Kasajima R[5], Wada Y[6], Yamaguchi R[4], Imoto S[5], Furukawa Y[3], Inoue-Nagamura T[6], Miyano S[4], Tojo A[1,2]: [1]Division of Molecular Therapy, [2]Department of Hematology/Oncology, IMSUT Hospital, [3]Department of Applied Genomics, IMSUT Hospital, [4]Laboratory of Genome Database, [5]Division of Health Medical Data Science, [6]Department of Cell Processing and Blood Transfusion, IMSUT Hospital**

A growing body of evidence suggests that tumor-derived fragmentary DNA, known as circulating tumor DNA (ctDNA), has the potential to serve as a non-invasive biomarker for disease monitoring. However, in the setting of hematological malignancy, few published studies support the utility of ctDNA. We retrospectively investigated ctDNA levels of 17 patients with various hematological malignancies who had achieved remission after first-line therapy. We identified somatic driver mutations by next-generation sequencing, and designed droplet digital PCR assays for each mutation to measure ctDNA. Variant allele frequencies of ctDNA changed in association with clinical response in all patients. Eight patients clinically relapsed after a median of 297 days post-first-line therapy (termed, "relapsed group"); the remaining nine patients remained disease-free for a median of 332 days (termed, "remission group"). Among patients in the relapsed group, ctDNA levels increased more than twofold at paired serial time points. In marked contrast, ctDNA levels of all patients in the remission group remained undetectable or stable during clinical remission. Notably, ctDNA-based molecular relapse demonstrated a median 30-day lead time over clinical relapse. In summary, ctDNA monitoring may help identify hematologic cancer patients at risk for relapse in advance of established clinical parameters.

### 6. Histone deacetylase inhibitors with or without AKT inhibition potentially increase the efficacy of daratumumab in multiple myeloma by enhancing the antibody-dependent cell-mediated cytotoxicity as well as apoptosis

**Hirano M[1], ImaiY[2], Futami M[1], Tojo A[1]: [1]Division of Molecular Therapy, The Advanced Clinical Research Center, [2]Department of Hematology/Oncology, Research Hospital**

Multiple myeloma (MM) is one of the many types of hematological malignancies, and many novel drugs, including histone deacetylase (HDAC) inhibitors are currently undergoing preclinical and clinical evaluation. Daratumumab, an anti-CD38 monoclonal antibody, is a promising agent showing high activity in relapsed/refractory MM. However, some patients become resistant to daratumumab. In our data, exposure of panobinostat for 24 h did not change expression of CD38. On the other hand, the expression of MICA (NK cell-activating ligand) increased after exposure of all the examined HDAC inhibitors for 24 h. Antibody-dependent cell-mediated cytotoxicity (ADCC) was enhanced after exposure of all the examined HDAC inhibitors for 24 h. These results suggest that induction of MICA plays an important role in the enhancement of ADCC by HDAC inhibitors.

Afuresertib is an oral AKT inhibitor, which has been clinically tested in patients with advanced hematological malignancies. In a proliferation assay, we found that afuresertib combined with HDAC inhibitors showed higher cytotoxicity than that of each agent used singly. Expression of cleaved caspase 3 and caspase 7 was induced more in myeloma cells treated with a combination of HDAC and AKT inhibitors than in those treated with only one agent. These results suggested that the combination of HDAC and AKT inhibitors strongly induce the apoptosis of MM cells. Thus, the addition of HDAC and Akt inhibitors to daratumumab could be an effective therapy for relapsed/refractory MM.

### 7. Semaphorin signaling via MICAL3 induces symmetric cell division of breast cancer stem-like cells

**Tominaga K[1], Tojo A[1], and Gotoh N[1,2]: [1]Division of Molecular Therapy, [2]Division of Cancer Cell Biology, Cancer Research institute of Kanazawa University**

Cancer stem-like cells (CSCs) are expanded in the CSC niche by increased frequency of symmetric cell divisions at the expense of asymmetric cell divisions. The symmetric division of CSCs is important for the malignant properties of cancer; however, underlying molecular mechanisms remain largely elusive. Here, we show a cytokine, semaphorin 3 (Sema3), produced from the CSC niche, induces symmetric divisions of CSCs to expand the CSC population. Our findings indicate that stimulation with Sema3 induced sphere formation in breast cancer cells through neuropilin 1 (NP1) receptor that was specifically expressed in breast CSCs (BCSCs). Knockdown of *MICAL3*, a cytoplasmic Sema3 signal transducer, greatly decreased tumor sphere formation and tumor-initiating activity. Mechanistically, Sema3 induced interaction among MICAL3, collapsin response mediator protein 2

(CRMP2), and Numb. It appears that activity of MICAL3 monooxygenase (MO) stimulated by Sema3 is required for tumor sphere formation, interaction between CRMP2 and Numb, and accumulation of Numb protein. We found that knockdown of *CRMP 2* or *Numb* significantly decreased tumor sphere formation. Moreover, *MICAL3* knockdown significantly decreased Sema3-induced symmetric divisions in NP1/Numb-positive BCSCs and increased asymmetric division that produces NP1/Numb negative cells without stem-like properties. In addition, breast cancer patients with NP1-positive cancer tissues show poor prognosis. Therefore, the niche factor Sema3-stimulated NP1/MICAL3/CRMP 2/Numb axis appears to expand CSCs at least partly through increased frequency of MICAL3-mediated symmetric division of CSCs.

## 8. Cancer stem-like properties and drug resistance are dependent on purine synthetic metabolism mediated by the mitochondrial enzyme MTHFD2

**Nishimura T[1,2], Tojo A[1], Gotoh N[1,2]: [1]Division of Molecular Therapy, [2]Division of Cancer Cell Biology, Cancer Research institute of Kanazawa University**

Tumor recurrence is attributable to cancer stem-like cells (CSCs), the metabolic mechanisms of which currently remain obscure. Here, we uncovered the critical role of folate-mediated one-carbon (1C) metabolism involving mitochondrial methylenetetrahydrofolate dehydrogenase 2 (MTHFD2) and its downstream purine synthesis pathway. MTHFD 2 knockdown greatly reduced tumorigenesis and stem-like properties, which were associated with purine nucleotide deficiency, and caused marked accumulation of 5-aminoimidazole carboxamide ribonucleotide (AICAR)-the final intermediate of the purine synthesis pathway. Lung cancer cells with acquired resistance to the targeted drug gefitinib, caused by elevated expression of components of the β-catenin pathway, exhibited increased stem-like properties and enhanced expression of MTHFD2. MTHFD2 knockdown or treatment with AICAR reduced the stem-like properties and restored gefitinib sensitivity in these gefitinib-resistant cancer cells. Moreover, overexpression of MTHFD2 in gefitinib-sensitive lung cancer cells conferred resistance to gefitinib. Thus, MTHFD2-mediated mitochondrial 1C metabolism appears critical for cancer stem-like properties and resistance to drugs including gefitinib through consumption of AICAR, leading to depletion of the intracellular pool of AICAR. Because CSCs are dependent on MTHFD2, therapies targeting MTHFD2 may eradicate tumors and prevent recurrence.

## 9. Critical roles of luminal progenitor cells for creating the cytokine-rich precancerous niche for mammary tumorigenesisis

**Kimura N[1], Murayama T[1], Machida Y[1], Nishimura T[1,2], Yoshida N[3], Inoue J-I[4], Tojo A[1], Gotoh N[1,2]: [1]Division of Molecular Therapy, [2]Division of Cancer Cell Biology, Cancer Research institute of Kanazawa University, [3]Laboratory of Developmental Genetics, [4]Division of Cellular and Molecular Biology**

The precancerous tissue microenvironment is thought to bring up cancer cells including cancer stem-like cells (CSCs). The underlying mechanisms, which should be targeted for preventing cancer, remain unclear. Here, we uncovered the critical roles of luminal progenitor cells expressing FRS2β, a cytoplasmic inhibitory adaptor of ErbB/ERK signaling, in creating the cytokine-rich inflammatory precancerous CSC niche in mammary tissues. Deficiency of FRS2β greatly decreased the mammary tumorigenesis with decreased tumor stroma in the mouse mammary tumor virus (MMTV)-ErbB2 mice. Moreover, wild type FRS2β but not FRS2β-deficient precancerous mammary tissues allowed tumorigenesis from xenografted wild type FRS2β tumor cells. Expression levels of insulin-like growth factor (IGF) 1 and CXC chemokine ligand (CXCL) 12, the stemness- and stroma-inducing cytokines, respectively, were decreased in FRS2β-deficient precancerous mammary cells. Treatment with inhibitors against these cytokines in wild type FRS2β precancerous mice greatly decreased the ability to support tumor growth. In addition, human breast cancer tissues in which FRS2β is highly expressed in tumor cells harbor more stroma and are associated with poor prognosis. Thus, FRS2β appears to induce cytokine-production in the precancerous luminal progenitor cells or in undifferentiated tumor cells. We provide a rational that the inflammatory precancerous CSC niche and tumor microenvironment created by FRS2β would be targetable for preventing or treating cancer.

## 10. EGFR-TKI-associated interstitial pneumonitis in nivolumab-treated patients with non-small cell lung cancer.

**Oshima Y[1], Tanimoto T[2], Yuji K[3], Tojo A[1]: [1]Division of Molecular Therapy, [2]Jyoban Hospital of Tokiwa Foundation, [3]Project Division of Advanced International Medical Research**

Nivolumab and epidermal growth factor receptor-tyrosine kinase inhibitors (EGFR-TKIs) are now the standard-of-care therapies in non-small cell lung cancer (NSCLC). Although EGFR-TKIs are well understood and have well-defined safety pro-

files, our experience with immune checkpoint inhibitors is still growing, particularly regarding the use of combinations of different classes of antitumor agents, including both the concomitant and sequential use of such agents. To determine whether nivolumab increases EGFR-TKI-associated interstitial pneumonitis (IP). A database study of 20 516 participants with NSCLC in the US Food and Drug Administration Adverse Event Reporting System (FAERS) database, performed between April 2015 and March 2017. We compared the incidence of EGFR-TKI-associated IP in patients receiving and not receiving nivolumab treatment. The mean (SD) age of participants treated with EGFR-TKI, with and without nivolumab, was 64.4 (15.5) and 68.9 (11.8) years, respectively, and the proportion of men was 40.0% and 53.8%, respectively. Of the 20 516 participants with NSCLC, 985 cases (4.80%; 95 % CI, 4.51-5.10) developed IP. Of 5777 patients treated with EGFR-TKI, 265 developed IP (4.59%; 95% CI, 4.06-5.16). Of 70 patients treated with both EGFR-TKI and nivolumab, 18 developed IP (25.7%; 95% CI, 16.0-37.6). The adjusted odds ratio for an interaction between EGFR-TKI and nivolumab was 4.31 (95% CI, 2.37-7.86; P<.001), suggesting the existence of an interaction. When we further stratified the patients by treatment with and without nivolumab, the odds ratio of EGFR-TKI-associated IP in cases with and without nivolumab treatment was 5.09 (95% CI, 2.87-9.03) and 1.22 (95% CI, 1.00-1.47), respectively. We found a higher proportion of reports of IP for nivolumab in combination with EGFR-TKI vs treatment with either drug alone. Owing to the limitations of this study, the results warrant further confirmation. However, careful consideration should be given to the possibility of an increased risk of IP when EGFR-TKI is administered in combination with nivolumab, including concomitant and sequential use, and careful monitoring for IP is recommended.

## Publications


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*Advanced Clinical Research Center*

# Division of Cellular Therapy
## 細胞療法分野


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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 targeted therapies, and (3) Elucidation of molecular basis of leukemia, hematological malignancioes.*

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

**Takeshi Fukushima, Yosuke Tanaka, Toshihiko Oki, Toshiyuki Kawashima, Kohtaro Nishimura, Susumu Goyama, and Toshio Kitamura.**

In search for key molecules that prevent murine M1 leukemic cells from undergoing IL-6-induced differentiation into macrophages, we previously isolated an antisense cDNA that encodes full-length mouse MgcRacGAP through functional cloning. In human HL-60 leukemic cells, overexpression of human MgcRacGAP induced differentiation to macrophages. Interestingly, MgcRacGAP localized to the nucleus in interphase, accumulated to the mitotic spindle in metaphase, and was condensed in the midbody during cytokinesis. 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. MgcRacGAP was found to harbor functional NLS and works as a nuclear chaperon together with Rac1.

We also found using an MgcRacGAP-GFP fusion protein that MgcRacGAP expression increased in the early G1 phase in parallel with or even earlier than Geminin, suggesting that MgcRacGAP may play roles in G1 check point. MgcRacGAP accumulates to the midbody during cytokinesis, and the midbody is included in one of the daughter cells after cell division. It was suggested by some researchers that the midbody is frequently released from the cells in stem cells. We therefore hypothesized that the cells with midbody tend to differentiate and the cells without midbody tend to self-renew or enter G0 phase. To test this hypothesis, we have recently generated a transgenic mouse expressing the MgcRacGAP-mVenus fusion protein in hematopoietic stem cells and/or progenitors.

## 2. Molecular targeting therapies using small molecule compounds

**Akiho Tsuchiya, Reina Nagase, Toshiyuki Kawashima, Yukinori Minoshima, Susumu Goyama, 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 established an efficient screening protocol for identification of STAT3 inhibitors. 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. 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 collaborated with a US-based biotech company in modification of RSJI-1 for optimization to develop anti-cancer drugs, and have developed JP1156 that kills the tumor cells more efficiently both in vitro and in vivo with much lower IC50.

In addition to STAT3 inhibitors, we have recently started a new project to develop STAT5 inhibitors in collaboration with a pharmaceutical company. To this end, we have developed a screening method to search for STAT5 inhibitors. In addition to STAT3/5 inhibitors, we have started several collaborations with several domestic and global pharmaceutical companies to evaluate the efficacies of a variety of molecular targeted therapies in our established mouse MDS/AML/MPN models.

## 3. Molecular basis of acute leukemia, myelodysplastic syndromes (MDS), MDS overt leukemia, and myeloproliferative neoplasms (MPN).

**Daichi Inoue, Reina Nagase, Takeshi Fujino, Yasutaka Hayashi, Shuji Asada, Reina Takeda, Kojin C Kawabata, Naoko Watanabe, Makoto Saika, Yukiko Komeno, Naoko Kato, Yutaka Enomoto, Toshihiko Oki, Yuka Harada[1], Hironori Harada[2], Tetsuya Nosaka[3], Jiro Kitaura[4], Yosuke Tanaka, Tomofusa Fukuyama, Susumu, Goyama, and Toshio Kitamura: [1]Department of Clinical Laboratory Medicine, Bunkyo Gakuin University, Laboratory of Oncology, Department of Medical Science Tokyo University of Pharmacy and Life Sciences, [3]Mie University School of Medicine, and [4]Allergy Center, Juntendo University.**

Recent progress using high-speed sequencing has identified mutations in genes that are not categorized to class I and class II mutations, including epigenetic factors, and splicing factors. We established two MDS models induced by ASXL1 mutations and EZH2 mutations; mice transplanted with bone marrow cells expressing C-terminal truncating mutants of ASXL1 derived from MDS patients or a catalytic domain (SET)-deleted mutant of EZH2 (EZH2-dSET) developed MDS-like diseases in a year or two. Concerning the molecular mechanisms, the ASXL1 mutant (ASXL1-MT) suppressed PRC2 and MLL fuctions, leading to the derepression of posterior HoxA genes and miR125a via inhibition of H3K27me3 and decreased expression of Id2 and TJP1 via inhibition of HeK4me3. In addition, ASXL 1-MT stabilizes and activate BAP1, leading to the derepression of IRF8 and Bcl2 via decreased H2AK 119Ub. Thus, ASXL1-MT changes cellular programs via reduced H3K4me3, H3K27me3 and H2AK119 Ub. ASXL1 mutations are frequently associated with SETBP1 mutations (SETBP1-MT) that stabilize SETBP1 and SET oncoprotein, leading to activation of the PI3K/Akt pathway. In the BMT model, combination of ASXL1-MT and SETBP1-MT induced AML with much shorter latencies. GSEA indicated that the TGF beta pathway was profoundly inhibited, implying the inhibition of the TGF beta pathway in leukemic transformation of MDS. Further experiment is now under way to clarify the molecular mechanisms by which the TGF beta pathway was inhibited.

We have recently established Rosa26-knock-in mice for ASXL1-MT. These KI mice did not develop MDS in their lives, but presented disturbed differentiation of erythroid cells and mild macrocytic anemia. Combination with other mutations (eg. Runx1 mutation) and insertional mutagenesis experiments have demonstrated that these mice are in the pre-leukemic states. Now we use the ASXL1-MT-KI mouse as a model for "clonal hematopoiesis". Clonal hematopoiesis is a state in which one or in rare case 2 leukemia-associated mutations are found in more than 1～2%blood cells in old (>65 years) healthy people. People with clonal hematopoiesis have 10-times higher risk for developing hematological malignancies. In addition, they have 2-times higher risks for developing stroke and acute myocardial infarction. In addition, in cancer patients after chemotherapy, clonal hematopoiesis is identified 20～30%. Importantly, cancer patients with clonal hematopoiesis have higher ratios of recurrence and poorer prognosis. Now we are characterizing ASXL1-MT-KI mice to clarify how people with clonal hematopoiesis develop a variety of diseases.

## 4. Investigating molecular pathogenesis of AML 1-MTG8/ETO and MLL-fusion acute myeloid leukemias.

**Tomofusa Fukuyama, Susumu Goyama, Keita**

**Yamamoto, Taishi Yonezawa, Joseph S. Palumbo[5], James C. Mulloy[5], Toshio Kitamura: [5]Cincinnati Children's Hospital Medical Center**

Using human and mouse models for AML1-MTG 8/ETO and MLL-fusion leukemias, we have been elucidating new molecular aspects in the pathogenesis and progression of acute myeloid leukemia (AML).

t(8;21) AML is the most common cytogenetic subtype of AML, and the resultant AML1-MTG8 chimeric protein is believed to play an important role in leukemogenesis. However, the role of AML1-MTG8 is still unclear because persistent existence of chimeric gene including chimeric fusion point detected by PCR is observed in complete remission or healthy persons, even in utero. In addition, full length of AML1-MTG8 by itself cannot cause leukemia in mouse models, suggesting that additional "events" should be required for leukemogenesis. Interestingly, AML1-ETO9a (AE9a) that lacks c-terminus of AML1-MTG8 is shown to possess leukemogenic potential in a mouse model of retroviral transduction-transplantation. Nonetheless, AE9a protein is barely expressed in t(8;21) cells and a recent report suggested that there was no impact on clinical outcome by AE9a. Now we have identified a new splicing variant that has a significant ability to induce leukemia in mouse models. Mechanisms of leukemogenesis by it as well as clinical significance are currently under investigation.

MLL-fusion leukemia is an aggressive form of leukemia carrying chimeric fusion of the MLL gene. We previously showed that the combined loss of Runx1/Cbfb inhibited the development of MLL-AF 9-induced leukemia. However, c-Kit + /Gr-1 − cells remained viable in Runx1/Cbfb-deleted cells, indicating that suppressing RUNX activity may not eradicate the most immature LSCs. We found upregulation of several hemostasis-related genes, including the thrombin-activatable receptor PAR-1 (protease-activated receptor-1), in Runx1/Cbfb-deleted MLL-AF9 cells. Similar to the effect of Runx1/Cbfb deletion, PAR-1 overexpression induced CDKN1A/p21 expression and attenuated proliferation in MLL-AF9 cells. To our surprise, PAR-1 deficiency also prevented leukemia development induced by a small number of MLL-AF9 leukemia stem cells (LSCs) in vivo. PAR-1 deficiency also reduced leukemogenicity of AML1-ETO-induced leukemia. Re-expression of PAR-1 in PAR-1-deficient cells combined with a limiting-dilution transplantation assay demonstrated the cell-dose-dependent role of PAR-1 in MLL-AF9 leukemia: PAR-1 inhibited rapid leukemic proliferation when there were a large number of LSCs, while a small number of LSCs required PAR-1 for their efficient growth. Mechanistically, PAR-1 increased the adherence properties of MLL-AF9 cells and promoted their engraftment to bone marrow. Taken together, these data revealed a multifaceted role for PAR-1 in leukemogenesis, and highlight this receptor as a potential target to eradicate primitive LSCs in AML.

### 5. Identification of E3 ubiquitin ligases for RUNX 1 and RUNX1-RUNX1T1

**Taishi Yonezawa, Hirotaka Takahashi[6], Shiori Shikata, Tomofusa Fukuyama, Yosuke Tanaka, Tatsuya Sawasaki[6], Toshio Kitamura, Susumu Goyama: [6]Proteo-Science Center (PROS), Ehime University**

RUNX1 is a member of RUNX transcription factors and plays important roles in hematopoiesis. Disruption of RUNX1 activity has been implicated in the development of hematopoietic neoplasms. Previous studies have shown that RUNX1 is an unstable protein and is subjected to proteolytic degradation mediated by the ubiquitin-proteasome pathway. However, the precise mechanisms of RUNX1 ubiquitination have not been fully understood. In this study, we identified several RUNX1-interacting E3 ubiquitin ligases using a novel highthroughput binding assay. Among them, we found that a nuclear ubiquitin ligase RNF38 induced ubiquitination of RUNX1. RNF38-induced RUNX1 ubiquitination did not promote RUNX1 degradation, but rather stabilized RUNX1 protein. We also showed that RNF38 enhanced RUNX1-mediated transcriptional repression of the erythroid master regulator KLF1 in K562 cells. Consequently, RNF38 cooperated with RUNX1 to inhibit erythroid differentiation of K562 cells. Thus, our study identified RNF38 as a novel E3 ligase that modifies RUNX1 function without inducing its degradation.

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*Advanced Clinical Research Center*

# Division of Infectious Diseases
## 感染症分野


| | | | |
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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. We are extending our research project to other viral diseases including viral hepatitis and associated morbidities, especially pathogenesis of co-infection with HIV and hepatitis B virus (HBV) or hepatitis C virus (HCV).*

### 1. Identification of preS/S sequence of HBV derived from patients co-infected with HIV and HBV.

**Takeya Tsutsumi, Nupur Nigam, Hidenori Sato[1], Eisuke Adachi[1], Tadashi Kikuchi[1], Michiko Koga, Tomohiko Koibuchi[1], Hiroshi Yotsuyanagi: [1]Department of Infectious Diseases and Applied Immunology, IMSUT hospital, IMSUT**

The number of patients infected with HBV dramatically decreased by successful prevention of mother-to-child transmission and post-transfusion infection, but horizontal transmission, especially of genotype A, is recently increasing. HBV vaccine is very useful for preventing infection, but about 10% of vaccinated adults cannot get an adequate neutralizing antibody. In addition, some vaccine-escape mutants (VEM) which cannot be controlled by vaccination have been identified. Considering these situations, a novel and more effective HBV vaccine are necessary and expected to be developed. In fact, some candidates which include preS as well as S region of HBV are under study. To develop a more effective vaccine, it is necessary to search sequence of this region of HBV derived from current patients with acute infection and also immune-compromised patients such as HIV co-infected patients. Therefore we extracted DNA from sera of HIV/HBV co-infected patients who visited IMSUT hospital, and cloned preS/S region of HBV for direct sequencing. Among 30 patients with successful analysis, G145A mutation in HBV-S region, which is reported to be VEM, is detected in 2 patients infected with genotype C. In addition, D144A mutation, known as another VEM, is also detected another patient. But all of the patients had no vaccination. The results suggest that VEM actually exist in some patients co-infected with HIV. In the future, we will analyze more patients as well as blood donors who were shown to be HBsAg-positive to examine whether VEM is also present in non-immunocompromised HBV carriers.

### 2. Identification of host factors associated with persistent infection of HBV in patients infected with HIV

**Takeya Tsutsumi, Hidenori Sato[1], Eisuke Adachi[1], Tadashi Kikuchi[1], Michiko Koga, Tomohiko Koibuchi[1], Hiroshi Yotsuyanagi: [1]Department of Infectious Diseases and Applied Immunology, IMSUT hospital, IMSUT**

Due to similar routes of transmission, HBV co-infection is frequently observed in patients infected with HIV. Compared to patients infected with only HBV, patients co-infected with HIV and HBV tend to develop persistent infection followed by advanced liver diseases, but what type of HIV-infected patients is at high risk of persistent HBV infection is not clearly demonstrated. Therefore, we examined HIV/HBV co-infected patients and tried to determine the host factors associated with persistent infection of HBV. Among HIV-infected patients who visited IMSUT Hospital between 1994 and 2017, 734 patients performed HBV-related blood tests and 472 patients (64.3%) were shown to have been infected with HBV. HBsAg sero-positivity was observed in 58 patients, and among them, 28 were cured (disappearance of HBsAg) and 21 continued to have HBsAg until now, while 9 were dropouts. By univariate analysis, cured patients were significantly younger, had higher CD4 and platelet counts, and presented higher transaminase levels after HBV infection. By multivariate analysis, only age is a determinant factor. We also examined SNPs in HLA-DPA1 and HLA-DBP1, which were reported to be associated with chronic HBV infection in non-HIV patients. Although the number of patients examined was small, both HLA SNPs were not associated as far as we examined. Considering these results, host immune status and degree of liver fibrosis are associated the persistent infection of HBV, and younger patients have better immune and hepatic conditions, which may be a reason for a determinant independent factor.

## 3. Suppression of mitophagy by HCV and exploration of drugs to restore mitophagy

**Takeya Tsutsumi, Kazuya Okushin[1], Kyoji Moriya[1], Kazuhiko Koike[2], Hiroshi Yotsuyanagi: [1]Department of Infectious Control and Prevention, Graduate School of Medicine, University of Tokyo, [2]Department of Gastroenterology, Graduate School of Medicine, University of Tokyo**

HCV infection is closely associated with hepatocellular carcinoma (HCC) development, and dysfunction of mitochondria and subsequent reactive oxygen species (ROS) accumulation by HCV, especially the core protein, may contribute to the pathogenesis. In hepatocytes of transgenic mice harboring the core protein, accumulation of morphologically abnormal mitochondria is observed, suggesting that mitophagy is suppressed by the core protein, leading to ROS accumulation. We examined the expression of several mitophagy-related proteins in core-expressing HepG2 cells and found that expression of Bnip3 (BCL2 and adenovirus E1B 19 kDa-interacting protein 3), localized to outermembrane of mitochondria as a mitophagy receptor, is decreased in core-expressing cells. Further analysis revealed that the core protein interacts with Bnip3 and impairs Bnip3 homodimerization and Bnip3-LC 3 interaction (manuscript in preparation). These results suggest that the core protein disrupts Bnip3 function which contributes to suppression of mitophagy, therefore it is possible that if Bnip3 function is restored by some chemicals, mitophagy will be recovered and ROS accumulation will be attenuated. For a screening we used NanoBiT system which detects protein-protein interaction quantitatively. We screened 1540 chemicals provided as FDA-approved drug library and found some chemicals enhanced Bnip3-Bnip3 interaction in the core-expressing cells by this system. Concerning some chemicals, we confirmed increased homodimerization of endogenous Bnip3 in vitro. We are now preparing HCV core-transgenic mice for administration of candidate chemicals to determine the effect in vivo. This study will lead to the future development of drugs for the prevention of HCC accompanied in HCV-infected patients.

## 4. Genetic analysis of hepatitis A virus (HAV)

**Nupur Nigam, Masato Ogishi, Takeya Tsutsumi, Hidenori Sato[1], Eisuke Adachi[1], Tadashi Kikuchi[1], Michiko Koga, Tomohiko Koibuchi[1], Tomoko Kiyohara[2], Ryosuke Suzuki[2], Hideki Aizaki[2], Masamichi Muramatsu[2], Hiroshi Yotsuyanagi: [1]Department of Infectious Diseases and Applied Immunology, IMSUT hospital, IMSUT, [2]Department of Virology II, National Institute of Infectious Diseases**

Since the end of 2017, in metropolitan areas in Japan, acute HAV infection is frequently observed in HIV-infected patients who are MSM (men sex with men). In IMSUT Hospital, we experienced 11 HIV-infected patients diagnosed as acute hepatitis by HAV. We obtained sera from 5 patients with informed consent and cloned HAV gene by PCR. Sequence analysis of VP1/2 region of HAV revealed that all of clones derived from these patients were very similar, belonging to RIVM-HAV16-090 cluster that was prevalent in Taiwan where outbreak of HAV was observed before that in Japan. In the future, we will plan to perform a multicenter study including the identification of factors associated with severe hepatitis.

### 5. Characteristics of Transmitted Drug-Resistant HIV-1 in Recently Infected Treatment-Naive Patients in Japan.

**Michiko Koga, Tomohiko Koibuchi[1], Eisuke Adachi[1], Tadashi Kikuchi, Takeya Tsutsumi, Hiroshi Yotsuyanagi, Yasumasa Iwatani[2], Kazuhisa Yoshimura[3]: [1]Department of Infectious Diseases and Applied Immunology, IMSUT hospital, IMSUT, [2]Clinical Research Center, National Nagoya Medical Center, [3]Nathional institute of infectious diseases**

Progress in antiretroviral treatment has led to fewer virological failure cases, but 10%-20% of treatment-naive HIV/AIDS cases are reported to harbor drug-resistant strains (RS), suggesting transmission of drug-resistant HIV. We have determined the trend in prevalence of transmitted drug-resistant (TDR) HIV in Japan from 2003.

Drug-resistance test had been performed on national-wide HIV-1-infected cases newly diagnosed. The overall prevalence of TDR was about 8%, ranging from 5.2% in 2004 to 10.0% in 2017. The prevalence of RS was significantly higher among cases who were male, Japanese, and men who have sex with men. Common mutations in both groups were M46I/L and T215 revertants. Furthermore, sequences with these mutations, K103N and D30N/N 88D formed clusters on phylogenetic trees. It was suggested that HIV with these mutations have become circulating strains.

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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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*Research Projects*
*We have been working on the following five projects, 1) understanding the role of Wnt/β-catenin signaling pathway in colorectal carcinogenesis, 2) discovery of molecular targeted anticancer drugs through a screening of large-scale chemical libraries, 3) establishment and investigation of mouse models of human cancer, 4) understanding the genetic features of rare cancers and mechanisms of their development, and 5) clinical sequencing for the implementation of genomic medicine. These projects are aimed to develop strategies for better diagnosis, effective treatment, and prevention of human cancer.*

### 1. Understanding the role of Wnt/β-catenin signaling pathway in colorectal carcinogenesis

**Kiyoshi Yamaguchi, Yoichi Furukawa.**

In colorectal cancer, constitutive activation of the Wnt/β-catenin signaling pathway that leads to an increase in β-catenin expression, is frequently observed. The β-catenin associates with Lef/Tcf transcription factors and activates the transcription of multiple genes including *MYC* and *CCND1*. We have also identified the target genes such as *RNF43, SP5, CLDN1, ENC1, APCDD1,* and *FRMD5,* which are up-regulated by the signaling. On the other hand, roles of down-regulated genes by the signaling remain largely unknown. Thus, we utilized transcriptome data of colorectal cancer cells introduced with β-catenin siRNAs or a dominant negative form of TCF7L2 (dnTCF7L2), and explored genes commonly up-regulated by the treatment with β-catenin siRNAs or dnTCF7L2. As a result, we identified interferon-induced protein with tetratricopeptide repeats 2 (*IFIT2*), whose expression was negatively regulated by the Wnt/β-catenin signaling pathway. Consistent with this result, expression of *IFIT2* was significantly lower in colorectal cancer tissues than that in normal tissues. Exogenous IFIT2 expression decreased cell proliferation and increased apoptosis of colorectal cancer cells. Through the analysis of *IFIT2* promoter, we further disclosed that a transcription factor, interferon regulatory factor 1 (IRF1), is involved in the expression of IFIT2 as a downstream of the Wnt/β-catenin signaling pathway. Interestingly, Wnt/β-catenin signaling suppressed the expression of IRF1 by its degradation through the ubiquitination-proteasome pathway. A new class of Wnt target genes that are indirectly or negatively regulated through the Wnt/β-catenin signal activation will provide a novel insight into the regulatory mechanism of Wnt signaling pathway involved in carcinogenesis.

### 2. Cancer drug discovery through a large chemical library screening

**Kiyoshi Yamaguchi, Yoichi Furukawa, Satoru Nagatoishi[1], Kohei Tsumoto[2]: [1]Project Division of Advanced Biopharmaceutical Science, [2]Medical**

**Proteomics Laboratory, IMSUT**

Establishment of well-designed high-throughput screening system is an essential for the identification of small molecules that inhibit a signaling pathway or a molecule of interest. Cell-based assays using TCF/LEF reporter (TOPFLASH), as readout of β-catenin/TCF-dependent transcriptional activity, have contributed to the discovery of small molecules that modulate Wnt signaling. Recently, we developed an efficient cell-based reporter assay system named "bidirectional reporter assay". Integrated transcriptome analysis identified a histidine ammonia-lyase gene (*HAL*) that was negatively regulated by β-catenin/TCF-dependent transcriptional activity. We leveraged a promoter region of *HAL* as another transcriptional readout of Wnt signaling. Cells stably expressing both an optimized HAL reporter and the TOPFLASH enabled bidirectional reporter activities in response to Wnt signaling. Indeed, increased HAL reporter activity and decreased TOPFLASH activity were observed simultaneously in the cells when β-catenin/TCF7L2 was functionally blocked. Most importantly, this method could decrease the number of false positives observed when screening an inhibitor library compared with the conventional TOPFLASH assay. Applying this assay system, we performed a high-throughput screening of Wnt inhibitors using small molecule compound libraries and natural compound libraries. Consequently, we have successfully identified several candidate small molecules that suppress Wnt signaling. We are currently working to elucidate the mode of action of these chemicals using approaches of chemical biology.

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

**Tsuneo Ikenoue, Yoichi Furukawa**

Genetically engineered mice are useful tools for studying human diseases, including cancer. In this project, we have demonstrated that liver-specific expression of oncogenic *Kras* cooperates homozygous *Pten* deletion induced intrahepatic cholangiocarcinoma (ICC) but not hepatocellular carcinoma (HCC) in mice. In contrast, oncogenic *Kras* cooperates with heterozygous *Pten* deletion caused both ICC and HCC, while oncogenic *Kras* expression alone induced only HCC. We are now studying the molecular mechanisms how *Kras* activation and *Pten* deletion induce ICC.

In addition, we are trying to establish novel mouse models of gastrointestinal, liver, and pancreatic cancer using mice carrying a cancer-associated mutant allele of *Fbxw7*, *Idh1/2*, and *Gnas* genes, which are frequently mutated in human cancers. Intensive analysis of these models should provide better understanding of their carcinogenesis and facilitate the development of new therapies to these cancers.

## 4. Elucidation of genetic characteristics of human tumors and mechanisms of their development

**Kiyoko Takane, Kiyoshi Yamaguchi, Tsuneo Ikenoue, Yoichi Furukawa, Rui Yamaguchi[3], Seiya Imoto[2], Satoru Miyano[1,3]: [1]Division of Health Medical Computational Science, [2]Division of Health Medical Data Science, Health Intelligence Center, [3]Laboratory of DNA Information Analysis, Human Genome Center, IMSUT.**

Whole genome and whole exome sequencing have disclosed comprehensive catalogues of various types of human cancer, and identified driver genes and pathways associated with their carcinogenesis. The information is helpful for the profound understanding of tumor biology, the development of novel therapeutic drugs, and the discovery of biomarkers. Pseudomyxoma peritonei (PMP) is a rare disease exhibiting a distinct clinical feature caused by cancerous cells that produce mucinous fluid in the abdominal cavity. Our previous analysis of 18 PMPs containing 10 low-grade tumors and 8 high-grade tumors determined that *KRAS* and/or *GNAS* mutations are common genetic features of PMP. Furthermore, we suggested that mutations in *TP53* and/or genes related to the PI3K-AKT pathway might provide malignant properties to PMP. To comprehensively understand genetic alterations in PMP, we extensively analyzed PMP tumors and matched normal colonic mucosa by the whole genome sequencing and RNA sequencing. Ongoing analysis of genetic and transcriptome data will facilitate the discovery of biomarkers of PMP, selection of effective anti-cancer drugs, and individualized medical care.

## 5. Clinical sequencing for the implementation of genomic medicine

**Kiyoko Takane, Kiyoshi Yamaguchi, Tsuneo Ikenoue, Yoichi Furukawa, Eigo Shimizu[1], Rui Yamaguchi[1], Tetsuo Shibuya[2], Satoru Miyano[1,2], Takanori Hasegawa[3], Seiya Imoto[3], Kazuaki Yokoyama[4], Arinobu Tojyo[4], Koichiro Yuji[5]: [1]Laboratory of DNA Information Analysis, [2]Laboratory of Sequence Analysis, Human Genome Center, [3]Division of Health Medical Data Science, Health Intelligence Center, [4]Division of Molecular Therapy, [5]Division of International Advanced Medical Research, Advanced Clinical Research Center, IMSUT.**

The application of Next-Generation Sequencing

(NGS) technology in clinical medicine has revolutionized molecular diagnostics by enabling multiple gene testing, or analysis of the entire exon or whole genome with a limited amount of DNA. In collaboration with Human Genome Center, Health Intelligence Center, and Advanced Clinical Research Center, we have been working on the determination of germline mutations in patients suspected of hereditary colon tumor and application of a cognitive computing system for the personalized medicine.

We have applied NGS technology for unexplained cases with familial colon cancer with adenomatous polyps. Whole genome sequencing successfully identified rare pathological mutations in the *APC* gene including mosaicism, a very rare mutation in the 3' terminal region, and a deletion in the promoter region of ~10 kb. In addition, we recently found a germline frameshift mutation in *POLE* in a patient with multiple adenomatous polyps and three synchronous colon cancers, which led to a genetic diagnosis of polymerase proofreading associated polyposis (PPAP). The precise understanding of genetics of inherited cancer is important for the patient's cancer surveillance, the identification of at-risk family members, and the development of therapeutic approaches and prevention strategies.

In the second project, we have been testing interpretation of genomic data using IBM Watson for Genomics (WfG). After written informed consent was obtained from the patients with colorectal, breast, uterine, gallbladder, pancreatic cancer, lymphoma, and hepatoblastoma, they were enrolled in this study. Genetic alterations in their tumors were determined by NGS and the data were subsequently analyzed by WfG. The results of NGS and interpretation of WfG are reviewed at the meeting of molecular tumor board, which consists of physicians, medical oncologists, genetic counsellors, geneticists, bioinformaticians, and experts of ethics.

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


| | | | | | |
|---|---|---|---|---|---|
| Professor | Tomoki Todo, M.D., Ph.D. | 教　授 | 博士(医学) | 藤　堂　具　紀 |
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| Senior Assistant Professor | Hiroyuki Momota, M.D., Ph.D. | 講　師 | 博士(医学) | 百　田　洋　之 |
| Assistant Professor | Lushun Chalise, M.D., Ph.D. | 助　教 | 博士(医学) | チャリセ　ルシュン |
| Assistant Professor | Yoshinori Sakata, M.D., Ph.D. | 助　教 | 博士(医学) | 坂　田　義　詞 |



*The major research topic of our laboratory is to develop oncolytic virus therapies 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. Three clinical trials using G47Δ are currently being conducted at IMSUT Hospital.*


### 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 rapid generation of multiple new recombinant HSV-1 with desired sequences inserted into a specific locus.

Application of oncolytic 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, malignant mesothelioma, tongue cancer, esophageal cancer, gastric cancer, colon cancer, lung cancer, breast cancer, nasopharyngeal cancer, cholangiocarcinoma, hepatic cancer, pancreatic cancer, malignant melanoma, and malignant lymphoma have also been used for testing efficacy and safety.

### Studies using surgical specimen derived cancer stem cells

There exists a small population of tumor-initiating, stem-like cells within the tumor. Because can-

cer 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. Novel oncolytic HSV-1 that exhibit high efficacy for tumors rich in CSCs have been created and are being evaluated.

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# 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　博士(医学) | 長　村　文　孝 |
| Associate Professor | Masanori Nojima, M.D., Ph.D., M.P.H. | 准教授　博士(医学) | 野　島　正　寛 |



*Division of Advanced Medicine Promotion was established in 2011. Our mission is to assist the clinical development and the conduct of clinical trials, especially for translational researches. For this purpose, it is critical to discover new "seeds" and to eradicate blockades until the clinical utilization. In this sense, our role is the translation from the results of basic research to the conduct of clinical trials at IMSUT Hospital. At IMSUT Hospital, we work together with staffs of Center for Translational Research. Concurrently, to concur blockades on translational researches, we engage in research on Regulatory Science.*


### 1. Assistance of Clinical Trials/TRs at Research Hospital

**Minako Kouno, Riyo Owada, Masanori Nojima, Fumitaka Nagamura**

In Research Hospital, we work together with staffs of Center for Translational Research. 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 Center for Translational Research. In 2014, we supported 4 investigator-initiated investigational new drug application (IND) clinical trials and 2 non-IND clinical studies.

### 2. Scholastic Program for the Graduate Students of Nurses in the Area of Translational Research.

**Minako Kouno, Riyo Owada, 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 planned 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.

### 3. Management of "Translational Research Network Program" of Japan Agency for Medical Research and Development.

**Makiko Karasawa, Hiroshi Yasui, Fumitaka Nagamura**

Ministry of Education, Culture, Sports, Science and Technology launched "Translational Research Network Program" to promote translational researches based on the results of basic science at academia. This grogram was transferred to Japan Agency for Medical Research and Development in 2015 and has been expected to support TRs from basic science to seek obtaining intellectual property to early stage of clinical trial. In 2018, we supported 22 basic researches, 15 preclinical studies, and 10 clinical studies. The number of studies we assist has been increasing year by year. Organization reinforcement is the urgent problem.

### 4. Approach for epigenome and multi-omics research by methodology of bioinformatics and biostatistics

**Masanori Nojima**

Epigenome and multi-omics research using clinical samples in collaborative study or public database of comprehensive omics-analysis. We are now focusing on the multi-omics approach integrating DNA methylation, mRNA expression, and miRNA, and building statistical models to assess functional linkage.

### 5. Statistical consulting for basic research

**Masanori Nojima**

For basic researchers, we suggest exploratory statistical approach and molecular epidemiological approach.

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# Division of Advanced Genome Medicine
## 先端ゲノム医学分野

Associate Professor　Yoshihiro Hirata, M.D., Ph.D.　准教授　博士(医学)　平　田　喜　裕

*The goals of our researches are to identify the mechanisms and to establish novel therapies especially for cancers and inflammatory diseases of the digestive system. One of the research fields is the inflammatory diseases, in which we investigated the molecular pathogenesis of gastritis, cholangitis and inflammatory bowel disease. Another research field is the malignancies. Using genetically engineered mice, we have elucidated the carcinogenic mechanisms driven by gene mutations.*

### 1. Pathogenesis of squamo-columnar junction cancer of the stomach

**Yoshihiro Hirata**

Squamo-columnar junction (SCJ) is one of the transitional zones in body where two different cell types merge. Barrett's adenocarcinoma and squamous cell carcinoma are two major tumors found in human gastric SCJ. The origin of SCJ tumors and the process of tumorigenesis are largely unknown. Using mouse models and lineage tracing, we try to identify cancer initiating cells as well as stem cells specific to gastric SCJ.

### 2. Dendritic cells as a therapeutic target for inflammatory bowel diseases

**Sozaburo Ihara[2], Yoshihiro Hirata, Yohko Hikiba[2], Aya Yamashita[1], Moyo Tsuboi[1], Masahiro Hata[1], Mitsuru Konishi[1], Nobumi Suzuki[2], Kosuke Sakitani[2], Hiroto Kinoshita[1], Yoku Hayakawa[1], Hayato Nakagawa[1], Hideaki Ijichi[1], Keisuke Tateishi[1], Kazuhiko Koike[1]: [1]Department of Gastroenterology, The University of Tokyo, [2]Division of Gastroenterology, Institute for Adult Diseases, Asahi Life Foundation**

Disturbance of intestinal homeostasis is associated with the development of inflammatory bowel disease (IBD), and TGF-β signaling impairment in dendritic cells (DCs) causes murine colitis with goblet cell depletion. We examined an organoid－DC co-culture system to study the role of DCs in intestinal epithelial differentiation and homeostasis. Intestinal organoids co-cultured with $CD11c^+$ lamina propria leukocytes or BMDCs from *CD11c-cre* $Tgfbr2^{fl/fl}$ mice showed morphological changes and goblet cell depletion with Notch signal activation, analogous to *CD11c-cre* $Tgfbr2^{fl/fl}$ colitis. E-cadherin was upregulated in $CD11c^+$ DCs. E-cadherin-mediated BMDC adhesion promoted Notch activation and cystic changes in organoids. Anti-E-cadherin antibody treatment attenuated colitis in *CD11c-cre* $Tgfbr2^{fl/fl}$ and T-cell-transferred mice. In addition, E-cadherin deletion in $CD11c^+$ cells attenuated colitis in both *CD11c-cre* $Tgfbr2^{fl/fl}$ and DSS-treated mice. E-cadherin-mediated DC－epithelium adhesion is associated with the development of colitis, and blocking these adhesions may have therapeutic potential for IBD.

### 3. Mechanism of gastric metaplasia development

**Hiroto Kinoshita, Yoku Hayakawa, Mitsuru Konishi, Masahiro Hata, Mayo Tsuboi, Yuki Hayata, Yoko Hikiba, Sozaburo Ihara, Hayato Nakagawa,**

**Tsuneo Ikenoue[3], Tetsuo Ushiku[4], Masashi Fukayama[4], Yoshihiro Hirata, Kazuhiko Koike: [3]Division of Clinical Genome Research, IMSUT, [4]Department of Pathology, The University of Tokyo**

Gastric metaplasia is recognized as a precursor of intestinal type gastric cancer. Its origin and pathogenesis is not defined so far. We examined the role of chief cells in this process using genetic or chemical ablation mouse model, which clearly demonstrated not mature chief cell, but stem/progenitor cell located in isthmus and neck region, is responsible for metaplasia formation. To examine the molecules involved in gastric metaplasia, we developed stomach epithelial cell specific genetic modification mouse (TFF1-cre mouse), which enabled gene manipulation predominantly in gastric pit cell lineage. Kras activation or Pten inactivation in gastric pit cell lineage leads to foveolar hyperplasia and metaplastic change of gastric gland. When cdh1 gene encoding E-cadherin is inactivated in gastric pit cells, transient signet ring cell formation and spontaneous pit cell shedding in gastric gland lead to metaplastic squamous cell expansion from squamo-columnar junction.

## 4. Molecular mechanism of the development and the progression of sclerosing cholangitis and biliary cancer

**Yoshihiro Hirata, Hayato Nakagawa, Yoku Hayakawa, Hiroto Kinoshita, Keisuke Tateishi, Kazuhiko Koike**

Primary sclerosing cholangitis is a rare form of biliary inflammation which can progress to cirrhosis and cancer. The cause of the disease is not clarified so far. Previously we have developed mouse models which mimic primary sclerosing cholangitis and extrahapatic cholangiocarcinoma by deletion of CDH1 gene in biliary tract and analyzed the mechanism of diseases induced by loss of E-cadherin. However the pathogenesis of these diseases still remains largely unsolved. Especially, the life style factors which affect the severity and the progression of biliary diseases are not well understood, and the mechanisms of disease modification are not clarified. We are currently investigating the effects of life style factors, such as smoking and obesity, on cholangitis and biliary cancer using mouse models.

## 5. Pathogenesis of of primary biliary cholangitis and novel therapy development targeting T cells

**Ryo Nakagawa[5], Yoshimi Kaise, Ryosuke Muroyama[5], Yasuo Matsubara[6], Naoya Kato[5], Yoshihiro Hirata: [5]Department of Gastroenterology, Chiba University, [6]Department of General Medicine, Research Hospital, IMSUT**

Primary biliary cholangitis (PBC) is an autoimmune liver disease, but the causes are unknown. We performed comprehensive expression analysis of mRNA and microRNA of T cells from PBC. Four microRNAs were identified as being decreased in PBC patients, leading to activation of T cell receptor signaling pathways, involved in inflammation. One particular target, N-Ras, could be an attractive and novel immunotherapeutic option for PBC. We are currently investigating the effect of Ras inhibitors as the potential novel therapy for PBC. We have screened the effect of Ras inhibitors using IL-2 promoter reporter cells. We also investigated cytokine production from T cells after Ras inhibitor treatment.

## 6. The role of fusion HBx from HBV integrant in the hepatocarcinogenesis

**Ryosuke Muroyama, Naoya Kato, Yoshihiro Hirata**

We identified fusion HBx translated from HBV integrant in human hepatocellular carcinoma cell line. The fusion HBx consisted of 1-140 amino acids of HBx followed by 61 amino acids from human genome. In KD cells, cell proliferation, invasion ability as well as tumor formation in nude mice, were significantly reduced compared to the parental cells. The fusion HBx had anchorage-independent growth ability in soft agar although the fusion HBx completely abrogated its transactivation ability. We also found that the fusion HBx dysregulated ER stress response via the modification of ATF3, ATF4, and ATF6 transcription. Interestingly, the effects of the fusion HBx on ER stress signaling pathway was similar to those of C-terminal truncated HBx but significantly different from those of wild HBx.

## 7. Novel zinc finger protein in gastrointestinal tract

**Yasuo Matsubara, Yoshihiro Hirata**

The gastrointestinal tract has definite anatomical and functional boundaries between its contiguous segments. Some genetic markers that delimit gastrointestinal boundaries have been reported, but it is still unknown how such boundaries are established and maintained. We identified novel zinc finger protein in the gastric biopsy specimen by mass spectrometry. Its mRNA sequence was determined by RACE. Currently we are analyzing the molecular function of this novel zinc finger protein.

## Publications

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*Advanced Clinical Research Center*

# Division of Bioethics
## 生命倫理研究分野

Associate Professor　Ayako Kamisato, Ph.D.

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*Division of Bioethics is a new laboratory established in 2017.*
*New ethical, legal and social issues (ELSI) may occur when conducting advanced clinical research or clinical practice. In our laboratory, we study about how and what decisions should be made by a nation, society, or individual when such issues arise.*

### 1. The REC Education project

**Ayako Kamisato, Kaori Muto[1], Fumitaka Nagamura[2], Kazuyo Arisawa: [1]Department of PublicPolicy, Human Genome Center, [2]Division of Advanced Medicine Promotion, Advanced Clinical Research Center**

Currently, there are more than 1,800 institutional Research Ethics Committees (RECs) in Japan. Since 2010, cases of research fraud have come to light (e. g., the scandal around the Novartis drug Diovan) and improving the quality of reviews by REC has become the need of the hour. Therefore, Japanese ethical guidelines regarding medical studies involving humans now mandate that institutions with established RECs should offer education and training programs to REC members at least once a year. However, the guidelines do not make any provisions regarding the contents of programs and the way to deliver. As implementation of programs require manpower and economic resources, most institutions are unable to provide high-quality education and training. To address this situation, we launched the REC Education project with support from the Japan Agency for Medical Research and Development (AMED) from FY 2016.

Our programs have the following salient features: 1) programs are animated, 2) in order to offer the learners how to review from their place, we created four characters: two experts in natural science and law, a lay member, and a secretariat, 3) each program has a subject of discussion, 4) an external expert committee evaluates each program prior to release, 5) each program is about 20 minutes long, 6) the programs are offered at no charge on the website, 7) REC which successfully complete the program receive a certificate of completion.

We have produced and released the following video programs on our website:

- Module 1. Revision of the Privacy Act
- Module 2. Procedure of Informed Consent for using human samples and information
- Module 3. Why REC is necessary? What is the role of each REC member?
- Module 4. Checklist for Effective Reviewing
- Module 5. Invasive Research and Interventional Study
- Module 6. Basic knowledge of clinical trials 1
- Module 7. Basic knowledge of clinical trials 2
- Module 8. Basic knowledge of clinical trials 3
- Module 9. Outline of "Clinical Research Act"
- Module 10. The points for reviewing on Informed Consent 1
- Module 11. The points for reviewing on Informed Consent 2
- Module 12. The points for reviewing on Informed Consent 3

- Module 13. The points for reviewing on Informed Consent 4
- Module 14. Handling of personal information

Currently, we have more than 920 members and 440 institutions registered with our project. We constantly assess our programs through questionnaires to get user feedback on each program. We have consistently received high scores from our users.

### 2. Large-scale survey on "lay person member" of Research Ethics Committees

**Ayako Kamisato, Kaori Muto[1]: [1]Department of PublicPolicy, Human Genome Center,**

We conducted survey on "lay person member" of Research Ethics Committees to know the actual condition, such as how they became a member of REC, how they feel about their role, or what kind of education they were provided before being a member. We sent out questionnaires to 1408 committees and got 538 answers (38%). We obtained important findings from this survey such as more than 60% people were not received any education before beginning their first review.

### 3. Policy making of human-animal chimeric embryos research

**Ayako Kamisato**

In Japan, there are guidelines called "Guidelines on the Handling of Specified Embryos "based on the Act on Regulation of Human Cloning Techniques. In these guidelines, there are some limitation such as; 1) the production of animal-human chimeric embryos shall be carried out only for the purpose of basic research for the production of human cell-derived internal organs that can be transferred to human body, 2) animal-human chimeric embryos shall be carried out limited to a period until a primitive streak appears or 14 days from the date of the embryos being produced if such primitive streak does not appear, 3) chimeric embryos may not be transferred to human or animal uterus.

Following the recent great scientific achievements in this field, discussions have started in the Ministry of Education, Culture, Sports, Science and Technology (MEXT) since 2013. Dr. Kamisato participated in these discussions as a member of the council and contributed for policy making.

### 4. Policy making of research using genome editing technology for human embryos

**Ayako Kamisato**

The Expert Panel on Bioethics of Council for Science, Technology and Innovation at Cabinet Office set research using genome editing technology for human embryos as an agenda. Dr. Kamisato participated as a member of the Expert Panel and contributed for policy making. Dr. Kamisato is also a member of the Council on research using genomic editing technology for human fertilized embryos set by the Ministry of Education, Culture, Sports, Science and Technology (MEXT) and Health, Labour and Welfare Ministry (MHLW) and contributed for developing guideline.

### 5. Production of Common IC Form for "Center of Healthy Aging Innovation project"

**Ayako Kamisato, Kazuyo Arisawa**

"Center of Healthy Aging Innovation project" promoted by Hirosaki University is one of the projects of JST Center of Innovation (COI) Program. One goal of this project is to build a platform of big data on medical and health. In order to achieve this goal, it is necessary to integrate data obtained from multiple cohort studies. In order to accelerate data integration, Dr. Kamisato produced a common IC form.

## Publications


・神里彩子．ゲノム編集と生殖医療―「ヒト受精胚に遺伝情報改変技術等を用いる研究に関する倫理指針（案）」策定までの経緯と概要．遺伝医学27号．メディカルドゥ，2018.

・神里彩子．第８章　研究対象者の個人情報保護のための基礎知識．超簡単！！研究倫理審査と申請～適正な臨床・疫学研究の推進に向けて～．飯嶋久志，氏原淳，内田直樹，神里彩子，佐藤愛美編集．薬事日報社. 2018.

・神里彩子．出生前診断，特にNIPTは社会にどのような影響を与えるか．倫理的に考える医療の論点．浅井篤・小西恵美子・大北全俊編集．日本看護協会出版会．2018.