Center for Stem Cell Biology and Regenerative Medicine

Division of Stem Cell and Molecular Medicine
幹細胞分子医学分野

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Stem cells have the remarkable capacity to both self-renew and give rise to many types of more specialized cells in the body, which explains their great therapeutic potential in regenerative medicine. But that's not the only reason stem cells have become such a hotbed of scientific inquiry. These cellular transformers also offer an invaluable research tool for probing the disease mechanisms that underpin cancer, aging and a host of other health problems. Our major interest is to elucidate the mechanisms of self-renewal and multi-lineage differentiation of hematopoietic stem cells (HSCs). We are also interested in how the deregulated HSC functions are associated with aging of our body and the development of age-related hematological malignancies. We approach these issues mainly from the view point of epigenetics.

1. The impact of hematopoietic insults on bone marrow niche

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Hematopoietic stem cells (HSCs) are exposed to various insults, such as genotoxic stress, inflammation, and infection, which directly affect HSCs. These insults deplete HSCs, cause a functional decline in HSCs, and promote their aging and transformation. However, the impact of hematopoietic insults on niche cells remains largely unknown. We have previously reported that p53 is activated in blood vessels by various stresses, including hypoxia, inflammation, and aging, and contributes to tissue dysfunction and metabolic abnormalities. We hypothesized that hematopoietic insults also affect bone marrow vascular niche. Indeed, we found that p53 becomes activated in BM endothelial cells upon hematopoietic stresses such as irradiation and chemotherapeutic treatments. The conditional activation of p53 in VE-cadherin^{+} vascular niche cells by deleting *Mdm2* induced the expression of p53 target genes specifically in vascular endothelial cells, resulting in the dilation and collapse of vascular endothelial cells and reductions in perivascular mesenchymal stromal cell numbers. Consequently, HSCs failed to maintain dormancy, mobilized to the periphery, and were significantly depleted. Our results indicate that various hematopoietic insults affect HSCs not only directly, but also indirectly by altering vascular integrity, which is critical for perivascular niche formation and the maintenance of HSCs.

2. Epigenetic dysregulation in the pathogenesis of myelodysplastic syndrome

Shiro Tara[1,2], Yusuke Isshiki[1], Kazumasa Aoyama[1], Yaeko Nakajima-Takagi, Motohiko Oshima, Tomoyuki Tanaka[1,3], Daisuke Shinoda[1], Shuhei Koide[1], Atsunori Saraya[1], Satoru Miyagi[1], Ichiro Manabe[1], Hirotaka Matsui[2], Haruhiko Koseki[4], Vivian J. Bardwell[5], and Atsushi Iwama: [1]Graduate School of Medicine, Chiba University, [2]Kumamoto University Graduate School of Medicine, [3]Niigata University, [4]RIKEN Research Center for Integrative Medical Sciences, [5]Masonic Cancer Center and Developmental Biology Center, University of Minnesota.

BCOR is a co-repressor for BCL6, a key transcriptional factor required for the development of germinal center (GC) B cells. Recent extensive analyses of the BCOR complex revealed that BCOR also functions as a component of PRC1.1, a non-canonical PRC1, which monoubiquitinates histone H2A. *BCOR* is X-linked and targeted by somatic mutations in various hematological malignancies including myelodysplastic syndrome (MDS). In order to understand the pathophysiological functions of *BCOR* mutants in hematological malignancies, we analyzed mice lacking *Bcor* exons 9 and 10 ($Bcor^{\Delta E9-10/y}$), which express a carboxyl-terminal truncated BCOR that fails to interact with core effector components of polycomb repressive complex (PRC) 1.1. $Bcor^{\Delta E9-10}$ hematopoietic cells showed a growth advantage in the myeloid compartment that was further enhanced by the concurrent deletion of *Tet 2*. $Tet2^{\Delta/\Delta}Bcor^{\Delta E9-10}$ mice developed lethal MDS with progressive anemia and leukocytopenia, inefficient hematopoiesis, and the morphological dysplasia of blood cells. $Tet2^{\Delta/\Delta}Bcor^{\Delta E9-10}$ MDS cells reproduced MDS or evolved into lethal MDS/MPN in secondary recipients. Transcriptional profiling revealed the de-repression of myeloid regulator genes of the *Cebp* family and *Hoxa* cluster genes in $Bcor^{\Delta E9-10}$ progenitor cells and the activation of p53 target genes specifically in MDS erythroblasts where massive apoptosis occurred. Our results reveal a tumor suppressor function of BCOR in myeloid malignancies and highlight the impact of *Bcor* insufficiency on the initiation and progression of MDS.

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Center for Stem Cell Biology and Regenerative Medicine

Division of Stem Cell Transplantation
幹細胞移植分野

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We are conducting clinical stem cell transplantation, especially using cord blood as a promising alternative donor for clinical use and investigating optimal strategies to obtain the best results in this area. We are also generating pre-clinical study to utilize virus-specific CTL for immune competent patients such as post-transplantation. Our goal is as allogeneic transplantation to be safer therapeutic option and to extend for older patients.

1. The prognostic impact of pretransplant inflammatory and nutritional status in adult patients after myeloablative single cord blood transplantation.

Miyashita E[1], Konuma T[2], Kataoka J[1], Oiwa-Monna M[2], Mizusawa M[2], Isobe M[2], Kato S[2], Sato T[1], Takahashi S[2,3], Tojo A[2,3]: [1]Department of Nursing, IMSUT Hospital, [2]Department of Hematology/Oncology, IMSUT Hospital, [3]Division of Stem Cell Transplantation

The markers of inflammatory and nutritional status, such as the controlling nutritional status (CONUT) score, prognostic nutritional index, Glasgow prognostic score, and C-reactive protein-albumin ratio (CAR) has been demonstrated to be associated with poor prognosis in patients with various cancers. Although the relatively low cell dose of a single cord blood unit restricts the indication for cord blood transplantation (CBT) to pediatric and relatively smaller and lighter adult patients, the impact of malnutrition on transplant outcomes after CBT is unclear. We retrospectively analyzed 165 adult patients who received myeloablative single-unit CBT in our institute. In multivariate analysis, a higher CONUT score, which is indicative of poor inflammatory and nutritional status, was significantly associated with poor outcomes including low neutrophil engraftment, and development of extensive chronic graft-versus-host disease. A higher CAR, which is also suggestive of poor inflammatory and nutritional status, was significantly associated with poor neutrophil engraftment and higher overall mortality. Body mass index (BMI) was not associated with transplant outcomes. These data suggest that poor pretransplant inflammatory and nutritional status, rather than lower BMI, might be a practical evaluation method of predicting transplant outcomes after single CBT for adults.

2. Risk factors and survival impact of readmission after single-unit cord blood transplantation for adults.

Yamagishi Y[1], Konuma T[2], Miwa Y[1], Oiwa-Monna M[2], Tanoue S[2], Isobe M[2], Jimbo K[2], Narita H[1], Kobayashi K[1], Kato S[2], Takahashi S[2,3], Tojo A[2,3]: [1]Department of Nursing, IMSUT Hospital, [2]Department of Hematology/Oncology, IMSUT Hospital, [3]Division of Stem Cell Transplantation

Hospital readmissions have been used as a prognostic indicator for patients receiving allogeneic hematopoietic cell transplantation (HCT). However, the impact of readmission during early and mid-

phase of cord blood transplantation (CBT) on long-term outcomes has not been fully investigated. We retrospectively analyzed 156 adult patients who received single-unit CBT in our institute. Among this cohort, thirteen patients (8%) were readmitted within 30 days after discharge, and 27 (17%) were readmitted within 90 days after discharge. The most common causes for readmission within 30 and 90 days of discharge were infection, chronic graft-versus-host disease, and relapse. Higher cryopreserved cord blood $CD34^+$ cell count was only significantly associated with lower readmission within 90 days after discharge. The probabilities of overall survival were significantly lower in patients readmitted within 90 days after discharge compared with those who were not readmitted within 90 days after discharge in univariate and multivariate analysis. These data suggest that readmission within 90 days after discharge may have a significant impact on long-term mortality after single-unit CBT.

3. Reduced-toxicity myeloablative conditioning consisting of fludarabine/ busulfan/low-dose TBI/G-CSF-combined cytarabine in single cord blood transplantation for elderly patients with non-remission myeloid malignancies.

Konuma T[1], Kato S[1], Isobe M[1], Mizusawa M[1], Oiwa-Monna M[1], Takahashi S[1,2], Tojo A[1,2]: [1]Department of Hematology/Oncology, IMSUT Hospital, [2]Division of Stem Cell Transplantation

The optimal intensity of a conditioning regimen might be dependent on not only age and comorbidities but also disease activity and the type of graft source. We evaluated the outcome of unrelated single cord blood transplantation (CBT) using a conditioning regimen of fludarabine 180 mg/m^2, i. v. busulfan 9.6 mg/kg, 4 Gy total body irradiation, granulocyte colony-stimulating factor-combined high-dose cytarabine (12 g/m^2) in 23 elderly patients (median, 64 years) with nonremission myeloid malignancies between 2013 and 2018 in our institution. All but 1 patient achieved neutrophil engraftment at a median of 23.5 days (range, 18 to 50). With a median follow-up of 28 months, the probabilities of overall survival (OS), disease-free survival (DFS), and cumulative incidence of relapse at 2 years were 62%, 52%, and 26%, respectively. The cumulative incidences of nonrelapse mortality at 100 days and 2 years were 9% and 22%, respectively. In the univariable analysis a higher proportion of blasts in bone marrow and in peripheral blood and a monosomal or complex karyotype were significantly associated with inferior OS and DFS. Poor cytogenetics were significantly associated with inferior DFS and increased relapse incidence. These data demonstrate that this reduced-toxicity myeloablative conditioning regimen was tolerable and effective in terms of engraftment, relapse, and survival in single CBT for elderly patients with nonremission myeloid malignancies.

4. Platelet Transfusion Refractoriness in Single-Unit Cord Blood Transplantation for Adults: Risk Factors and Clinical Outcomes.

Tanoue S[1], Konuma T[1], Kato S[1], Oiwa-Monna M[1], Isobe M[1], Jimbo K[1], Takahashi S[1,2], Tojo A[1,2]: [1]Department of Hematology/Oncology, IMSUT Hospital, [2]Division of Stem Cell Transplantation

Platelet transfusion refractoriness (PTR) is frequently observed after allogeneic hematopoietic cell transplantation (HCT). However, the incidence of and risk factors for PTR, and impact of PTR on transplant outcomes after cord blood transplantation (CBT) have not been fully investigated. We retrospectively analyzed 185 adult patients who received single-unit CBT in our institute. The mean 16-hour corrected count increment (CCI) for the 5840 platelet transfusions was 3.68×10^9/L. Among them, 3196 transfusions (54.7%) were associated with a PTR with 16-hour-CCI $<4.5 \times 10^9$/L. Results of multivariate analysis indicated that the following factors were significantly associated with decreased platelet transfusion responses: female sex with pregnancy history, male sex, the presence of HLA class I antibody, lower cord blood total nucleated cell dose, lower cord blood $CD34^+$ cell dose, 3 locus HLA disparities, body temperature ≥38℃, C-reactive protein ≥10 mg/dL, cytomegalovirus reactivation, use of foscarnet, and use of liposomal amphotericin B. By contrast, graft-versus-host disease prophylaxis including methotrexate, ABO minor mismatch, use of ganciclovir, and use of linezolid were significantly associated with better platelet transfusion responses. PTR had a significant effect on poor neutrophil and platelet recovery, and overall mortality after CBT. These data suggest that early phase PTR may be predictive of engraftment and mortality after single-unit CBT for adults.

5. Circulating monocyte subsets and phenotypes in chronic graft-versus- disease.

Konuma T[1], Kohara C[2], Watanabe E[3], Mizukami M[4], Nagai E[4], Tanoue S[1], Isobe M[1], Jimbo K[2], Kato S[1], Ohno N[1], Takahashi S[1,2], Tojo A[1,2]: [1]Department of Hematology/Oncology, IMSUT Hospital, [2]Division of Molecular Therapy, [3]IMSUT Clinical Flow Cytometry Laboratory, [4]Department of Laboratory Medicine, IMSUT Hospital

Chronic graft-versus-host disease (cGVHD) is a major cause of late morbidity and mortality after allogeneic hematopoietic cell transplantation (HCT).

Monocytes/ macrophages play a central role in inflammation, tissue repair, and fibrosis, which are the main clinical features of cGVHD. Here, we examined the expression levels of activation markers, chemokine receptors, and scavenger receptors for each circulating monocyte subset in 145 patients without disease recurrence at least 12 months after undergoing allogeneic HCT. There were no significant differences in the numbers and the proportions of each monocyte subset between patients without cGVHD and those with mild or moderate/severe cGVHD. Lower expression of CCR5 on classical monocytes, and higher expression of CD204 and lower expression of CX_3CR1 on non-classical monocytes were associated with joint, and lung cGVHD, respectively. These data showed that alterations of activation markers and chemokine and scavenger receptors in each circulating monocyte subset were associated with the development of organ-specific cGVHD. Alterations of surface markers in each circulating monocyte subset may be candidate biomarkers for cGVHD.

Publications

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Center for Stem Cell Biology and Regenerative Medicine

Division of Stem Cell Processing
幹細胞プロセシング分野

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Stem cells are well recognized as a key player in the field of regenerative medicine. Hematopoietic stem cells represent a valuable cell source for transplantation medicine, with which various diseases including primary immunodeficiency and hematologic malignancies can expect life-long cure by reconstitution of healthy hematopoiesis. Our eventual goal is to establish safe and efficacious transplantation strategies in a form of either allogeneic transplantation or gene therapy using autologous hematopoietic stem cells after gene correction.

1. Status of KRAS in iPS cells impacts upon self-renewal and differentiation propensity

Kenji Kubara, Kazuto Yamazaki, Yasuharu Ishihara, Huan-Ting Lin, Ken Nishimura, Manami Ohtaka, Mahito Nakanishi, Masashi Ito, Kappei Tsukahara, Masatoshi Takagi, Makoto Otsu

Oncogenic KRAS mutations in hematopoietic stem cells cause RAS-associated autoimmune lymphoproliferative syndrome-like disease (RALD). KRAS plays essential roles in stemness maintenance in some types of stem cells. However, its roles in pluripotent stem cells (PSCs) are poorly understood. Here, we investigated roles of KRAS on stemness in the context of induced PSCs (iPSCs). We used KRAS mutant (G13C/WT) and wild-type isogenic (WT/WT) iPSCs from the same RALD patients, as well as wild-type (WTed/WT) and heterozygous knockout (Δed/WT) iPSCs, both obtained by genome-editing from the same G13C/WT clone. Compared with wild-type iPSCs, G13C/WT iPSCs displayed enforced retention of self-renewal and suppressed capacity for neuronal differentiation, whilst Δed/WT iPSCs showed normalized cellular characteristics similar to those of isogenic WTed/WT cells. The KRAS－ERK pathway, but not the KRAS－PI3K pathway, was shown to govern these G13C/WT-specific phenotypes, indicating the strong impact of the KRAS－ERK signaling upon self-renewal and differentiation propensity in human iPSCs.

2. Enhanced selective inhibition of KRAS mutant hematopoietic progenitor cell expansion by MEK and Bcl-2 inhibition.

Huan-Ting Lin, Kenji Kubara, Kazuto Yamazaki, Masatoshi Takagi, Takuya Naruto, Tomohiro Morio, Takashi Okumura, Makoto Otsu

Amidst the complex genetic landscape of cancer, the mutagenic and oncogenic effects caused by RAS mutations alone can be overlooked particularly in the context of hematopoietic malignancies. Therefore identifying drugs that could modulate aberrant RAS-MAPK signaling is clinically important. Here we demonstrate the utilization of an iPS cell-based system that could be adapted for compound screening to identify inhibitors with selective inhibition activity against the expansion of KRAS-mutant hematopoietic progenitor cells (HPCs).

RAS-associated autoimmune lymphoproliferative disorder (RALD) is a leukemia-like disease caused by a single acquired RAS mutation. In this study, BM CD34＋ cells from RALD patients were reprogrammed into iPS cells. KRAS-mutant cells could be obtained without necessitating the use of gene

editing techniques. Non-mutant cells were used as isogenic controls thus permitting the assessment of unwanted toxicity against healthy cells. Constitutive activation of the RAS-MAPK pathway was confirmed by western blotting in mutant iPS cells. As expected, whole exome sequencing (WES) showed that mutant KRAS (G13C) was the only mutation present in these cells. Therefore, the inherent characteristics of mutant cells expansion can be attributed to this mutation only.

Previously we reported using these patient-derived iPS cells that oncogenic KRAS enforces retention of self-renewal in human pluripotent cells (Kubara et al. Stem Cell Reports. 2018) due to constitutive activation of the KRAS-MAPK signaling pathway. Of note, mutant iPS cells retained pluripotency marker expression even in the absence of bFGF, but not isogenic control cells. When we tested compounds with different mechanisms of action, direct MEK inhibition was the most effective in triggering a loss in pluripotency marker expression in mutant iPS cells in the absence of bFGF. This method permits rapid determination of compounds that could modulate aberrant KRAS-MAPK signaling. However, further verification is required to determine whether inhibitory effects on mutant HPC expansion could also be observed.

Next, differentiated multi-potent (Lin-34 + 43 +) HPCs from iPS cells were expanded in "standard" ex vivo culture conditions using SCF, TPO Flt3L and IL-3. This favored the expansion of control cells. We also found "selective" culture conditions that could selectively expand mutant HPCs. RNA-seq analysis on pre-expanded and expanded HPCs revealed the unique molecular signature of expanding mutant cells, meaning genes that showed upregulation in both "standard" and "selective" culture conditions. There was upregulation of CCND1 and CDK4 in mutant HPCs, which facilitates progression through the G1 phase of the cell cycle. This was also accompanied by upregulation p16INK4a and p21Cip1, which is characteristic of senescent cells. Indeed, it could be shown that expanded mutant HPCs acquired senescence features such as increased p16 INK4a and Bcl-xL expression at the protein level and increased SA-b-gal activity compared with control cells. Gene correction of KRAS (G13C) using CRISPR/Cas9 reversed these phenotypes. Therefore CCND1/CDK4 and Bcl-2 may be potential inhibitory targets.

We developed a high-throughput screening method whereby differentiated HPCs were sorted directly into 96-well plates and following expansion, cell numbers were quantified by luminescence detection of ATP. First we screened drugs for selective activity, where the IC50 value for mutant cells is lower than control cells. Consistent with iPS cell pluripotency assay, we identified Trametinib (MEK), as having selective effects against mutant HPCs in addition to Palbociclib (CCND1/CDK4) and Navitoclax (Bcl-2). When used in combination, the selective inhibitory effect was enhanced without compromising the viability of control cells.

To test these drugs in an in vivo setting, highly purified transduced (KRAS or control) mouse HSCs (Lin-CD34-KSL) were transplanted in a competitive repopulation assay. Recipient mice were given Trametinib, Palbociclib and Navitoclax. In the KRAS group, donor cells contributed to a lower level of chimerism. We also developed the use of droplet digital PCR (ddPCR) to monitor the percentage of mutant cells in bulk BM CD34 + cells (mutant and isogenic control) when cultured in the presence of inhibitors. Taken together, our study offers novel insights into mutant KRAS-driven HPC expansion and demonstrate an efficient platform for drug screening.

3. Designing Motif-Engineered Receptors To Elucidate Signaling Molecules Important for Proliferation of Hematopoietic Stem Cells

Shuta Ishizuka, Chen-Yi Lai, Makoto Otsu, Hiromitsu Nakauchi, Teruyuki Nagamune, Masahiro Kawahara

The understanding of signaling events is critical for attaining long-term expansion of hematopoietic stem cells ex vivo. In this study, we aim to analyze the contribution of multiple signaling molecules in proliferation of hematopoietic stem cells. To this end, we design a bottom-up engineered receptor with multiple tyrosine motifs, which can recruit multiple signaling molecules of interest. This is followed by a topdown approach, where one of the multiple tyrosine motifs in the bottom up engineered receptor is functionally knocked out by tyrosine-to-phenylalanine mutation. The combination of these two approaches demonstrates the importance of Shc in cooperation with STAT3 or STAT5 in the proliferation of hematopoietic stem cells. The platform developed herein may be applied for analyzing other cells and/or other cell fate regulation systems.

4. Infantile onset primary alveolar proteinosis with hypogammaglobulinemia caused by heterozygous mutations of 2'-5'-oligoadenylate synthase 1.

Tsubasa Okano, Kazutoshi Cho, Shunsuke Kawamura, Nobuyuki Onai, Shigeru Kakuta, Masami Kanai-Azuma, Toshiaki Ohteki, Kohsuke Imai, Hirokazu Kanegane, Makoto Otsu, Tadashi Ariga, and Tomohiro Morio

2'-5'-oligoadenylate synthase 1 (OAS1) is one of the interferon stimulated genes (ISG) which plays a

critical role in innate anti-viral immunity. Stimulated by viral double strand RNA (dsRNA), OAS1 produces 2'-5' linked oligomer of adenylates (2'-5'A) and the produced 2'-5'A activates ribonuclease-L (RNAse-L) which results in degradation of viral RNA and inhibition of viral replications.

We identified autosomal inherited OAS1 missense mutations in three Japanese pedigrees with infantile onset primary alveolar proteinosis with hypogammaglobulinemia. Patients' peripheral blood monocytes and CD11c+ myeloid dendritic cells (mDC) were progressively decreased; CD27+ memory B cells were lacking. The alveolar macrophages showed decreased phagocytosis activity; and the enlarged and foamy form was scarcely observed, which were typical in conventional pulmonary alveolar proteinosis (PAP). Their bone marrow monocyte progenitors were decreased and showed reduced colony forming potentials. One of the patients is currently alive without PAP after hematopoietic stem cell transplantation.

Recombinant mutant OAS1 protein had unaltered dsRNA binding activity. Patient-derived lymphoblastoid cell lines showed normal expression of OAS1 protein and mRNA, and unaltered function in OAS1 - RNAse-L pathway.

We generated model OAS1 knock-in mice by CRISPR/Cas9 based gene editing, and analyzed immune-phenotypes. The mice show normal B and DC numbers and normal serum immunoglobulin levels so far. Detailed functional study on mutant OAS1 protein with multi-Omics approach is currently under way.

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Center for Stem Cell Biology and Regenerative Medicine

Division of Stem Cell Signaling
幹細胞シグナル制御分野

Professor Toshio Kitamura, M.D., D.M.Sc.

教授 医学博士 北 村 俊 雄

Our major interest is to elucidate the mechanisms of pluripotency, self-renewal and the control of cell division and differentiation of hematopoietic stem and progenitor cells. We have developed the retrovirus-mediated efficient gene transfer and several functional expression cloning systems, and utilized these system to our experiments. We are now conducting several projects related to stem cells to clarify underling mechanisms of maintenance of pluripotency and differentiation of stem cells.

1. Developing Analysis Tools for Cell Cycle and Cell Division of Hematopoietic Stem Cells: MgcRacGap - hmKusabiraOrange2 (MRG - hmKuO2) fusion protein for midbody marker.

Yosuke Tanaka, Tsuyoshi Fukushima, Toshihiko Oki, Kotarou Nishimura, Asako Sakaue-Sawano[1], Atsushi Miyawaki[1], Toshio Kitamura: [1]Laboratory for Cell Function Dynamics, RIKEN, Wako, Saitama and ERATO Miyawaki Life Function Dynamics Project, JST.

Previously, we reported that MgcRacGap is a marker for midbody and that MgcRacGap-mVenus fusion protein visualized asymmetric inheritance and release of midbody during cytokinesis (Nishimura et al., 2013). We retrovirally introduced MRG-hmKuO2 into hematopoietic stem cells (HSCs), in order to examine whether asymmetric inheritance and release of midbody is involved in asymmetric division of HSCs. HSCs showed high frequency of midbody release during cytokinesis in culture. Interestingly, one daughter cell releasing midbody differentiated earlier than the other daughter cell inheriting midbody. We generated Cre-inducible MRG-hmKuO2 mouse line. Briefly, the MRG-hmKuO2 fusion gene is inserted into Rosa26 locus following a loxP-NEO-STOP-loxP cassette, in order to visualize asymmetric inheritance and release of midbody in vivo without retroviral infection. By Crossing MRG-hmKuO2 mice with Vav-Cre mice, MRG-hmKuO2 nicely marked asymmetric inheritance and release of midbody in HSCs in culture. We are planning to do paired-daughter assay using HSCs from MRG-hmKuO2 mice to examine whether inheritance and release of midbody link to asymmetric division of HSCs. Given that some problems (eg. weakness of the color) were found in this new mouse line, we are planning to establish surrogate experimental models for this.

2. Developing Analysis Tools for Cell Cycle and Cell Division of Hematopoietic Stem Cells: A novel G0 marker, mVenus-p27K- and its transgenic mouse

Tsuyoshi Fukushima, Yosuke Tanaka, Toshihiko Oki, Kotarou Nishimura, Asako Sakaue-Sawano[1], Atsushi Miyawaki[1], Toshio Kitamura: [1]Laboratory for Cell Function Dynamics, RIKEN, Wako, Saitama and ERATO Miyawaki Life Function Dynamics Project, JST.

One of the common features of the stem cells is that they are in quiescent (G0) phase of cell cycle. Several reports indicate that hematopoietic stem cells and cancer stem cells are in G0 phase, like tissue-specific normal stem cells.

We developed a novel G0 marker, mVenus-p27K- (Oki et al, 2013). The mVenus-p27K- clearly marked G0 and very early G1 in NIH3T3 cells. To examine G0 status in HSCs, we generated a Cre-inducible mVenus-p27K- mouse line that carried mVenus-p27K- fusion gene in Rosa26 locus following a loxP-NEO-STOP-loxP cassette. After crossing with Vav1-Cre mice, we analyzed mVenus-p27K- expression in HSCs. We expected that most of the HSCs are mVenus-p27K- positive because most of the HSCs are in G0 phase. However, three different fractions (mVenus-p27K-high (70%), mVenus-p27K-low (20%), mVenus-p27K-negative (10%)) were identified in the HSC fraction (CD150 + CD48-cKit + Sca-1 + Lineage-). These three fractions were in G0 stage judged by Pyronin Y/Hoechst double-staining. The bone marrow reconstitution assay showed that the mVenus-p27K-high/low fractions showed bone marrow reconstitution ability, but not mVenus-p27K-negative fraction. BrdU-label retaining assay, a method for detection of dormant cells in various tissues, showed that the mVenus-p27K-high population contained dormant transplantable HSCs and the mVenus-p27K-low population contained active transplantable HSCs. Single-cell RNA Sequence (scRNASeq) analysis showed that highly expressed genes in mVenus-p27K-high cells included well-known HSC-related genes, such as *Hlf*, *Ifitm1*, *Mpl* and *Ly6a*, providing evidence that this population contains the 'true' HSCs. On the other hand, highly expressed genes in mVenus-p27K-low cells included genes associated with cell cycle or differentiation, such as *Gata1*, *Itga2b* and *Cdk6*. Small-cell Mass Spec analysis showed that Cdk6 protein was detected in the mVenus-p27K-low fraction, but not in the mVenus-p27K-high fraction. Taken together, these data clearly showd that mVenus-p27K- could discriminate dormant and active transplantable HSCs in the conventional HSC fraction. We are planning to cralify molecular mechanisms regulating dormant and active states of transplantable HSCs.

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Center for Stem Cell Biology and Regenerative Medicine

Division of Stem Cell Dynamics
幹細胞ダイナミクス解析分野

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Proteases perform highly selective and limited cleavage of specific substrates including growth factors and their receptors, cell adhesion molecules, cytokines, apoptotic ligand and angiogenic factors. The goal of our laboratory is to identify novel therapeutic targets for diseases like cancer or inflammatory diseases by studying the role of proteases and growth factors during inflammation, tissue regeneration, and in stem cell biology. In 2018, we focused on understanding how proteases like tissue type plasminogen activator (tPA) promote melanoma cell spreading. We propose a novel way to block melanoma metastasis and growth by preventing the serine protease tPA to connect with low density lipoprotein receptor-related protein 1 (LRP1).

1. The fibrinolytic factor tPA drives LRP1-mediated melanoma growth and metastasis

Yousef Salama, Shiou-Yuh Lin, Douaa Dhahri, Koichi Hattori, Beate Heissig

The multifunctional endocytic receptor low-density lipoprotein receptor-related protein 1 (LRP1) has recently been identified as a hub within a biomarker network for multi-cancer clinical outcome prediction. The mechanism how LRP1 modulates cancer progression is poorly understood. Here, we show that LRP1 and one of its ligands tissue type plasminogen activator (tPA) are expressed in melanoma cells and control melanoma growth and lung metastasis *in vivo*.

Mechanistic studies were carried out on two melanoma cancer cell lines, the B16F10 and the B16 F1 cells, both of which form primary melanoma tumors, but only B16F10 cells are able to metastasize to the lungs. Tumor-, but not niche cell-derived tPA enhanced melanoma cell proliferation as shown in $tPA^{-/-}$ mice. Gain-of-function studies revealed that melanoma LRP1 is critical for tumor growth, and recruitment of mesenchymal stem cell into the tumor bed and metastasis. Melanoma LRP1 was found to enhance extracellular signal-regulated kinase activation, resulting in increased matrix metalloproteinase (MMP)9 RNA, protein, and secreted activity, a well-known modulator of melanoma metastasis. Restoration of LRP1 and tPA in the less aggressive, poorly metastatic B16F1 tumor cells enhanced tumor cell proliferation and led to massive lung metastasis in murine tumor models. Anti-melanoma drug treatment induced tPA and LRP1 expression. tPA or LRP1 knockdown enhanced chemo-sensitivity in melanoma cells. Our results identify the tPA-LRP1 pathway as a key switch that drives melanoma progression in part by modulating the cellular composition and proteolytic makeup of the tumor niche. Targeting this pathway might be a novel treatment strategy in combination therapies in melanoma.

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<Beate Heissig Group>

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Center for Stem Cell Biology and Regenerative Medicine

Division of Stem Cell Cellomics
幹細胞セロミクス分野

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Single cell analysis has become increasingly important for cellular biologists doing basic, translational, and clinical research. It was once believed that cell populations were homogeneous, but the latest evidence shows that heterogeneity does in fact exist even within small cell populations. Gene expression measurements based on the homogenized cell population are misleading averages and don't account for the small but critical changes occurring in individual cells. Individual cells can differ dramatically in size, protein levels, and expressed RNA transcripts, and these variations are key to answering previously irresolvable questions in cancer research, stem cell biology, immunology, and developmental biology. We are also trying to develop new advanced techniques by the integration of photonics, chemistry, electrical engineering, mechanical engineering, bioinformatics, and others.

1. Ultrafast confocal fluorescence microscopy beyond the fluorescence lifetime limit.

Hideharu Mikami[1], Jeffrey Harmon[1], Hirofumi Kobayashi[1], Syed Hamad[1], Yisen Wang[1,2], Osamu Iwata[3], Kengo Suzuki[3], Takuro Ito[4], Yuri Aisaka[5], Natsumaro Kutsuna[5], Kazumichi Nagasawa[6], Hiroshi Watarai[6], Yasuyuki Ozeki[7], Keisuke Goda[1,4,8]: [1]Department of Chemistry, The University of Tokyo, [2]College of Precision Instrument and Optoelectronic Engineering, Tianjin University, [3]Euglena Co., Ltd., [4]Japan Science and Technology Agency, [5]LPixel Inc., [6]Division of Stem Cell Cellomics, Center for Stem Cell Biology and Regenerative Medicine, Institute of Medical Science, The University of Tokyo, [7]Department of Electrical Engineering and Information Systems, The University of Tokyo, [8]Department of Electrical Engineering, University of California.

Laser-scanning confocal fluorescence microscopy is an indispensable tool for biomedical research by virtue of its high spatial resolution. Its temporal resolution is equally important, but is still inadequate for many applications. Here we present a confocal fluorescence microscope that, for the first time to our knowledge, surpasses the highest possible frame rate constrained only by the fluorescence lifetime of fluorophores (typically a few to several nanoseconds). This microscope is enabled by integrating a broadband, spatially distributed, dual-frequency comb or spatial dual-comb and quadrature amplitude modulation for optimizing spectral efficiency into frequency-division multiplexing with single-pixel photodetection for signal integration. Specifically, we demonstrate confocal fluorescence microscopy at a record high frame rate of 16,000 frames/s. To show its broad biomedical utility, we use the microscope to demonstrate 3D volumetric confocal fluorescence microscopy of cellular dynamics at 104 volumes/s and confocal fluorescence imaging flow cytometry of hematological and microalgal cells at 2 m/s.

2. Generation and validation of novel anti-bovine CD163 monoclonal antibodies ABM-1A9 and ABM-2D6.

Yoshinori Shimamoto[1], Junko Nio-Kobayashi[4], Hiroshi Watarai[5], Masashi Nagano[6], Natsuko Saito[2],

Eiki Takahashi[7], Hidetoshi Higuchi[3], Atsushi Kobayashi[6], Takashi Kimura[6], Hiroshi Kitamura[2]: [1]Laboratory of Animal Therapeutics, Department of Veterinary Science, Rakuno Gakuen University, [2]Laboratory of Veterinary Physiology, Department of Veterinary Medicine, Rakuno Gakuen University, [3]Laboratory of Animal Health, Department of Veterinary Medicine, Rakuno Gakuen University, [4]Laboratory of Histology and Cytology, Department of Functional Morphology, Graduate School of Medical Sciences, Hokkaido University, [5]Division of Stem Cell Cellomics, The Institute of Medical Science, The University of Tokyo, [6]Laboratory of Theriogenology and Laboratory of Pathology, Department of Veterinary Clinical Sciences, Graduate School of Veterinary Medicine, Hokkaido University, [7]Research Resources Center, RIKEN Brain Science Institute.

The scavenger receptor CD163 is widely used as a cell signature of alternatively active "M2" macrophages in mammals. In this study, we generated two monoclonal antibodies, namely ABM-1A9 and ABM-2D6, against the extracellular region of bovine CD163. Conventional Western blotting using the antibodies gave immune reactive bands corresponding to the transmembrane (～150 kDa) and soluble (～120 kDa) forms of bovine CD163 when tested using spleen protein lysate extracted from cattle with mastitis. The minimum limit of detectable concentration of both antibodies was relatively lower (5.0 ng/mL) than that of the anti-human CD163 monoclonal antibody AM-3K (>1.0 μg/mL), which has been used previously for the detection of bovine CD163. An immunohistochemical study using formalin-fixed paraffin-embedded sections indicated that ABM-1A9 and ABM-2D6 clearly stained some Iba1＋macrophages in the lymph nodes of cattle with mastitis. Moreover, the CD163-stained macrophages were frequently observed engulfing leukocytes. ELISA using ABM-2D6 distinguished levels of circulating soluble CD163 between healthy cattle (<16.9 pmol/mL) and cattle with mastitis (>33.7 pmol/mL). These new monoclonal antibodies can be used for the diagnosis and evaluation of prognosis of inflammatory diseases in cattle, using immunohistological analysis and blood test applications.

3. New genetically manipulated mice provide insights into the development and physiological functions of invariant natural killer T cells.

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Invariant natural killer T (iNKT) cells are a unique T cell subset that exhibits characteristics of both innate immune cells and T cells. They express Vα14-Jα18 (*Trav11-Traj18*) as an invariant chain of the T cell receptor (TCR) and are restricted to the MHC class I-like monomorphic antigen presenting molecule CD1d. iNKT cells are known as immune regulators that bridge the innate and acquired immune systems by rapid and massive production of a wide range of cytokines, which could enable them to participate in immune responses during various disease states. Thus, Traj18-deficient mice, *Cd1d* deficient mice, or iNKT cell-overexpressing mice such as iNKT TCRα transgenic mice and iNKT cell cloned mice which contain a Vα14-Jα18 rearrangement in the TCRα locus are useful experimental models for the analysis of iNKT cells in vivo and in vitro. In this review, we describe the pros and cons of the various available genetically manipulated mice and summarize the insights gained from their study, including the possible roles of iNKT cells in obesity and diabetes.

4. Liver-primed immune cells regulate pre- and post- metastatic niche in the lung.

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Primary tumours establish metastases by interfering with distinct organs. In pre-metastatic organs, a tumour-friendly microenvironment supports metas-

tatic cells and is prepared by many factors including tissue resident cells, bone marrow-derived cells and abundant fibrinogen depositions. However, other components are unclear. Here, we show that a third organ, originally regarded as a bystander, plays an important role in metastasis by directly affecting the pre-metastatic soil. In our model system, the liver participated in lung metastasis as a leucocyte supplier. These liver derived leucocytes displayed liver-like characteristics and, thus, were designated hepato-entrained leucocytes (HepELs). HepELs had high expression levels of coagulation factor X (FX) and vitronectin (Vtn) and relocated to fibrinogen-rich hyperpermeable regions in pre-metastatic lungs; the cells then switched their expression from Vtn to thrombospondin, both of which were fibrinogen- binding proteins. Cell surface marker analysis revealed that HepELs contained B 220^+ $CD11c^+$ $NK1.1^+$ cells. In addition, an injection of $B220^+$ $CD11c^+$ $NK1.1^+$ cells successfully eliminated fibrinogen depositions in pre-metastatic lungs via FX. Moreover, $B220^+$ $CD11c^+$ $NK1.1^+$ cells demonstrated anti-metastatic tumour ability with IFNγ induction. These findings indicate that liver primed $B220^+$ $CD11c^+$ $NK1.1^+$ cells suppress lung metastasis.

5. Intelligent Image-Activated Cell Sorting.

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A fundamental challenge of biology is to understand the vast heterogeneity of cells, particularly how cellular composition, structure, and morphology are linked to cellular physiology. Unfortunately, conventional technologies are limited in uncovering these relations. We present a machine-intelligence technology based on a radically different architecture that realizes real-time image-based intelligent cell sorting at an unprecedented rate. This technology, which we refer to as intelligent image-activated cell sorting, integrates high-throughput cell microscopy, focusing, and sorting on a hybrid software-hardware data-management infrastructure, enabling real-time automated operation for data acquisition, data processing, decision-making, and actuation. We use it to demonstrate real-time sorting of microalgal and blood cells based on intracellular protein localization and cell-cell interaction from large heterogeneous populations for studying photosynthesis and atherothrombosis, respectively. The technology is highly versatile and expected to enable machine-based scientific discovery in biological, pharmaceutical, and medical sciences.

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Center for Stem Cell Biology and Regenerative Medicine

Stem Cell Bank
ステムセルバンク

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Stem cells represent a valuable cell source in the field of regenerative medicine. Pluripotent stem cells are newly emerging types of stem cells that have been utilized either for the basic research or to develop curative treatment for various diseases. We have been focusing especially on the utilization of induced pluripotent stem cells as a research platform to elucidate pathophysiology of intractable diseases based on their proper modeling. Our eventual goal is to establish safe and efficacious treatment for the patients suffering from various types of diseases currently with no curative treatment available

1. Status of KRAS in iPS cells impacts upon self-renewal and differentiation propensity

Kenji Kubara, Kazuto Yamazaki, Yasuharu Ishihara, Huan-Ting Lin, Ken Nishimura, Manami Ohtaka, Mahito Nakanishi, Masashi Ito, Kappei Tsukahara, Masatoshi Takagi, Makoto Otsu

Oncogenic KRAS mutations in hematopoietic stem cells cause RAS-associated autoimmune lymphoproliferative syndrome-like disease (RALD). KRAS plays essential roles in stemness maintenance in some types of stem cells. However, its roles in pluripotent stem cells (PSCs) are poorly understood. Here, we investigated roles of KRAS on stemness in the context of induced PSCs (iPSCs). We used KRAS mutant (G13C/WT) and wild-type isogenic (WT/WT) iPSCs from the same RALD patients, as well as wild-type (WTed/WT) and heterozygous knockout (Δed/WT) iPSCs, both obtained by genome-editing from the same G13C/WT clone. Compared with wild-type iPSCs, G13C/WT iPSCs displayed enforced retention of self-renewal and suppressed capacity for neuronal differentiation, whilst Δed/WT iPSCs showed normalized cellular characteristics similar to those of isogenic WTed/WT cells. The KRAS-ERK pathway, but not the KRAS-PI3K pathway, was shown to govern these G13C/WT-specific phenotypes, indicating the strong impact of the KRAS-ERK signaling upon self-renewal and differentiation propensity in human iPSCs.

2. Enhanced selective inhibition of KRAS mutant hematopoietic progenitor cell expansion by MEK and Bcl-2 inhibition.

Huan-Ting Lin, Kenji Kubara, Kazuto Yamazaki, Masatoshi Takagi, Takuya Naruto, Tomohiro Morio, Takashi Okumura, Makoto Otsu

Amidst the complex genetic landscape of cancer, the mutagenic and oncogenic effects caused by RAS mutations alone can be overlooked particularly in the context of hematopoietic malignancies. Therefore identifying drugs that could modulate aberrant RAS-MAPK signaling is clinically important. Here we demonstrate the utilization of an iPS cell-based system that could be adapted for compound screening to identify inhibitors with selective inhibition activity against the expansion of KRAS-mutant hematopoietic progenitor cells (HPCs).

RAS-associated autoimmune lymphoproliferative disorder (RALD) is a leukemia-like disease caused by a single acquired RAS mutation. In this study, BM CD34+ cells from RALD patients were repro-

grammed into iPS cells. KRAS-mutant cells could be obtained without necessitating the use of gene editing techniques. Non-mutant cells were used as isogenic controls thus permitting the assessment of unwanted toxicity against healthy cells. Constitutive activation of the RAS-MAPK pathway was confirmed by western blotting in mutant iPS cells. As expected, whole exome sequencing (WES) showed that mutant KRAS (G13C) was the only mutation present in these cells. Therefore, the inherent characteristics of mutant cells expansion can be attributed to this mutation only.

Previously we reported using these patient-derived iPS cells that oncogenic KRAS enforces retention of self-renewal in human pluripotent cells (Kubara et al. Stem Cell Reports. 2018) due to constitutive activation of the KRAS-MAPK signaling pathway. Of note, mutant iPS cells retained pluripotency marker expression even in the absence of bFGF, but not isogenic control cells. When we tested compounds with different mechanisms of action, direct MEK inhibition was the most effective in triggering a loss in pluripotency marker expression in mutant iPS cells in the absence of bFGF. This method permits rapid determination of compounds that could modulate aberrant KRAS-MAPK signaling. However, further verification is required to determine whether inhibitory effects on mutant HPC expansion could also be observed.

Next, differentiated multi-potent (Lin-34 + 43 +) HPCs from iPS cells were expanded in "standard" ex vivo culture conditions using SCF, TPO Flt3L and IL-3. This favored the expansion of control cells. We also found "selective" culture conditions that could selectively expand mutant HPCs. RNA-seq analysis on pre-expanded and expanded HPCs revealed the unique molecular signature of expanding mutant cells, meaning genes that showed upregulation in both "standard" and "selective" culture conditions. There was upregulation of CCND1 and CDK4 in mutant HPCs, which facilitates progression through the G1 phase of the cell cycle. This was also accompanied by upregulation p16 INK4a and p21Cip1, which is characteristic of senescent cells. Indeed, it could be shown that expanded mutant HPCs acquired senescence features such as increased p16 INK4a and Bcl-xL expression at the protein level and increased SA-b-gal activity compared with control cells. Gene correction of KRAS (G13C) using CRISPR/Cas9 reversed these phenotypes. Therefore CCND1/CDK4 and Bcl-2 may be potential inhibitory targets.

We developed a high-throughput screening method whereby differentiated HPCs were sorted directly into 96-well plates and following expansion, cell numbers were quantified by luminescence detection of ATP. First we screened drugs for selective activity, where the IC50 value for mutant cells is lower than control cells. Consistent with iPS cell pluripotency assay, we identified Trametinib (MEK), as having selective effects against mutant HPCs in addition to Palbociclib (CCND1/CDK4) and Navitoclax (Bcl-2). When used in combination, the selective inhibitory effect was enhanced without compromising the viability of control cells.

To test these drugs in an in vivo setting, highly purified transduced (KRAS or control) mouse HSCs (Lin-CD34-KSL) were transplanted in a competitive repopulation assay. Recipient mice were given Trametinib, Palbociclib and Navitoclax. In the KRAS group, donor cells contributed to a lower level of chimerism. We also developed the use of droplet digital PCR (ddPCR) to monitor the percentage of mutant cells in bulk BM CD34 + cells (mutant and isogenic control) when cultured in the presence of inhibitors. Taken together, our study offers novel insights into mutant KRAS-driven HPC expansion and demonstrate an efficient platform for drug screening.

3. Designing Motif-Engineered Receptors To Elucidate Signaling Molecules Important for Proliferation of Hematopoietic Stem Cells

Shuta Ishizuka, Chen-Yi Lai, Makoto Otsu, Hiromitsu Nakauchi, Teruyuki Nagamune, Masahiro Kawahara

The understanding of signaling events is critical for attaining long-term expansion of hematopoietic stem cells ex vivo. In this study, we aim to analyze the contribution of multiple signaling molecules in proliferation of hematopoietic stem cells. To this end, we design a bottom-up engineered receptor with multiple tyrosine motifs, which can recruit multiple signaling molecules of interest. This is followed by a topdown approach, where one of the multiple tyrosine motifs in the bottom up engineered receptor is functionally knocked out by tyrosine-to-phenylalanine mutation. The combination of these two approaches demonstrates the importance of Shc in cooperation with STAT3 or STAT5 in the proliferation of hematopoietic stem cells. The platform developed herein may be applied for analyzing other cells and/or other cell fate regulation systems.

4. Infantile onset primary alveolar proteinosis with hypogammaglobulinemia caused by heterozygous mutations of 2′-5′-oligoadenylate synthase 1.

Tsubasa Okano, Kazutoshi Cho, Shunsuke Kawamura, Nobuyuki Onai, Shigeru Kakuta, Masami Kanai-Azuma, Toshiaki Ohteki, Kohsuke Imai, Hirokazu Kanegane, Makoto Otsu, Tadashi Ariga, and Tomohiro Morio

2′-5′-oligoadenylate synthase 1 (OAS1) is one of the interferon stimulated genes (ISG) which plays a critical role in innate anti-viral immunity. Stimulated by viral double strand RNA (dsRNA), OAS1 produces 2′-5′ linked oligomer of adenylates (2′-5′A) and the produced 2′-5′A activates ribonuclease-L (RNAse-L) which results in degradation of viral RNA and inhibition of viral replications.

We identified autosomal inherited OAS1 missense mutations in three Japanese pedigrees with infantile onset primary alveolar proteinosis with hypogammaglobulinemia. Patients' peripheral blood monocytes and CD11c+ myeloid dendritic cells (mDC) were progressively decreased; CD27+ memory B cells were lacking. The alveolar macrophages showed decreased phagocytosis activity; and the enlarged and foamy form was scarcely observed, which were typical in conventional pulmonary alveolar proteinosis (PAP). Their bone marrow monocyte progenitors were decreased and showed reduced colony forming potentials. One of the patients is currently alive without PAP after hematopoietic stem cell transplantation.

Recombinant mutant OAS1 protein had unaltered dsRNA binding activity. Patient-derived lymphoblastoid cell lines showed normal expression of OAS1 protein and mRNA, and unaltered function in OAS1 - RNAse-L pathway.

We generated model OAS1 knock-in mice by CRISPR/Cas9 based gene editing, and analyzed immune-phenotypes. The mice show normal B and DC numbers and normal serum immunoglobulin levels so far. Detailed functional study on mutant OAS1 protein with multi-Omics approach is currently under way.

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Center for Stem Cell Biology and Regenerative Medicine

FACS Core Laboratory
FACSコアラボラトリー

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The FACS Core Laboratory provides high quality, cost effective state-of-art flow cytometry services for internal and external researcher. The facility has three BD FACSAria cell sorters, SONY SH800 cell sorter for sorting, BD FACSCalibur, BD FACSVerse for analysis. We offer assistance in the following areas, (1) initial project planning (2) antibody panel design and optimization (3) instrument operation and maintenance (4) data analysis.

Yumiko Ishii, Yukiko Komiya, Azusa Fujita