Center for Stem Cell Biology and Regenerative Medicine

Division of Stem Cell Therapy
幹細胞治療分野

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Recent great progress in stem cell biology has brought about increase in the prospect for application of stem cell-based therapy. Especially the discovery of iPSCs, a great step forward in stem-cell research, holds out the promise of development of novel therapeutic strategies by generating iPSCs from patients. The goal of this laboratory is to provide new insights into stem cell biology as well as approaches to novel therapeutic intervention for various intractable diseases.

1. Effective treatment against severe Graft-versus-Host Disease with allele-specific anti-HLA monoclonal antibody in a humanized-mouse model.

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Graft-versus-host disease (GVHD), mediated by donor-derived alloreactive T cells, is a major cause of non-relapse mortality in allogeneic hematopoietic stem-cell transplantation (allo-HSCT). Its therapy is not well-defined. We established allele-specific anti-HLA monoclonal antibodies (ASHmAbs) that specifically target HLA molecules, with steady death of target-expressing cells. One such ASHmAb, against HLA-A*02:01 (A2-kASHmAb), was examined in a xenogeneic GVHD mouse model. To induce fatal GVHD, non-irradiated NOD/Shi-scid/IL-2Rγ^{null} (NOG) mice were injected with healthy-donor human peripheral blood mononuclear cells (PBMCs), some expressing HLA-A*02:01, some not. Administration of A2-kASHmAb promoted the survival of mice injected with HLA-A*02:01-expressing PBMCs ($p<0.0001$) and, in humanized NOG mice, immediately cleared HLA-A*02:01-expressing human blood cells from mouse peripheral blood. Human PBMCs were again detectable in mouse blood 2-4 weeks after A2-kASHmAb administration, suggesting that kASHmAb may be safely administered to GVHD patients without permanently ablating the graft. This approach, different from those of existing GVHD pharmacotherapy, may open a new door for treatment of GVHD in HLA-mismatched allo-HSCT.

2. Gene targeting study reveals unexpected expression of brain-expressed X-linked 2 in endocrine and tissue stem/progenitor cells in mice.

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Identification of genes specifically expressed in stem/progenitor cells is an important issue in developmental and stem cell biology. Genome-wide gene expression analyses in liver cells performed in this study have revealed a strong expression of X-linked genes that include members of the brain-expressed X-linked (Bex) gene family in stem/progenitor cells. Bex family genes are expressed abundantly in the neural cells and have been suggested to play important roles in the development of nervous tissues. However, the physiological role of its individual members and the precise expression pattern outside the nervous system remain largely unknown. Here, we focused on Bex2 and examined its role and expression pattern by generating knock-in mice; the enhanced green fluorescence protein (EGFP) was inserted into the Bex2 locus. Bex2-deficient mice were viable and fertile under laboratory growth conditions showing no obvious phenotypic abnormalities. Through an immunohistochemical analysis and flow cytometry-based approach, we observed unique EGFP reporter expression patterns in endocrine and stem/progenitor cells of the liver, pyloric stomach, and hematopoietic system. Although Bex2 seems to play redundant roles in vivo, these results suggest the significance and potential applications of Bex2 in studies of endocrine and stem/progenitor cells.

3. Clock gene Bmal1 is dispensable for intrinsic properties of murine hematopoietic stem cells.

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Circadian rhythms are known to influence a variety of biological phenomena such as cell cycle, sleep-wake rhythm, hormone release and other important physiological functions. Given that cell cycle entry of hibernating hematopoietic stem cells (HSCs) plays a critical role in controlling hematopoiesis, we asked functional significance of the clock gene Bmal1, which plays a central role in regulating circadian rhythms as a transcription factor. Here we investigated the necessity of Bmal1 for HSC functions using Bmal1 deficient ($Bmal1^{-/-}$) mice.

Using colony-forming assays in vitro, we found that the frequency of mixed colony formation between $Bmal1^{+/+}$ and $Bmal1^{-/-}$ CD34-KSL cells does not differ significantly. Competitive bone marrow assays also revealed that $Bmal1^{-/-}$ bone marrow cells competed normally with wild-type cells and displayed long-term multi-hematopoietic lineage reconstitution. In addition, there were no significant differences in the frequencies and hibernation state of bone marrow HSCs between $Bmal1^{+/+}$ and Bmal $1^{-/-}$ mice, suggesting that they are independent of circadian rhythms.

This paper discusses the necessity of circadian rhythms for HSC functions. Our data clearly shows that a key circadian clock gene Bmal1 is dispensable for intrinsic functions of HSCs, such as differentiation, proliferation and repopulating ability.

4. Generation of mouse functional oocytes in rat by xeno-ectopic transplantation of primordial germ cells.

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Primordial germ cells (PGCs) are germ cell progenitors in the fetal genital ridge; female PGCs give rise to definitive oocytes that contribute to the next generation. Artificial PGCs have been induced in

vitro from pluripotent stem cells and gonad-like tissue has been induced in vivo by cotransplantation of PGCs with PGC-free gonadal cells. To apply these technologies to human infertility treatment or conservation of rare species, PGC transplantation must be established in xenogenic animals. Here, we established a xenogeneic transplantation model by inducing ovary-like tissue from PGCs in xenogenic animals. We transplanted enzymatically dispersed PGCs with PGC-free gonadal cells under the kidney capsule of xenogenic immunodeficient animals. The transplanted cells formed ovary-like tissues under the kidney capsule. These tissues were histologically similar to the normal gonad and expressed the oocyte markers Vasa and Stella. In addition, mouse germinal vesicle-stage oocyte-like cells collected from ovary-like tissue in rats matured to metaphase II via in vitro maturation and gave rise to offspring by intracytoplasmic sperm injection. Our studies show that rat/mouse female PGCs and PGC-free gonadal cells can develop and reconstruct ovary-like tissue containing functional oocytes in an ectopic xenogenic microenvironment.

5. Successful reprogramming of epiblast stem cells by blocking nuclear localization of β-catenin

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Epiblast stem cells (EpiSCs) in mice and rats are primed pluripotent stem cells (PSCs). They barely contribute to chimeric embryos when injected into blastocysts. Reprogramming (r) of EpiSCs to ES-cell-like cells (rESCs) may occur in response to LIF-STAT3 signaling; however, low reprogramming efficiency hampers potential use of rESCs in generating chimeras. Here we describe dramatic improvement of conversion efficiency from primed to naïve-like PSCs through up-regulation of *E-cadherin* in the presence of the cytokine LIF. Analysis revealed that blocking nuclear localization of β-CATENIN with small-molecule inhibitors significantly enhances reprogramming efficiency of mouse EpiSCs. Although activation of Wnt / β-catenin signals has been thought desirable for maintenance of naïve PSCs, this study provides the evidence that inhibition of nuclear translocation of β-CATENIN enhances conversion of mouse EpiSCs to naïve-like PSCs (rESCs). This affords better understanding of gene regulatory circuits underlying pluripotency and reprogramming of PSCs.

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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. Hematopoietic stem cells provide a good example of such usefulness of stem cell research, showing many successful cases in both hematopoietic cell transplantation and gene therapy. Pluripotent stem cells have become another possibility of cell sources in regenerative medicine that may be utilized either for the basic research or to cure the diseases. Our eventual goal is to establish safe and efficacious treatment for the patients suffering from various types of intractable diseases with no curative treatment available.

1. Recapitulation of pathophysiological features of Wiskott Aldrich Syndrome using induced pluripotent stem cells (iPSCs)

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Wiskott Aldrich Syndrome (WAS) is an X-linked disorder, which is characterized by thrombocytopenia, immunodeficiency, eczema and autoimmunity. Patients have mutations in the gene encoding WAS protein (WASp), leading to the absence or dysfunction of this molecule specifically in their hematopoietic cells. As severe reduction in platelet numbers with their small sizes is one of the most obvious features for the disease, which is associated with a significant risk of life-threatening hemorrhage, elucidation of its precise cause has been the subject of interest for researchers for many years. Although both platelet production and consumption are reportedly affected by WASp-deficiency, it has been an issue of debate whether defective proplatelet release from megakaryocytes and/or destruction of platelets in the spleen constitutes major cause of this phenomena. Because murine WASp-null models are poor phenocopies of the disease, showing relatively unaffected numbers and sizes of platelets comparing to those in wild type mice, more accurate investigation for the mechanisms underlying thrombocytopenia needs a reliable model, which can mimic the disease features as precisely as possible. Induced Pluripotent Stem Cells (iPSC) have recently been successful to model several monogenic diseases *in vitro*, we thus sought to utilize this technology for our purpose. We first established iPSC lines of two WAS patients (with different mutations) using hematopoietic progenitor cells in peripheral blood and the Sendai virus (SeV) vector harboring four reprogramming factors (OCT3/4, SOX2, KLF4 and c-MYC), then investigated possible defects in the processes of megakaryocyte and platelet production in patient cells and their underlying mechanisms. In brief, iPSCs were allowed to differentiate into hematopoietic progenitors (HPCs) for 14 days. Megakaryocyte/erythrocyte progenitor cells (CD34 + CD41 +) were then sorted by flow cytometry, and cultured on feeder cells with fixed input numbers for another 9 days in the presence of appropriate cytokines. Absolute counts of megakaryocytes and platelets were estimated by flow cytometry analysis using fluorescent beads. To avoid the issue of clonal variation that may signifi-

cantly compromise the results, we have used at least five different iPSC clones from each patient and those from healthy individuals. First, we did not detect any considerable difference in the efficiency of iPSC generation between patient and control samples. Both patients' iPSCs were shown to exhibit the pluripotent states by detailed characterization, and were confirmed to maintain each patient-specific gene mutation after the process of somatic cell reprogramming. In the process of HPC generation, both patient-iPSCs and control-iPSCs showed comparable capability to form cells with hematopoietic cell lineages. As expected, the numbers of both platelets and megakaryocytes obtained from WAS iPSCs showed a significant decrease comparing to those from healthy iPSCs. Although still preliminary, the data so far demonstrate that there is an intrinsic defect in megakaryocyte and platelet production machineries in WAS patient cells and that the reduced platelet number seems to be due to a proplatelet production deficiency. This finding so far can support the utility of iPSC-based approaches in a pathophysiological studies for WAS, for which proper disease-modeling is otherwise not feasible, thus providing a promising measure to develop effective treatment with maximal safety for this intractable genetic disorder.

2. Alpharetroviral vectors: demonstration of safe and efficacious gene therapy using X-CGD iPSCs.

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Alpharetroviral vectors are of a newly developed design that is both replication-deficient and self-inactivating. Comparative integrome analysis has revealed its integration profile to be more neutral than that of lenti- or gammaretroviral vectors in addition to having minimal genotoxic risk. However, previous studies were carried out using either animal models or immortalized cell lines the physiological relevance of which to humans may be questionable. Using induced pluripotent stem cells (iPSCs) it is possible to recapitulate disease phenotypes whilst permitting precise cell manipulation in vitro. Chronic granulomatous disease (CGD) is a condition characterized by impaired neutrophil (NEU) functionality. It represents a "worst case" scenario in hematopoietic gene therapy given that no clinical trial has successfully achieved sustained persistence gene marked cells and a certain number of patients even developed myelodysplastic changes. In this study, the objective is to demonstrate safe and efficacious alpharetroviral mediated gene therapy using iPSCs as a modeling platform. To generate patient autologous iPSCs, peripheral blood (PB) CD34+ cells were reprogrammed using a Sendai virus vector expressing Oct4/Sox2/Klf4/c-Myc. Alpharetroviral vectors were used to insert codon optimized gp91 cDNA into iPSCs. Neutrophil differentiation was induced using G-CSF. The maturation status was determined by assessing cell morphology and immunophenotype. The ROS generating capacity was determined by the DHR flow cytometry assay and the formation of neutrophil extracellular traps (NETs). Identification of insertion sites was attempted using PCR-based methods and the vector integration profile was assessed through Southern blotting. Overall, the validity of using iPSCs as a disease model was established by demonstrating that differentiated NEUs (control) are functionally comparable to PB NEUs. Addition of the gp91 transgene to CGD-iPSCs led to functional recovery. Using these techniques, it was possible to correlate the efficiency of transduction to the level of transgene expression with the extent of functional recovery. Similar to previous findings, it was found that transgene expression could be sustained in the absence of any clonal expansion. Taken together, it is hope these data may act as an important pre-clinical assessment step that facilitates the application of these vectors in clinical trials. In the future, it is hope that alpharetroviral vectors may also be utilized to treat other congenital hematological disorders through hematological gene therapy.

3. KLRG+ invariant natural killer T cells are long-lived effectors.

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Immunological memory has been regarded as a unique feature of the adaptive immune response mediated in an antigen-specific manner by T and B lymphocytes. However, natural killer (NK) cells

and γδT cells, which traditionally are classified as innate immune cells, have been shown in recent studies to have hallmark features of memory cells. Invariant natural killer T cell (iNKT cell)-mediated antitumor effects indicate that iNKT cells are activated in vivo by vaccinationwith iNKT cell ligand-loaded CD1d + cells, but not by vaccination with unbound iNKT cell ligand. In such models, it previously was thought that the numbers of IFN-γ-producing cells in the spleen returned to the basal level around 1 wk after the vaccination. In the current study, we demonstrate the surprising presence of effector memory-like iNKT cells in the lung. We found long-term antitumor activity in the lungs of mice was enhanced after vaccination with iNKT cell ligand-loaded dendritic cells. Further analyses showed that the KLRG1 + (Killer cell lectin-like receptor subfamily G, member 1-positive) iNKT cells coexpressing CD49d and granzyme A persisted for several months and displayed a potent secondary response to cognate antigen. Finally, analyses of CDR3β by RNA deep sequencing demonstrated that some particular KLRG1 + iNKT-cell clones accumulated, suggesting the selection of certain T-cell receptor repertoires by an antigen. The current findings identifying effector memory-like KLRG1 + iNKT cells in the lung could result in a paradigm shift regarding the basis of newly developed extrathymic iNKT cells and could contribute to the future development of antitumor immunotherapy by uniquely energizing iNKT cells.

4. Generation of induced pluripotent stem cell-derived mice by reprogramming of a mature iNKT cell.

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iNKT cells are characterized by their expression of an iNKT-cell-specific invariant antigen-receptor α chain encoded by Vα14Jα18 gene segments. These iNKT cells bridge the innate and acquired immune systems to mediate effective and augmented responses; however, the limited number of iNKT cells in vivo hampers their analysis. Here, two lines of induced pluripotent stem cell-derived mice (iNKT-iPSC-derived mice) were generated by reprogramming of mature iNKT cells, where one harbors both rearranged Vα14Jα18 and Vβ7 genes and the other carries rearranged Vα14Jα18 on both alleles but germline Vβ loci. The analysis of iNKT-iPSC-derived mice showed a significant increase in NKT cell numbers with relatively normal frequencies of functional subsets, but significantly enhanced in some cases, and acquired functional iNKT cell maturation in peripheral lymphoid organs. iNKT-iPSC-derived mice also showed normal development of other immune cells except for the absence of γδT cells and disturbed development of conventional CD4 αβT cells. These results suggest that the iNKT-iPSC-derived mice are a better model for iNKT cell development and function study rather than transgenic mouse models reported previously and also that the presence of a pre-rearranged Vα14 Jα18 in the natural chromosomal context favors the developmental fate of iNKT cells.

5. Natural Killer T cells are essential for the development of contact hypersensitivity in BALB/c Mice.

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Contact hypersensitivity (CHS) has been widely used to study cutaneous immune responses, as a prototype of delayed-type hypersensitivity. Although iNKT cells have been assumed to have an important role in CHS, their role is controversial. Here, we report the role of iNKT cells in the sensitization phase of CHS, by promoting the survival and maturation of dendritic cells (DCs) in the draining lymph nodes (LNs). The CHS response was attenuated with *Cd1d1*$^{-/-}$ and *Traj18*$^{-/-}$ BALB/c mice in which iNKT cells were absent. In the draining LNs, the number of effector T cells and cytokine production were significantly reduced with iNKT cell-deficient mice. iNKT cells activated and colocalized with DCs in the draining LNs after sensitization. The number of migrated and mature DCs was reduced in iNKT cell-deficient mice 72hours after FITC application. In in vitro experiments, activated iNKT cells enhanced bone marrow-derived DC (BMDC) survivability via tumor necrosis factor (TNF) production from BMDCs. In addition, TNF production from BMDCs was partially suppressed by the neutralizing anti-CD54 or CD154 antibodies. Our data demonstrate that DC-iNKT interaction has a pivotal role in the sensitization phase of CHS.

6. Synthesis of RCAI-172 (C6 epimer of RCAI-147) and its biological activity.

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RCAI-147 is one of the hydroxylated analogues of KRN7000 which is known as a ligand for the activation of CD1d mediated iNKT cells and releases both T helper 1 (Th1) cytokines such as IFN-γ and T helper 2 (Th2) cytokines such as IL-4. KRN7000 has been anticipated as an antitumor drug or an adjuvant for viral infection such as influenza, because of its strong secretion of IFN-γ. In an interesting twist, it has been obvious in our previous paper that RCAI-147 induces much more Th2 cytokines (IL-4) than Th1 cytokines (IFN-γ) from iNKT cells compared to KRN7000, and shows fairly good result in the experimental autoimmune encephalomyelitis (EAE) test. Therefore, synthesis of RCAI-172 (C6-OH epimer of RCAI-147) was attempted to examine the biological activity. As a result, RCAI-172 was synthesized and its biological activity biased to Th2 response largely compared to that of KRN7000. However, this level decreased to approximately 61 % compared to that of RCAI-147. And the clinical score of RCAI-172 for EAE suppression was disappointing. There exist seven chiral centers in the aglycon part of RCAI-172, and even though the change of configuration is just one position (C6-OH), the effect on both Th1/Th2 response and EAE test is fairly large.

7. Exacerbation of invasive Candida albicans infection by commensal bacteria or a glycolipid through IFN-γ produced in part by iNKT cells.

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The commensal yeast Candida albicans is a major cause of invasive fungal infections. Despite treatment with antifungal agents, the mortality rate attributed to these types of infection is high. Although numerous cases have been reported regarding a poor outcome for patients with bacterial and C. albicans coinfection, the mechanisms by which the coinfecting bacteria exacerbate the C. albicans infection remain elusive.

We evaluated how glycolipid-mediated activation of iNKT cells affects the clearance of C. albicans. Surprisingly, C. albicans-infected, glycolipid-treated mice exhibited significantly lower survival rates, increased fungal burden, and higher interleukin (IL)-6 production in the kidneys compared with control mice. Glycolipid-induced exacerbation of C. albicans infection was not observed in interferon-gamma knockout (IFN-γ KO) mice. In the C. albicans-infected, glycolipid-treated mice, the number of neutrophils in the blood and bone marrow dramatically decreased in an IFN-γ-dependent manner. Furthermore, mice that were coinfected with C. albicans and nonfermentative gram-negative commensal bacteria exhibited increased fungal burden and inflammatory cytokine production in the kidneys that were dependent on IFN-γ and iNKT cells. Our results indicate that coinfecting commensal bacteria exacerbate C. albicans infection through IFN-γ produced, in part, by iNKT cells.

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Center for Stem Cell Biology and Regenerative Medicine

Laboratory of Stem Cell Regulation
幹細胞制御領域

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The goal of our laboratory is to identify novel therapeutic targets for diseases like cancer or inflammatory diseases by studying the role inflammatory and adult stem cells. Persistent inflammation is associated with diseases, including cancer, atherosclerosis, arthritis and autoimmune diseases. Recently, we identified plasmin as a novel therapeutic target for the treatment of inflammatory diseases like inflammatory bowel disease, sepsis and chronic graft-versus host disease after bone marrow transplantation.

1. Inhibition of Plasmin Protects Against Colitis in Mice by Suppressing Matrix Metalloproteinase 9-mediated Cytokine Release From Myeloid Cells

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BACKGROUND & AIMS: Activated proteases such as plasmin and matrix metalloproteinases (MMPs) are activated in intestinal tissues of patients with active inflammatory bowel diseases. We investigated the effect of plasmin on progression of acute colitis.

METHODS: Colitis was induced in $Mmp9^{-/-}$, $Plg^{-/-}$, and C57BL/6 (control) mice by administration of dextran sulfate sodium, trinitrobenzene sulfonic acid, or CD40 antibody. Plasmin was inhibited in control mice by intraperitoneal injection of YO-2, which blocks its active site. Mucosal and blood samples were collected and analyzed by reverse transcription polymerase chain reaction and immunohistochemical analyses, as well as for mucosal inflammation and levels of cytokines and chemokines.

RESULTS: Circulating levels of plasmin were increased in mice with colitis, compared with controls. Colitis did not develop in control mice injected with YO-2 or in $Plg^{-/-}$ mice. Colons from these mice had reduced infiltration of Gr1+ neutro-

phils and F4/80+ macrophages, and reduced levels of inflammatory cytokines and chemokines. Colonic inflammation and colitis induction required activation of endogenous MMP9. Following colitis induction, mice given YO-2, $Plg^{-/-}$ mice, and $Mmp9^{-/-}$ mice had reduced serum levels of tumor necrosis factor and CXCL5, compared to control mice.
CONCLUSIONS: In mice, plasmin induces a feedback mechanism in which activation of the fibrinolytic system promotes development of colitis, via activation of MMP9 or proteolytic enzymes. The proteolytic environment stimulates influx of myeloid cells into the colonic epithelium and production of tumor necrosis factor and CXCL5. In turn, myeloid CD11b+ cells release the urokinase plasminogen activator, which accelerates plasmin production. Disruption of the plasmin-induced chronic inflammatory circuit might therefore be a strategy for treatment of colitis.

2. Inhibition of plasmin attenuates murine acute graft-versus-host disease mortality by suppressing the matrix metalloproteinase-9-dependent inflammatory cytokine storm and effector cell trafficking

Aki Sato[1], Chiemi Nishida[2], Kaori Sato-Kusubata[2], Makoto Ishihara[1], Yoshihiko Tashiro[1], Ismael Gritli[2], Hiroshi Shimazu[1], Shinya Munakata[1], Hideo Yagita[3], Ko Okumura[3], Yuko Tsuda[4], Yoshio Okada[4], Arinobu Tojo[5], Hiromitsu Nakauchi[6], Satoshi Takahashi[5], Beate Heissig[2], Koichi Hattori[1,7]: [1]Department of Stem Cell Regulation, Center for Stem Cell Biology and Regenerative Medicine, Institute of Medical Science at the University of Tokyo (IMSUT), Tokyo, [2]Department of Stem Cell Dynamics, Center for Stem Cell Biology and Regenerative Medicine, Institute of Medical Science at the University of Tokyo (IMSUT), Tokyo, [3]Department of Immunology, Atopy (Allergy) Research Center, Juntendo University School of Medicine, Tokyo, [4]Faculty of Pharmaceutical Sciences, Kobe Gakuin University, Kobe, [5]Department of Hematology and Oncology, Center for Stem Cell Biology and Regenerative Medicine, Tokyo, [6]Department of Stem Cell Therapy, Center for Stem Cell Biology and Regenerative Medicine, Institute of Medical Science at the University of Tokyo (IMSUT), Tokyo, [7]Center for Genome and Regenerative Medicine, Juntendo University School of Medicine, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo, 113-8421, Japan.

The systemic inflammatory response observed during acute graft-versus-host disease (aGVHD) is driven by proinflammatory cytokines, a 'cytokine storm'. The function of plasmin in regulating the inflammatory response is not fully understood, and its role in the development of aGVHD remains unresolved. Here we show that plasmin is during the early phase of aGVHD in mice, and its activation correlated with aGVHD severity in humans. Pharmacological plasmin inhibition protected against aGVHD-associated lethality in mice. Mechanistically, plasmin inhibition impaired the infiltration of inflammatory cells, the release of membrane-associated proinflammatory cytokines including tumor necrosis factor-α (TNF-α) and Fas-ligand directly, or indirectly via matrix metalloproteinases (MMPs) and alters monocyte chemoattractant protein-1 (MCP-1) signaling. We propose that plasmin and potentially MMP-9 inhibition offers a novel therapeutic strategy to control the deadly cytokine storm in patients with aGVHD, thereby preventing tissue destruction.

3. Hes1 promotes blast crisis in chronic myelogenous leukemia through MMP-9 upregulation in leukemic cells

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High levels of HES1 expression are frequently found in $BCR\text{-}ABL^{+}$ chronic myelogenous leukemia

in blast crisis (CML-BC). In mouse bone marrow transplantation (BMT) models, co-expression of BCR-ABL and Hes1 induces CML-BC-like disease; however, the underlying mechanism remained elusive. Here, based on gene expression analysis, we show that MMP-9 is upregulated by Hes1 in common myeloid progenitors (CMPs). Analysis of promoter activity demonstrated that Hes1 upregulated MMP-9 by activating NF-κB. Analysis of 20 samples from CML-BC patients showed that MMP-9 was highly expressed in three, with two exhibiting high levels of HES1 expression. Interestingly, MMP-9 deficiency impaired the cobblestone area-forming ability of CMPs expressing BCR-ABL and Hes1 that were in conjunction with a stromal cell layer. In addition, CMPs expressing BCR-ABL and Hes1 secreted MMP-9, promoting the release of soluble Kit-ligand (sKitL) from stromal cells, thereby enhancing proliferation of the leukemic cells. In accordance, mice transplanted with CMPs expressing BCR-ABL and Hes1 exhibited high levels of sKitL as well as MMP-9 in the serum. Importantly, MMP-9 deficiency impaired the development of CML-BC-like disease induced by BCR-ABL and Hes1 in mouse BMT models. The present results suggest that Hes1 promotes the development of CML-BC, partly through MMP-9 upregulation in leukemic cells.

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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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Our major goal is to cure patients suffering from life-threatening diseases by the treatment with processing of various stem cells. Currently our efforts are directed toward the establishment of novel therapies using human pluripotent stem cells (hPSC), such as embryonic stem cells and induced pluripotent stem cells (ESC and iPSC, respectively), and the analysis of pathogenesis of a variety of disorders based on disease-specific iPS cells.

1. Novel method for efficient production of multipotential hematopoietic progenitors from human pluripotent stem cells

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ESC are pluripotent cells derived from the inner cell mass of preimplantation embryos, and iPSC are induced from somatic cells by nuclear reprogramming. Since both have the ability to be maintained in culture indefinitely as undifferentiated cells, yet they are capable of forming more differentiated cell types, they are expected as a novel source of human transplantable cells for the regenerative medicine. We then planed to produce hematopoietic stem cells (HSC) for therapeutic HSC transplantation and functional blood cells for transfusion medicine from these human pluripotent stem cells. In result, we developed a novel method for the efficient production of hematopoietic progenitor cells (HPC) from hESC and hiPSC by co-culture with AGMS-3 stromal cells, which originate from murine aorta-gonad-mesonephros (AGM) region at 11 to 12 dpc. In the co-culture, various hematopoietic progenitors were generated, and this hematopoietic activity was concentrated in cobblestone-like (CS) cells within differentiated human ESC or iPSC colonies. A fraction of CS cells expressed CD34 and retained a potential for endothelial cells. They also contained HPC, especially erythroid and multipotential HPC at high frequency. The multipotential HPC abundant among the CS cells produced all types of mature blood cells, including adult type β globin-expressing erythrocytes and tryptase and chymase-double positive mast cells (MC). They showed neither immature properties of PSC nor potentials to differentiate into endoderm and ectoderm at a clonal level. The developed co-culture system of hPSC can provide a novel source for hematopoietic and blood cells applicable to cellular therapies and drug screenings.

2. Derivation of blood cells from human pluripotent stem cells in culture without animal serum or cells

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It is inevitable to establish an *in vitro* culture method for the induction of hPSC, such as hESC or hiPSC, to differentiate into mature blood cells without animal serum and cells. To achieve this, we first induced hPSC to differentiate into mesenchymal stem cells (MSC). When human ES or iPS cells cultured on murine embryonic fibroblast (MEF) feeder cells were recultured on gelatin-coated culture dishes with platelet lysate (PL)-containing media in the absence of MEF feeder cells. Cells were passaged several times with PL containing media, and then MSC were induced after 6 to 8 weeks. The MSC were spindle-like shaped, revealed a phenotype of CD45−, CD34−, CD14−, CD105+, CD166+, CD31−, and SSEA-4−, and had the ability to differentiate into mesenchymal tissues such as bone, cartilage and fat in vitro. Murine MEF and undifferentiated hPSC were undetectable in the hPSC-derived MSC by reverse transcription polymerase chain reaction analysis. We then cocultured hPSC with MSC derived from hPSC themselves under serum-free condition. Two weeks later, a number of HPC appeared in the coculture. These HPC were cultured in hematopoietic colony assay using human serum. In result, hPSC-derived HPC produced various hematopoietic colonies, such as myeloid, erythroid and multilineage colonies, including all types of blood cells. The novel culture method must be useful for the clinical application of hPSC-derived blood cells.

3. Recapitulation of pathophysiological features of Wiskott Aldrich Syndrome using induced pluripotent stem cells (iPSCs)

Mozhgan Khalaj Amirhosseini[6], Haruna Takagi[6], Chieko Konishi[6], Huang-Ting Lin[6], Takashi Ishida[6], Chen-Yi Lai[6], Tomohiro Morio[7], Kohsuke Imai[7], Hiromitsu Nakauchi[4], Makoto Otsu: [6]Division of Stem Cell Bank, Center for Stem Cell Therapy and Regenerative Medicine, [7]Department of Pediatrics Perinatal and Maternal Medicine, Tokyo Medical and Dental University

Wiskott Aldrich syndrome (WAS) is an X-linked disease, which is caused by mutations in the gene encoding the WAS protein (WASp). As thrombocytopenia is the most typical feature of this disease, often exposing patients to a significant risk of life-threatening hemorrhage, it has been the main subject of study for many researchers. Because of limitations in disease modeling, precise mechanisms of the platelet abnormality remain to be elucidated. Here we established induced pluripotent stem cell (iPSC) lines from two XLT and one WAS patients as disease models to address the issues. We first confirmed that these disease-specific iPSCs retained gene mutations characteristic to each patient. Using our differentiation culture system, we demonstrated that numbers of both megakaryocyte (MK)s and platelets obtainable from both XLT- and WAS-iPSCs were significantly smaller than those from healthy iPSCs. Detailed analysis revealed that the observed defects were mainly due to insufficient production of proplatelet-bearing cells, but not to impaired platelet production per MK. Lentiviral-mediated gene transfer led to appearance of WASp expression in patient iPSC-derived MKs. The expression of WASp, however, did not reach the normal level that was seen in control-iPSC-MKs; yields of platelets showed some increase after gene transfer, but only marginally. Although further investigation is necessary, these results indicate the utility of iPSC-based disease modeling for WAS. We are now in the process of addressing how critically expression levels of WASp will affect the efficacy in platelet number recovery after gene transfer.

4. Demonstration of safe and efficacious gene therapy using X-CGD iPSCs.

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X-linked chronic granulomatous disease (XCGD) is caused by gp91phox deficiency. This compromises neutrophil (NEU) killing of phagocytosed pathogens due to impaired production of reactive oxygen species (ROS). In previous XCGD gene therapy clinical trials, the sustained persistence of gene marked cells could not be achieved without adversely triggering insertional mutagenesis. Additionally cellular recovery (gp91phox and ROS) had been incomplete on a per cell basis in comparison with healthy controls. This led to the hypothesis that the expression of ectopic gp91phox in developing NEUs could impede further differentiation into mature NEUs. To investigate this theory, a modeling system was established by generating patient autologous XCGD-iPSCs and its differentiation into NEUs. In this culture system, the hierarchical transition of NEU differentiation could be demonstrated from developing ($CD64^{dull}CD15^{dull}$) to mature ($CD64^{high}CD15^{high}$). Alpharetroviral vectors were used to transduce XCGD-iPSCs with the expression of codon optimized gp91phox cDNA driven by the ubiquitous EF1α short promoter. In healthy iPSC-derived NEUs, gp91phox expression and ROS production could only be detected in the mature fraction. In NEUs derived from transduced XCGD-iPSCs, functional recovery in the mature fraction was incomplete. Ectopic gp91phox expression could be detected in the developing fraction through in-

tracellular staining but not in healthy control cells. Most importantly, cell death was most prominent in developing NEUs ectopically expressing gp91 phox. Mechanistic studies are under way to investigate the role of non-physiological ROS production in inducing endoplasmic reticulum (ER) stress resulting in cell apoptosis. Therefore, affording cellular protection from the detrimental effects of non-physiological ROS production may improve XCGD clinical outcomes.

5. A New Strategy To Overcome The Cell Dose Barrier To Umbilical Cord Blood Transplants

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Umbilical cord blood (UCB) serves as a suitable donor source in hematopoietic stem cell transplantation (HSCT). However, UCB has the major drawback that is delayed engraftment due to its low graft cell numbers, which often limits its use in HSCT. To overcome this cell dose barrier, double units UCB transplantation (UCBT) has been attempted, but the time to engraftment is still relatively delayed. Based on the report by Japan Red Cross Society, the majority of UCB units remain unused clinically because of their insufficient graft cell doses. Overall, these facts prompted us to seek for a new strategy to improve UCBT by using multiple (more than three) units. We here provide a proof of feasibility of such an approach using mouse transplantation models.

To mimic a clinical setting of UCBT, we first established an insufficient cell dose model by using mouse BM KSL (c-Kit$^+$, Sca-1$^+$, lineage-marker-negative) cells. In this model, C57BL/6 (B6-Ly5.2, H2^b) mice were the recipients, and congenic B6-Ly5.1 mice (H2^b) were the primary donors of cell grafts. The recipient mice were lethally irradiated, and thus could not survive with only the "insufficient cell dose" of B6-Ly5.1 graft. The outcomes in HSCT were tested by the addition of mixed allogeneic KSL cells (multi-allo HSPCs) in comparison with the addition of B6-Ly5.1 KSL cells (congenic HSPCs) with the equivalent doses. The effects of multi-allo HSPC transplants were evaluated by recipients' survival rate and complete blood counts over time. Detailed donor cell contribution in peripheral blood and BM was also determined by flow cytometry analysis.

Interestingly, addition of multi-allo HSPCs rescued otherwise lethal recipients as effectively as congenic-HSPCs with the equivalent acceleration of hematopoietic recovery. Chimerism analysis, however, revealed that this "KSL cells alone" transplantation led to long-term existence of multi-donor hematopoiesis, which was not ideal for a clinical setting. We then replaced B6-Ly5.1 KSL grafts with the whole BM (WBM) grafts. Titration experiments determined 50,000 WBM cells as a single unit, mimicking an "insufficient dose" of unmanipulated UCB. Addition of multi-allo HSPCs in this model also showed complete protection of recipients from lethality and enhanced early hematopoietic recovery. Remarkably, dominant B6-Ly5.1 chimerism was established and maintained in this modified model. Experiments using subfractionation of the B6-Ly5.1 grafts demonstrated that small numbers of T cells were responsible to single-donor chimerism formation possibly through graft versus graft reactions. Finally, to maximize transient early hematopoietic reconstitution by multi-allo HSPCs (we call this "bridging effect"), we tested whether cultured hematopoietic stem cells (HSC, defined as CD34$^{negatvie/low}$ KSL cells) worked better than uncultured KSL cells. Using our defined protocol compatible with stem cell amplification, we demonstrated that a mixture of cultured allo-HSCs exhibited the bridging effect even superior to that of an uncultured KSL cell mixture.

6. Stage-specific roles for Cxcr4 signaling in murine hematopoietic stem/progenitor cells in the process of bone marrow repopulation

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Hematopoietic cell transplantation has proven beneficial for various diseases, but low-level engraftment would be problematic leading to patient mortality, requiring elucidation of the molecular determinant for successful engraftment. It remains unclear how hematopoietic stem/progenitor cells (HSPCs) home to the bone marrow (BM) microenvironment, initiate hematopoietic reconstitution, and maintain life-long hematopoiesis. By monitoring the *in vivo* kinetics of transplanted donor cells, we demonstrated that modification of Cxcr4 signaling in murine HSPCs did not affect homing/lodging events, but led to alteration in subsequent BM repopulation kinetics, with observations confirmed by both gain- and loss-of-function approaches. With the use of C-terminal truncated Cxcr4 as a gain-of-function effector, we showed that signal augmentation through Cxcr4 led to favorable *in vivo* repopulation of primitive cell populations in BM. These Cxcr4-augmented HSPCs exhibited *in vitro* enhanced seeding efficiencies in stromal cell co-cul-

tures and altered ligand-mediated phosphorylation kinetics of Extracellular signal-regulated kinases. Sustained signal enhancement, however, even with wild-type Cxcr4 overexpression resulted in poor peripheral blood reconstitution due to blunted release of donor hematopoietic cells from BM. We thus conclude that timely regulation of Cxcr4/CXCR4 signaling will provide donor HSPCs with enhanced repopulation potential, with preserving their ability to release progeny into peripheral blood for improved transplantation outcomes.

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Center for Stem Cell Biology and Regenerative Medicine

Division of Stem Cell Transplantation
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We are conducting clinical stem cell transplantation, especially using unrelated 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 preclinical 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. Myeloablative unrelated cord blood transplantation for Philadelphia chromosome-positive acute lymphoblastic leukemia: comparison with other graft sources from related and unrelated donors.

Konuma T, Kato S, Ooi J, Oiwa-Monna M, Tojo A, Takahashi S.

Philadelphia chromosome (Ph)-positive acute lymphoblastic leukemia (ALL) is a distinct clinical entity among ALL and is associated with adverse outcomes and higher rates of relapse when conventional chemotherapy is used alone. Although allogeneic hematopoietic stem cell transplantation (allo-HSCT) is a potentially curative therapy for patients with Ph + ALL, the impact of graft sources, particularly cord blood transplantation (CBT), on allo-HSCT for patients with Ph + ALL has yet to be clarified. We retrospectively compared clinical outcomes after unrelated CBT (n=20), unrelated bone marrow transplantation (n=7), and related bone marrow and peripheral blood stem cell transplantations (n=13) following myeloablative conditioning in 40 patients with Ph + ALL. Although graft source had no significant impact on survival or relapse, disease status at transplantation did significantly affect outcomes. These data suggest that unrelated CBT is feasible and should be considered early in the course of patients with Ph + ALL when HLA-compatible related and unrelated donors are not available.

2. Comparable long-term outcome of unrelated cord blood transplantation with related bone marrow or peripheral blood stem cell transplantation in patients aged 45 years or older with hematologic malignancies after myeloablative conditioning.

Konuma T, Kato S, Ooi J, Oiwa-Monna M, Kawamata T, Tojo A, Takahashi S.

We investigated whether bone marrow or peripheral blood stem cells from older sibling donors or cord blood from unrelated donors provided a better outcome in allogeneic hematopoietic stem cell transplantation for relatively older patients who were candidates for myeloablative conditioning. Clinical outcomes of 97 patients aged 45 years or older with hematologic malignancies who received unrelated cord blood transplantation (CBT) (n=66) or bone marrow transplantation (BMT) or peripheral blood stem cell transplantation (PBSCT) from related donors (n=31) were compared. The cumulative incidences of grades III to IV acute and exten-

sive chronic graft-versus-host diseases were similar between both groups. Although transplant-related mortality was significantly lower after CBT compared with BMT/PBSCT from related donors (hazard ratio [HR], .29, P=.04), overall mortality (HR, .72, P=.47) and relapse (HR, 2.02, P=.23) were not significantly different after CBT and BMT/PBSCT from related donors. These data suggest that CBT could be as safe and effective as BMT/PBSCT from older related donors for relatively older patients when it is used as a primary unrelated stem cell source.

3. Impact of sex incompatibility on the outcome of single-unit cord blood transplantation for adult patients with hematological malignancies.

Konuma T, Kato S, Ooi J, Oiwa-Monna M, Ebihara Y, Mochizuki S, Yuji K, Ohno N, Kawamata T, Jo N, Yokoyama K, Uchimaru K, Tojo A, Takahashi S.

Donor-recipient sex incompatibility has been associated with transplant outcomes in allogeneic hematopoietic SCT. Such outcomes might be because mHA encoded by Y chromosome genes could be immunological targets for allogeneic T cells and B cells to induce GVHD, GVL effect and graft failure. However, its effect on the outcome of cord blood transplantation (CBT) is yet to be clarified. We retrospectively analyzed 191 adult patients who received single-unit CBT after myeloablative conditioning for malignant disease in our institute. In multivariate analysis, male recipients with female donors had a higher incidence of extensive chronic GVHD (hazard ratio (HR) 2.97, P=0.02), and female recipients with male donors had a lower incidence of platelet engraftment (HR 0.56, P=0.02) compared with female recipients with female donors as the reference. Nevertheless, there was no increase in mortality following sex-incompatible CBT. These data suggested that donor-recipient sex compatibility does not have a significant impact on survival after myeloablative CBT for hematological malignancies.

4. Generation of multivirus-specificT Cells by a single stimulation of PBMCs with a peptide mixture utilizing serum-free medium.

Fujita Y, Tanaka Y, Takahashi S.

To meet the requirement for the viral infections after HSCT by broad viral antigens and in terms of regulation by the Japanese FDA, we generated mutivirus-specific T cells targeting 7 viruses (CMV, EBV, AdV, HHV-6, BKV, JCV, and VZV) in serum-free medium. PBMCs were stimulated with peptide mixture spanning the target antigens of 3(CMV, EBV, AdV) or 7 viruses as above and cultured in RPMI＋5％human serum (HS) or serum-free medium with cocktail of IL4 and IL7 for 9-12 days. The single-stimulated and cultured cells were analyzed with cell number, surface markers and IFNγ production by intracellular staining (ICS) or ELISpot assay. Starting from 20×10^6 of PBMCs, 3 viruses' antigens stimulated and cultured cells increased to average of 144.9×10^6 cells after 9-12 days of culture in RPMI＋5％HS versus average of 92.0×10^6 cells in serum-free medium (n=4). The phenotypes of these cells revealed CD3＋/CD4＋ central memory phenotype in both medium. Viral antigens-specific IFNγ productions were average of 9.8％ in RPMI＋5％HS versus average of 7.9％ in serum-free medium measured by ICS (n=3). Then, 20×10^6 of PBMCs were stimulated with peptide mixture spanning the target antigens of 7 viruses and cultured in serum-free medium with cocktail of IL4 and IL7 for 9-12 days to obtain 112.7×10^6 cells (n=9). These cells were mostly CD3＋ (average 95.6％), which contained both CD4＋ (average 74.1 ％) and CD8＋ (average 20.8％) and they expressed central memory markers (average 80％ of CD3＋ CD62L＋CD45RO＋ cells). These single stimulated and cultured cells showed specificity toward all the 7 virus antigens measured by IFNγ ELISpot. The average of spot forming cells (SFCs) toward CMV, EBV, AdV, BKV, HHV6, JCV, VZV and negative control were 1214, 989, 2606, 697, 811, 751, 821, 77 SFCs per 2×10^5 cells respectively (n=7). We could rapidly and easily generate 7 viruses-specific T cells with a single stimulation of PBMCs without using any serum products. This system is ready to go to the clinical trials after allogeneic transplantation setting.

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Center for Stem Cell Biology and Regenerative Medicine

Division of Stem Cell Signaling
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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 experiment. We are now conducting several projects related to stem cells to characterize stem cells, clarify underling mechanisms of maintenance of pluripotency, and differentiation.

1. RasGRP family proteins and Leukemia

Toshihiko Oki, Jiro Kitaura, Koutarou Nishimura, Akie Maehara, Tomoyuki Uchida, Fumio Nakahara, and Toshio Kitamura

The Ras guanyl nucleotide-releasing proteins (RasGRPs) are a family of guanine nucleotide-exchange factors, with four members (RasGRP1-4), which positively regulate Ras and related small GTPases. In the previous study, we identified RasGRP4 using expression cloning as a gene that fully transformed IL-3-dependent HF6 cells, and demonstrated that in a mouse bone marrow transplantation (BMT) model, RasGRP4 induced acute myeloid leukemia (AML) and/or T-ALL. On the other hand, it has been reported that RasGRP1 transgenic mice developed thymic lymphoma or skin tumors.

However, the roles of RasGRP family proteins in leukemogenesis have not been investigated in detail. We have recently characterized leukemogenicity of RasGRP1 and 4 in details using a BMT model (Oki et al. Leukemia 2012).

RasGRP1 exclusively induced T-cell acute lymphoblastic leukemia/lymphoma (T-ALL) after a shorter latency when compared to RasGRP4. Accordingly, Ba/F3 cells transduced with RasGRP1 survived longer under growth factor withdrawal or phorbol ester stimulation than those transduced with RasGRP4, presumably due to the efficient activation of Ras. Intriguingly, *NOTCH1* mutations resulting in a gain of function were found in 77% of the RasGRP1-mediated mouse T-ALL samples. In addition, gain-of-function *NOTCH1* mutation was found in human T-cell malignancy with elevated expression of RasGRP1. Importantly, RasGRP1 and NOTCH1 signaling cooperated in the progression of T-ALL in the murine model. The leukemogenic advantage of RasGRP1 over RasGRP4 was attenuated by the disruption of a PKC phosphorylation site (RasGRP1(Thr184)) which RasGRP4 is lacking. In summary, cooperation between aberrant expression of RasGRP1, a strong activator of Ras, and secondary gain-of-function mutations of *NOTCH1* plays an important role in T-cell leukemogenesis.

2. Development of new retroviral vectors.

Toshikhiko Oki, Jiro Kitaura, Yutaka Enomoto, Tomoyuki Uchida, Fumi Shibata-Minoshima, and Toshio Kitamura:

We previously developed an effective retroviral transduction system consisted of vectors named as pMXs, pMYs, pMZs and pMCs and packaging cells named as PLAT-E, PLAT-A, and PLAT-F. Based on this system, we developed new vectors including

vectors with luciferase maker (pMX-IL), vectors for GFP or RFP fusion proteins, vectors with lox sequences for deletion of inserted genes with Cre-loxP, Tet-On and Tet-Off systems, vectors for expression, inhibition, and monitoring the expression of miroRNA (pMXe series). We utilized these vectors in studying stem cell biology and also in developing the innovative tools for regenerative medicine

3. Co-ordinate control of cell division and cell fate of by the Rho family small GTPases.

Toshihiko Oki, Kohtaro Nishimura, Toshiyuki Kawashima, and Toshio Kitamura:

We previously identified MgcRacGAP through functional cloning as a protein that enhances or induces macrophage differentiation of leukemic cell lines M1 and HL60. Interestingly, MgcRacGAP plays distinct roles depending on the cell cycle. In the interphase, it plays critical roles in activation and nuclear translocation of STAT3 and STAT5 as a Rac-GAP. In the mitotic phase, MgcRacGAP plays essential roles in completion of cytokinesis as a Rho-GAP. Interestingly, Aurora B-mediated phosphorylation of S387 converts MgcRacGAP from Rac-GAP to Rho-GAP.

We have recently shown that expression of MgcRacGAP is regulated by a cell cycle- dependent manner: MgcRacGAP expression increases in S/G2/M phase and decreases in early G1 phase, suggesting that MgcRacGAP may play some roles in G1 check point. In addition to the transcriptional control, MgcRacGAP protein levels are controlled by ubiquitin-dependent degradation, leading to its decrease in G1 phase. Using the proteome analysis and retroviral transduction, we identified APC/CDH1 as an E3 ligase involved in regulation of MgcRacGAP and the degron in MgcRacGAP. Now we are investigating the physiological roles of this regulation. In summary, our results implicate MgcRacGAP in coordination of cell cycle progression and cell fate determination.

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

Toshiyuki Kawashima, Akiho Tsuchiya, Toshihiko Oki, Jiro Kitaura, and Toshio Kitamura:

STAT3 is frequently activated in many cancers and leukemias, and is required for transformation of NIH3T3 cells. Therefore, we have started searching for STAT3 inhibitors. We already established an efficient screening protocol for identification of STAT3 inhibitors, and identified several compounds that inhibit STAT3 activation. Through the screening of a library of small molecule compounds, we found the compounds RJSI-1 and RJSI-2 that inhibited STAT3 activation. RJSI-2 also inhibited activation of STAT1, STAT5, JAK1 and JAK2, however RJSI-2 is not a kinase inhibitor. On the other hand, RJSI-1 inhibited nuclear transport of phosphorylated STAT proteins, implicating a novel mechanism in inhibiting STAT proteins. We have also shown that these compounds are effective in a tumor-burden mouse model. In addition, we collaborate with a US biotech venture company in modification of RSJI-1 for optimization to develop anticancer drugs, and have developed JP1156 which kill the tumor cells with much lower IC50. In addition to STAT3 inhibitors, we have started a new project to develop STAT5 inhibitors in collaboration with a pharmaceutical company. To this end, we are now in the process to establish a screening method to search for STAT5 inhibitors.

5. Development of G0 indicator

Toshihiko Oki, Kotarou Nishimura, Jiro Kitarura, Fumio Nakahara, Asako Sakaue-Sawano[2], Atsushi Miyawaki[2], Toshio Kitamura: [2]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 tissue specific stem cells like hematopietic stem cells and cancer stem cells with tumor initiating potentials are in G0 phase.

Recently we have developed the system to indicate cells in G0 phase. It is a system to monitor the amount of p27, which is destructed during G0 to G1 phase and is not expressed in S/G2/M phase, using the cells retrovirally trasduced with the fusion protein between a fluorescent protein like mVenus and p27K- (a p27 mutant lacking CDK inhibitory activities) as a similar cell cycle indicator system, fluorescent, ubiquitination-based cell cycle indicator, (Fucci). mVenus-p27K- positive cells are Ki67 negative quiescent cells and mVenus-p27K- signals are enhanced when the cycling cell enter G0 phase in response to serum starvation or contactinhibition.

Using this system, we identified genetic signatures of G0 cells. Several genes specifically expressed in G0 cells are now being investigated in terms of their functions and biological significance in G0 phase. The mVenus-p27K- trasgenic mice have also been generated to track several kinds of tissue specific stem cells in vivo. Now we are generating a Rosa26-knock in mouse.

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Center for Stem Cell Biology and Regenerative Medicine

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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. We demonstrated that the matrix metalloproteinase-9 is activated during leukemic cell progression. In addition, the serine proteinase plasmin plays a role in the myeloid cell recruitment in inflamed tissues during inflammatory bowel disease, sepsis and graft versus host disease after bone marrow transplantation.

1. Inhibition of plasmin attenuates murine acute graft-versus-host disease mortality by suppressing the matrix metalloproteinase-9-dependent inflammatory cytokine storm and effector cell trafficking

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The systemic inflammatory response observed during acute graft-versus-host disease (aGVHD) is driven by proinflammatory cytokines, a 'cytokine storm'. The function of plasmin in regulating the inflammatory response is not fully understood, and its role in the development of aGVHD remains unresolved. Here we show that plasmin is activated during the early phase of aGVHD in mice, and its activation correlated with aGVHD severity in humans. Pharmacological plasmin inhibition protected against aGVHD-associated lethality in mice. Mechanistically, plasmin inhibition impaired the infiltration of inflammatory cells, the release of membrane-associated proinflammatory cytokines including tumor necrosis factor-α (TNF-α) and Fas-ligand directly, or indirectly via matrix metalloproteinases (MMPs) and alters monocyte chemoattractant protein-1 (MCP-1) signaling. We propose that plasmin and potentially MMP-9 inhibition offers a novel therapeutic strategy to control the deadly cytokine storm in patients with aGVHD, thereby preventing tissue destruction.

2. Inhibition of plasmin protects against colitis in mice by suppressing matrix metalloproteinase 9-mediated cytokine release from myeloid cells

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Activated proteases such as plasmin and matrix metalloproteinases (MMPs) are activated in intestinal tissues of patients with active inflammatory bowel diseases. We investigated the effect of plasmin on progression of acute colitis. Colitis was induced in Mmp9−/−, Plg−/−, and C57BL/6 (control) mice by administration of dextran sulfate sodium, trinitrobenzene sulfonic acid, or CD40 antibody. Plasmin was inhibited in control mice by intraperitoneal injection of YO-2, which blocks its active site. Mucosal and blood samples were collected and analyzed by reverse transcription polymerase chain reaction and immunohistochemical analyses, as well as for mucosal inflammation and levels of cytokines and chemokines. We showed that circulating levels of plasmin were increased in mice with colitis, compared with controls. Colitis did not develop in control mice injected with YO-2 or in Plg−/− mice. Colons from these mice had reduced infiltration of Gr1+ neutrophils and F4/80+ macrophages, and reduced levels of inflammatory cytokines and chemokines. Colonic inflammation and colitis induction required activation of endogenous MMP9. Following colitis induction, mice given YO-2, Plg−/− mice, and Mmp9−/− mice had reduced serum levels of tumor necrosis factor and CXCL5, compared to control mice. In summary, in mice, plasmin induces a feedback mechanism in which activation of the fibrinolytic system promotes development of colitis, via activation of MMP9 or proteolytic enzymes. The proteolytic environment stimulates influx of myeloid cells into the colonic epithelium and production of tumor necrosis factor and CXCL5. In turn, myeloid CD11b+ cells release the urokinase plasminogen activator, which accelerates plasmin production. Disruption of the plasmin-induced chronic inflammatory circuit might therefore be a strategy for treatment of colitis.

3. Hes1 promotes blast crisis in chronic myelogenous leukemia through MMP-9 upregulation in leukemic cells

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High levels of HES1 expression are frequently found in BCR-ABL$^+$ chronic myelogenous leukemia in blast crisis (CML-BC). In mouse bone marrow transplantation (BMT) models, co-expression of BCR-ABL and Hes1 induces CML-BC-like disease; however, the underlying mechanism remained elusive. Here, based on gene expression analysis, we show that MMP-9 is upregulated by Hes1 in common myeloid progenitors (CMPs). Analysis of promoter activity demonstrated that Hes1 upregulated MMP-9 by activating NF-κB. Analysis of 20 samples from CML-BC patients showed that MMP-9 was highly expressed in three, with two exhibiting high levels of HES1 expression. Interestingly, MMP-9 deficiency impaired the cobblestone area-forming ability of CMPs expressing BCR-ABL and Hes1 that were in conjunction with a stromal cell layer. In addition, CMPs expressing BCR-ABL and Hes1 secreted MMP-9, promoting the release of soluble Kit-ligand (sKitL) from stromal cells, thereby enhancing proliferation of the leukemic cells. In accordance, mice transplanted with CMPs expressing BCR-ABL and Hes1 exhibited high levels of sKitL as well as MMP-9 in the serum. Importantly, MMP-9 deficiency impaired the development of CML-BC-like disease induced by BCR-ABL and Hes1 in mouse BMT models. The present results suggest that Hes1 promotes the development of CML-BC, partly through MMP-9 upregulation in leukemic cells.

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