Center for Stem Cell Biology and Regenerative Medicine

Division of Stem Cell Therapy
幹細胞治療分野

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Stem cell based regenerative medicine has been a focus of attention worldwide. In particular, recent development of the iPS cell technology has enabled generation of patient's pluripotent stem cells (PSCs), opening up the way to regenerative medicine using patient's own PSC-derived cells. 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. Nonmyelinating Schwann cells maintain hematopoietic stem cell hibernation in the bone marrow niche.

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Hematopoietic stem cells (HSCs) reside and self-renew in the bone marrow (BM) niche. Overall, the signaling that regulates stem cell dormancy in the HSC niche remains controversial. Here, we demonstrate that TGF-β type II receptor-deficient HSCs show low-level Smad activation and impaired long-term repopulating activity, underlining the critical role of TGF-β/Smad signaling in HSC maintenance. TGF-β is produced as a latent form by a variety of cells, so we searched for those that express activator molecules for latent TGF-β. Nonmyelinating Schwann cells in BM proved responsible for activation. These glial cells ensheathed autonomic nerves, expressed HSC niche factor genes, and were in contact with a substantial proportion of HSCs. Autonomic nerve denervation reduced the number of these active TGF-β-producing cells and led to rapid loss of HSCs from BM. We propose that glial cells are components of a BM niche and maintain HSC hibernation by regulating activation of latent TGF-β.

2. Generation of germline-competent rat induced pluripotent stem cells.

Sanae Hamanaka[1,2], Tomoyuki Yamaguchi[1,2*],

Toshihiro Kobayashi[1,2], Megumi Kato-Itoh[2], Satoshi Yamazaki[2], Hideyuki Sato[2], Ayumi Umino[2], Yukiko Wakiyama[2], Mami Arai[2], Makoto Sanbo[3], Masumi Hirabayashi[3,4], Hiromitsu Nakauchi[1,2*]: [1]Japan Science Technology Agency, Exploratory Research for Advanced Technology (ERATO), Nakauchi Stem Cell and Organ Regeneration Project, Tokyo, Japan, [2]Division of Stem Cell Therapy, Center for Stem Cell Biology and Regenerative Medicine, Institute of Medical Science, University of Tokyo, Tokyo, Japan, [3]Center for Genetic Analysis of Behavior, National Institute for Physiological Sciences, Okazaki, Japan, [4]School of Life Science, The Graduate University for Advanced Studies, Okazaki, Japan

BACKGROUND: Recent progress in rat pluripotent stem cell technology has been remarkable. Particularly salient is the demonstration that embryonic stem cells (ESCs) in the rat (rESCs) can contribute to germline transmission, permitting generation of gene-modified rats as is now done using mouse ESCs (mESCs) or mouse induced pluripotent stem cells (iPSCs; miPSCs). However, determinations of whether rat iPSCs (riPSCs) can contribute to germ cells are not published. Here we report the germline competency of riPSCs.

METHODOLOGY/PRINCIPAL FINDINGS: We generated riPSCs by transducing three mouse reprogramming factors (Oct3/4, Klf4, and Sox2) into rat somatic cells, followed by culture in the presence of exogenous rat leukemia inhibitory factor (rLIF) and small molecules that specifically inhibit GSK3, MEK, and FGF receptor tyrosine kinases. We found that, like rESCs, our riPSCs can contribute to germline transmission. Furthermore we found, by immunostaining of testis from mouse-rat interspecific chimeras with antibody against mouse vasa homolog, that riPSCs can contribute to embryonic development with chimera formation in mice (rat-mouse interspecific chimeras) and to interspecific germlines.

CONCLUSIONS/SIGNIFICANCE: Our data clearly demonstrate that using only three reprogramming factors (Oct3/4, Klf4, and Sox2) rat somatic cells can be reprogrammed into a ground state. Our generated riPSCs exhibited germline transmission in either rat-rat intraspecific or mouse-rat interspecific chimeras.

3. Prospective isolation and characterization of bi-potent progenitor cells in early mouse liver development.

Ken Okada, Akihide Kamiya, Keiichi Ito, Ayaka Yanagida, Hidenori Ito, Hiroki Kondou, Hiroshi Nishina and Hiromitsu Nakauchi

Outgrowth of the foregut endoderm to form the liver bud is considered the initial event of liver development. Hepatic stem/progenitor cells (HSPCs) in the liver bud are postulated to migrate into septum transversum mesenchyme at around embryonic day (E) 9 in mice. The studies of liver development focused on the mid-fetal stage (E11.5-14.5) have identified HSPCs at this stage. However, the *in vitro* characteristics of HSPCs before E11.5 have not been elucidated. This is probably partly because purification and characterization of HSPCs in early-fetal livers have not been fully established. To permit detailed phenotypic analyses of early-fetal HSPC candidates, we developed a new co-culture system, using mouse embryonic fibroblast cells. In this co-culture system, $CD13^{+}Dlk^{+}$ cells purified from mouse early-fetal livers (E9.5 and E10.5) formed colonies composed of both albumin-positive hepatocytic cells and cytokeratin (CK) 19-positive cholangiocytic cells, indicating that early-fetal $CD13^{+}Dlk^{+}$ cells have properties of bi-potent progenitor cells. Inhibition of signaling by Rho-associated coiled-coil containing protein kinase (Rock) or by non-muscle myosin II (downstream from Rock) was necessary for effective expansion of early-fetal $CD13^{+}Dlk^{+}$ cells *in vitro*. In sorted $CD13^{+}Dlk^{+}$ cells, expression of the hepatocyte marker genes albumin and α-fetoprotein increased with fetal liver age, while expression of CK19 and Sox17, endodermal progenitor cell markers, was highest at E9.5 but decreased dramatically thereafter. These first prospective studies of early-fetal HSPC candidates demonstrate that bi-potent stem/progenitor cells exist before E11.5 and implicate Rock-myosin II signaling in their development.

4. *In vitro* expansion and functional recovery of mature hepatocytes from mouse adult liver.

Hidenori Ito, Akihide Kamiya, Keiichi Ito, Ayaka Yanagida, Ken Okada, and Hiromitsu Nakauchi

Mature hepatocytes retain the ability to regenerate the liver lobule fully *in vivo* following injury. Several cytokines and soluble factors (hepatocyte growth factors, epidermal growth factors, insulin, and nicotinamide) are known to be important for proliferation of mature hepatocytes *in vitro*. However, hepatocytes monolayer-cultured on extracellular matrices have gradually lost their specific functions, particularly those in drug metabolism. We have explored

and established a new culture system for expansion of functional hepatocytes. We evaluated two approaches to efficient expansion of mature hepatocytes: 1. Co-culture with mouse embryonic fibroblasts (MEF); 2. Addition to culture of inhibitors of cell signals involved in liver regeneration. After expansion steps, 3-dimensional spheroid-forming culture was used to re-induce mature hepatocellular function. The addition of inhibitors for tumor growth factor (TGF) β and glycogen synthase kinase (GSK) 3β efficiently induced *in vitro* expansion of mature hepatocytes. Although expression of hepatocellular functional genes decreased after expansion in monolayer culture, their expression and the activity of cytochrome P450 enzymes significantly increased with spheroid formation. Furthermore, when hepatocytes were co-cultured with MEF, addition of a MAPK/ERK kinase (MEK) inhibitor at the spheroid formation step enhanced drug-metabolism-related gene expression. Combination of the MEF co-culture system with the addition of inhibitors of TGFβ and GSK3β induced *in vitro* expansion of hepatocytes. Moreover, expression of mature hepatocellular genes and the activity of drug-metabolism enzymes in expanded hepatocytes were re-induced after spheroid culture.

5. Generation of antigen specific functional T cells from human induced pluripotent stem cells of a single T cell origin.

Toshinobu Nishimura[1], Shin Kaneko[1], Ai Kawana-Tachikawa[2], and Hiromitsu Nakauchi[1]: [1]Division of Stem Cell Therapy, Center for Stem Cell Biology and Regenerative Medicine, [2]Division of Infectious Disease, The Advanced Clinical Research Center, Institute of Medical Science, The University of Tokyo, Tokyo, Japan

Adoptive immunotherapy with antigen-specific T cells expressing a limited TCRs repertoire is a promising approach to fight against various types of cancers or chronic viral infections. However, its effectiveness declines due to the exhaustion of antigen-specific T cells. To overcome this problem, we have explored the potential of induced pluripotent stem (iPS) cell technology and generated T cell-derived iPS (T-iPS) cells. Furthermore, we have succeeded in redifferentiation of T-iPS cells into functional T cells specific to the original antigen. As was expected, invariance of the antigen-specificity was evidenced by the fact that TCR gene rearrangement patterns in the re-differentiated T cells were identical to those found in their original T cell. Taken together, regeneration of antigen-specific immune systems using iPS cell technology will shed new light on the field of adoptive immunotherapy.

6. A pluripotent stem cell specific cell-suicide system for prophylaxis and treatment of teratocarcinoma in autologous iPSC-based cell and gene therapy

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Induced pluripotent stem cells (iPSCs) have been expected for their potential use as cell sources of regenerative medicine. iPSC-based therapy, however, would have a latent risk of teratomagenesis by contamination of undifferentiated iPSCs in the graft. Aiming at realizing safer iPSC-based therapies by reducing the risk, we've been trying to develop an inducible "cell-suicide" system which enables undifferentiated cells to be eliminated *in vitro* and *in vivo*.

We have developed a lentiviral vector NpTk to express herpes simplex virus thymidine kinase (HSV-TK) gene under the control of *Nanog* promoter. The vector was designed to work with prodrug ganciclovir (GCV) to induce cell-suicide only in undifferentiated iPSCs. C57BL/6 mouse tail-tip fibroblast derived murine iPSCs were transduced by the vector and tested for GCV sensitivity. Under a leukemia inhibitory factor (LIF) containing culture condition, NpTk/iPSCs showed clear cell death within 48 hours with GCV. On the contrary, differentiating NpTk/iPSCs in embryoid body (EB) showed GCV resistance as they decrease *Nanog* expression. In addition, when NpTk/iPSCs have differentiated into pre-hematopoietic stem cells in the presence of GCV, functional hematopoietic cells were successfully obtained without contamination of significant number of undifferentiated cells. These data suggested that iPSC-differentiation with the cell-suicide system works well for reduceing the risk of undifferentiated cell contamination during cell processing.

Next, we inoculated 1×10^6 NpTk/iPSCs to B6 mice with or without GCV treatment to observe whether the undifferentiated cell specific suicide system can prevent teratomagenesis *in vivo*. The group starting 2-week GCV treatment simultaneously with iPSC-innoculation showed effective prevention of teratomagenesis and the group starting 4-week GCV after teratoma formation showed effective elimination of pathologically immature cells in teratoma.

In conclusion, the results indicate the feasibility of "pluripotent cell specific promoter-driven cell-suicide system" *in vitro* and *in vivo*, as a safety switch for prevention and treatment of teratocarcinoma in iPSC-based therapies.

7. The entities and molecular pathogenesis of Primary Myelofibrosis

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The gain-of-function JAK 2 mutation, JAK2V617F, is the most common molecular abnormality in Myeloproliferative neoplasms (MPNs) and appears in most of the patients with polycythemia vera (PV) and in half of the patients with essential thrombocythemia (ET) and primary myelofibrosis (PMF). A number of JAK2V617F mediated mouse models with features of PV or ET have been reported, however, typical PMF model with the JAK2V617F had not been reported. We have succeeded to generate PMF mouse model by transplantation of hematopoietic stem cells (HSCs) with STAT5a(1*6), the constitutive active STAT5a, a direct downstream molecule of JAK2. Clinical conditions of the model were closely resemble to that of the human and fulfilled the WHO criteria.

Through the detail analysis of the model, we revealed that intensive STAT5a activity inducing c-Myc expression leads to increment of atypical Megakaryocytes (at-Mks). The at-Mks producing TGF-β, Oncostatin M and VEGF evoke mesenchymal stem cells (MSCs) abnormalities, which leads to aberrant fibrogenesis and angiogenesis in bone marrow (BM). These microenvironment failure, especially loss of nestin+ MSCs, resulted in reduction of HSC niches and followed HSC-exhaustion even to genetically unmodified HSC.

Taken together, the specific entity of PMF is HSCs exhaustion associated to MSCs abnormalities.

8. Elucidation of the mechanism of the development of pulmonary alvelor proteinosis accompanied with aberrant productions of Th1 cytokines.

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Mononuclear phagocyte system plays an important role in maintaining homeostasis and, under the inflammations and infections, triggers immune response to combat against invading pathogens. To date, it has been shown that altered regulations of the mononuclear phagocyte system involved in the pathogenesis of certain diseases. Among them, the pathogenesis of nearly 90% of congenital and acquired pulmonary alveolar proteinosis (PAP) patients has been identified to the disrupted granulocyte/macrophage-colony stimulating factor (GM-CSF) signaling in alveolar macrophages. However, little is known about the pathogensis of secondary PAP. In this study, we found that CD2-T-bet$^{tg/tg}$ mice developed PAP independent of the disruption of GM-CSF signaling under the influence of severe infiltration of T lymphocytes producing aberrant amount of type 1 helper T lymphocyte (Th1) cytokines in lung. Further study on the hematopoietic system of the mice together with the results of splenocyte transplantation experiment indicated that the over-expression of T-bet in T lymphocytes induces inflammatory condition and increases influx of inflammatory monocytes from bone marrow into the peripheral tissues of T-bet$^{tg/tg}$ mice. Moreover, *in vitro* BM colony assay of T-bet$^{tg/tg}$ raised the possibility that there could have been defects in monocyte/macrophage lineage commitment and the defect was partially corrected with the addition of M-CSF. Combined together, these results suggest that the imbalance in the activations of IFN-γ, M-CSF, and GM-CSF signalings in lung triggered by the Th1/Th2 cytokine production imbalance results in the development of pulmonary alveolar proteinosis. Our findings propose a new pathway in the pathogenesis of pulmonary alveolar proteinosis.

Publications

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Center for Stem Cell Biology and Regenerative Medicine

Laboratory of Diagnostic Medicine
病態解析領域

Project Associate Professor Nobukazu Watanabe, M.D., Ph.D.

特任准教授 医学博士 渡 辺 信 和

The Laboratory of Diagnostic Medicine was established in January 2009 as a division of the Center for Stem Cell Biology and Regenerative Medicine. Our major purpose is to conduct clinical research and develop analytical methods of pathogenic conditions during infectious disease, cancer and hematopoietic stem cell and organ transplantations. Through collaborations with hospitals in Japan, we have performed several problem-based clinical studies to tackle the issues of adult T cell leukemia (ATL) and pathogenic conditions after transplantation, e.g. cytomegalovirus infection, graft failure, acute graft versus host disease (GVHD), relapse of leukemia, and recurrence of hepatitis. In addition, we also started a new project to make allele-specific anti-human leukocyte antigen (HLA) monoclonal antibodies (ASHmAbs) using HLA-transgenic mouse and HLA-tetramers for the purpose of a flow cytometry-based method of chimerism analysis (HLA-Flow method).

1. Clinical studies through collaborations with hospitals

a) Phenotypic analysis of ATL cells and prediction of the onset of ATL from human T-lymphotropic virus type 1 (HTLV-1) asymptomatic carriers

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Among the one million HTLV-1 carriers in Japan, approximately one thousand progress to ATL every year. Through collaborations with the Research Hospital and two laboratories at IMSUT, we are analyzing ATL cells using a flow cytometry-based method of phenotypic analysis [HTLV-1 associated system (HAS)-Flow method] to monitor disease condition. In addition, we are analyzing peripheral blood from HTLV-1 carriers to find a predictable phenotypic change of immune cells just before ATL onset in order to begin more effective treatment.

b) Analysis of engraftment, ATL cells, and T cell activation after cord blood transplantation in patients with ATL.

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In a Japanese study group of cell therapy for ATL, cord blood transplantation is planned for patients with acute ATL. We are joining this study group and analyzing engraftment and ATL cells using HLA-Flow method and HAS-Flow method. In addition, we are analyzing T cell activation using intracellular cytokine staining for the purpose of regulation of immunosuppressants, e.g. cyclosporine A.

c) Studies for the mechanisms underlying persistent chimerism and late rejection after cord blood transplantation in patients with severe combined immunodeficiencies (SCID).

Eri Watanabe, Nobukazu Watanabe, Tomohiro Morio[5]: [5]Department of Pediatrics, Tokyo Medical Dental University

Although T cells and NK cells are lacked in patients with SCID, persistent chimerism and late rejection sometimes occur after cord blood transplantation. We analyze subpopulation-specific chimerism using HLA-Flow method and investigate the underlying mechanisms of these pathogenic conditions.

d) Studies for the mechanisms underlying recurrence of type C hepatitis and rejection after living-donor liver transplantation

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Since the 2004 approval of insurance coverage for living-donor liver transplantations (LDLT), more than 6,000 LDLTs have been performed in Japan. Although most recipients have a good prognosis, patients with hepatitis C virus (HCV) infection still face the recurrence of hepatitis after transplantation. In addition, rejection is an important issue because immunosuppressive agents are needed to suppress anti-graft immune reactions. Long-term use of immunosuppressants, however, can worsen HCV infections and future malignancies. To understand the mechanism underlying these pathologic conditions, we are investigating the following: chimerism analysis/HLA-Flow method, detection of regulatory T cells and allospecific T cells, and identification of HCV-specific $CD8^+$ T cells using tetramers.

2. Generation of allele-specific anti-HLA monoclonal antibodies (ASHmAbs)

Yusuke Nakauchi, Stephanie C. Napier, Satoshi Yamazaki[8], Nobukazu Watanabe, and Hiromitsu Nakauchi[8]: [8]JST-ERATO, IMSUT.

The difficulty in generating ASHmAbs is well known. We recently established a novel method for generating ASHmAb. Our strategy involves suppressing the production of non-allele-specific anti-HLA antibodies against xenogeneic determinants of HLA molecules by immunizing human HLA-A2, A24, and B51 transgenic mice against non-HLA-A2, A24, and B51 HLA tetramers. ASHmAbs generated in this manner will be useful for HLA typing and for clinical diagnoses, such as flow cytometry-based chimerism analysis for early detection of graft failure and relapse of leukemia after HLA-mismatched hematopoietic stem cell transplantation.

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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 precious cell source usable in various types of regenerative medicine. Hematopoietic stem cells provide a good example of the potential of stem cells as seen in successful cases in both the hematopoietic transplantation and gene therapy. Pluripotent stem cells are the emerging cell sources that may be utilized either for the basic research or to cure the diseases. Our aim is to establish safe and efficacious treatment for the patients suffering from diseases with no curative treatment available.

1. *In vitro* modeling of neutrophil development and function using iPSCs: clinical values in understanding the pathophysiology and applications in the treatment of chronic granulomatous disease

Lin Huan-Ting, Makoto Otsu, Naoya Takayama, Koji Eto, Hiromitsu Nakauchi

Neutrophil (NEU) differentiation is a dynamic process that can be recaptured *in vitro* using induced pluripotent stem cells (iPSCs). This process can be manipulated by cytokines, allowing for the study of these cells at specific stages in their development. Most importantly from a clinical perspective, by generating patient autologous iPSCs, it is possible to recapitulate a specific disease phenotype. Chronic granulomatous disease (CGD) is a congenital NEU disorder characterized by the impaired generation of reactive oxygen species (ROS). The transplantation of gene modified $CD34^+$ cells is the possible cure. In this study, we aim to highlight the clinical value of iPSCs as a disease model in elucidating the underlying pathophysiology and in assessing the effective recovery of cell functions following gene modification.

Peripheral blood (PB) $CD34^+$ cells were isolated from two CGD patients with gp91phox or p47phox deficiency and from a healthy donor. Cells were reprogrammed using a Sendai virus vector expressing Oct4/Sox2/Klf4/c-Myc. Self-inactivating lentiviral or alpharetroviral vectors were used to insert either gp91 or p47 cDNA into iPSCs. Neutrophil differentiation was induced using VEGF and G-CSF. WG, MPO and ALP staining was done to assess cell morphology. The immunophenotype profile of differentiating cells was assessed using macrophage and neutrophil specific antigens. Neutrophils were stimulated with PMA to analyze ROS production by the DHR flow cytometry assay. Neutrophil extracellular traps (NETs) were visualized by staining with SYTO 13 and anti-MPO antibody. Neutrophils from healthy donors served as the control.

Mature PB-NEUs and control iPSC-NEUs displayed the classic multi-lobed appearance of the nucleus. ROS was generated at comparable levels and both populations were able to form NETs. However, CGD iPSC-derived NEUs (gp91 phox and p47) appeared to show impaired development. This was suggested by the fact that at day 7 of the differentiation culture, only a proportion of the population stained positive for ALP, which is a protein found in secretory ves-

icles. At the same time point, nearly all control iPSC-NEUs stained positive for ALP. CGD iPSC-derived NEUs were ROS negative at all time points and displayed impaired formation of NETs. The loss of these functions however, was recovered in NEUs differentiated from gene modified CGD iPSCs.

This is the first report to show that *in vitro* differentiated NEUs have the capacity to form NETs. Only mature NEUs with a complete repertoire of cellular components and normal ROS generating capacity have this property. Along with other matching characteristics to PB NEUs, these results may be taken as validation of the accuracy with which this model can be utilised to mimic NEU physiology and development *in vivo*. Indeed, in this instance it has been utilized to uncover a previously unreported impairment in NEU development in CGD. Further work is required to determine its implications. Furthermore, we have shown that vector mediated gene transfer can recover the characteristic loss of function associated with CGD NEUs. It is possible that this disease model may also be used to study insertion site profiling for example. This allows therapeutic vectors to be evaluated for clinical safety thus minimizing the potential risk of genotoxicity and possible harm to patients.

2. Evidence for the involvement of CXCR4 signaling in *in vivo* self-renewal of transplanted hematopoietic stem cells

Chen-Yi Lai, Sachie Suzuki, Motohito Okabe, Satoshi Yamazaki, Makoto Otsu, Hiromitsu Nakauchi

Hematopoietic stem cells (HSCs) represent the unique cell population capable of self-renewal and multi-lineage differentiation, thereby lifelong sustainment of the hematopoiesis. HSC transplantation has proven beneficial for various diseases, it is therefore important to elucidate the molecular determinants for successful HSC engraftment. Signaling through the chemokine receptor CXCR4 has been implicated in HSC engraftment by the observation that transplantation of HSCs lacking this molecule results in poor hematopoietic reconstitution. Because this impairment, however, can be attributed to the defects in any of the post-transplantation processes that include bone marrow (BM)-homing, -repopulation, or -retention, it is still unclear whether CXCR4 plays an essential role in HSC self-renewal upon transplantation.

To elucidate the role of CXCR4 signaling in HSC self-renewal in conjunction with transplantation, we used a purified $CD34^{neg/low}$ c-Kit^{+} Sca-1^{+} Lineage-$marker^{neg}$ population as the defined stem cell source. As a loss-of-function study, CXCR4 was conditionally deleted in HSCs before transplantation. As a gain-of-function study, we generated the HSC populations overexpressing either wild-type (wt)- or C-terminal truncated (ΔC)-CXCR4, the latter of which is known to exhibit enhancement in the SDF-1 signaling, by gene transfer and subsequent cell sorting. We compared these cells with control HSCs in *in vitro* assays with regard to the biological characteristics including chemotaxis, proliferation, colony formation, and cobblestone-area (CA) forming ability. To dissect *in vivo* post-transplantation processes, we investigated hematopoietic repopulation kinetics in the recipient BM at the homing/lodging phase (within 1 wk) and the early repopulation phase (2-3 wks) for the above test HSCs. The self-renewal potential of each HSC population was estimated by competitive repopulation assay.

In vitro studies: HSCs overexpressing wt- or ΔC-CXCR4 exhibited enhanced chemotaxis and proliferation in response to SDF1, confirming the gain-of-function effects of these modifications. CA forming ability was greater in HSCs overexpressing ΔC-CXCR4 than control counterparts and absent in CXCR4-KO HSCs, suggesting the critical role of CXCR4-signaling in HSC proliferation in the presence of stromal support.

In vivo studies: 1) the homing/lodging phase. Unexpectedly, we did not find significant alteration in the numbers of early progenies detectable in recipient BM 3 days after transplantation of HSCs receiving either loss- or gain-of-function modification to CXCR4, indicating that this signaling is indispensable in HSC homing. 2) the early repopulation phase. Impairment of hematopoietic repopulation in BM became evident for CXCR4-KO HSCs through 2-3 wks. On the other hand, HSCs overexpressing CXCR4, more remarkably of ΔC-mutation, showed enhanced BM repopulation kinetics at ~3 wks post transplantation, suggesting the importance of CXCR4 signaling in HSC amplification at this post-transplantation phase. 3) long-term hematopoiesis. CXCR4-KO-HSCs showed poor hematopoietic reconstitution potentials, consistent with previous observations. Interestingly, impaired peripheral repopulation was also observed with HSCs overexpressing wt- or ΔC-CXCR4. Further characterization revealed that the recipients of CXCR4-overexpressing HSCs did retain their progenies, which showed multilineage differentiation, but exhibited impaired release of mature leukocytes from the BM to the peripheral blood. Most importantly, however, test-cell chimerism in the long-term HSC fraction was significantly higher in the mice receiving HSCs overexpressing CXCR4, especially of

ΔC-type, than those transplanted with control HSCs, indicating that the augmentation of CXCR4 signaling enhanced competitive repopulation ability of HSCs. These modified HSCs demonstrated repopulation abilities also in secondary recipients.

We demonstrated that CXCR4 signaling is indispensible for HSC homing and that continuous overexpression of CXCR4 cannot benefit the peripheral reconstitution in contrary to the expectation. More importantly, our studies showed that augmentation of CXCR4 signaling leads to HSC expansion *in vivo*. We thus conclude that CXCR4 signaling has a role in HSC self-renewal and that its regulation may find the approach that will improve HSC transplantation outcomes.

3. Platelet production system using an immortalized megakaryocyte cell line derived from human pluripotent stem cells

Sou Nakamura, Naoya Takayama, Hiromitsu Nakauchi, Koji Eto

Human induced pluripotent stem cells (hiPSCs) are a promising source of blood cells, including platelets, for transfusion. However, there remains a need for: 1) a method to obtain large numbers of cells, 2) a system to provide cells of a predefined quality, and 3) a method to overcome the storage limitations of platelets caused by their short shelf life.

To address these issues, we attempted to establish an immortalized megakaryocyte progenitor cell line derived from hiPSCs. We recently showed that the temporal profile of c-MYC activation during megakaryopoiesis is critical for normal platelet production from hiPSCs; that is, peak activation of c-MYC in megakaryocyte progenitors must be followed by a reduction of c-MYC expression for further maturation (Takayama et al. J Exp Med, 2010). Mechanistic analysis revealed that overexpression (O/E) of c-MYC increased megakaryocyte numbers but also induced apoptosis and senescence. Here we demonstrate that this phenomenon is primarily regulated by induction of the INK4A and ARF genes. When we examined the effects of a) c-MYC O/E and p53 knockdown, b) c-MYC O/E and BCL-XL (negative regulator of caspase family) O/E, and c) c-MYC O/E and BMI1 (negative regulator for both INK4A and ARF genes) O/E in hematopoietic progenitors derived from human embryonic stem cells (hESCs), we found that only c-MYC and BMI1 O/E (protocol b) increased numbers of $CD41a^+/CD42b^+$ non-polyploid megakaryocytes in an exponential manner for over 3 months. Neither c-MYC O/E and p53 knockdown (protocol a) nor c-MYC O/E and BCL-XL O/E (protocol c) were sufficient to maintain an increase in the megakaryocyte population, which suggests that down-regulation of INK4A and ARF contributes mainly to the prevention of excessive c-MYC-induced cell apoptosis and senescence. It thus appears that we were able to establish an immortalized megakaryocyte cell line (MKCL).

As mentioned, a decline in c-MYC activation during hiPSC-derived megakaryocyte maturation is required for generation of functional $CD41a^+/CD42b^+$ platelets *in vitro*. Excessively sustained c-MYC expression in megakaryocytes was accompanied by increased ARF and INK4A expression and decreased GATA1 and NF-E2 expression, eventually leading to megakaryocyte senescence and apoptosis, and $CD41a^+/CD42b^{low/-}$ platelet generation. By using an inducible expression vector system with c-MYC and BMI1 in this context, the MKCL was capable of generating polyploid megakaryocytes (>8N; 40%). The MKCL also subsequently showed proplatelet formation leading to the release of "functional" $CD41a^+/CD42b^+$ platelets. Furthermore, following transfusion of 6×10^8 platelets originally derived from the our immortalized MKCL into immunodeficient NOG mice, the platelets appeared to exhibit normal circulation a high degree of chimerism (human CD41a/ human CD41a+mouse CD41 was ~67% at 2 hrs and 26% at 24 hrs post transfusion). We therefore propose that establishment of immortalized MKPCs through gene manipulation could potentially provide a stable supply of platelets at a predefined quality and quantity for transfusion therapy.

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Center for Stem Cell and Regenerative Medicine

Laboratory of Stem Cell Regulation
幹細胞制御領域

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The major goal of our laboratory is to understand the role of mesenchymal and adult stem cells in regenerative and cancer biology. Stem cells from adult tissues have the unique capacity to repair damaged tissue, a process controlled in part by the microenvironment. Proteases, as part of the microenvironment act as processing enzymes that perform highly selective and limited cleavage of specific substrates including growth factors and their receptors, cell adhesion molecules, cytokines, chemokines, apoptotic ligands and angiogenic factors. We currently focused our scientific efforts on understanding the mechanism how the fibrinolytic pathway controls tumor cell growth (1), a process in part due to its ability altering blood cell formation (hematopoiesis) (2). The goal of this laboratory is to identify novel therapeutic targets for diseases like cancer or inflammatory diseases.

1. Plasmin inhibitor reduces lymphoid tumor growth by suppressing matrixmetallproteinase-9 dependent CD11 b$^+$/F4/80$^+$ myeloid cell recruitment.

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Activation of the fibrinolytic system during lymphoma progression is a well-documented clinical phenomenon. But the mechanism by which the fibrinolytic system can modulate lymphoma progression has been elusive. The main fibrinolytic enzyme, plasminogen (Plg)/plasmin (Plm), can activate matrix metalloproteinases (MMPs), like MMP-9, which has been linked to various malignancies. Here we provide the evidence that blockade of Plg reduces lymphoma growth by inhibiting MMP-9-dependent recruitment of CD11b$^+$F4/80$^+$ myeloid cells locally within the lymphoma tissue. Genetic plasminogen deficiency and drug-mediated Plm blockade delayed lymphoma growth and diminished MMP-9 dependent CD11b$^+$F4/80$^+$ myeloid cell infiltration into lymphoma tissues. A neutraliz-

ing antibody against CD11b inhibited lymphoma growth *in vivo*, which indicates that CD $11b^+$ myeloid cells play a role in lymphoma growth. Plg deficiency in lymphoma-bearing mice resulted in reduced plasma levels of the growth factors vascular endothelial growth-A and Kit ligand, both of which are known to enhance myeloid cell proliferation. Collectively, the data presented in this study demonstrate a previously undescribed role of Plm in lymphoproliferative disorders and provide strong evidence that specific blockade of Plg represents a promising approach for the regulation of lymphoma growth.

2. Tumor necrosis factor receptor-associated factor (TRAF) 2 controls homeostasis of the colon to prevent spontaneous development of murine inflammatory bowel disease.

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Fine-tuning of host cell responses to commensal bacteria plays a crucial role in maintaining homeostasis of the gut. Here, we show that tumor necrosis factor receptor-associated factor (Traf)2(−/−) mice spontaneously developed severe colitis and succumbed within 3 weeks after birth. Histological analysis revealed that apoptosis of colonic epithelial cells was enhanced, and B cells diffusely infiltrated into the submucosal layer of the colon of Traf2(−/−) mice. Expression of proinflammatory cytokines, including Tnfa, Il17a, and Ifng, was up-regulated, whereas expression of antimicrobial peptides was down-regulated in the colon of Traf2(−/−) mice. Moreover, a number of IL-17-producing helper T cells were increased in the colonic lamina propria of Traf2(−/−) mice. These cellular alterations resulted in drastic changes in the colonic microbiota of Traf2(−/−) mice compared with Traf2(+/+) mice. Treatment of Traf2 (−/−) mice with antibiotics ameliorated colitis along with down-regulation of proinflammatory cytokines and prolonged survival, suggesting that the altered colonic microbiota might contribute to exacerbation of colitis. Finally, deletion of Tnfr1, but not Il17a, dramatically ameliorated colitis in Traf2(−/−) mice by preventing apoptosis of colonic epithelial cells, down-regulation of proinflammatory cytokines, and restoration of wild-type commensal bacteria. Together, TRAF2 plays a crucial role in controlling homeostasis of the colon.

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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 hematopoetic 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 originates from mirine aorta-gonad-mesoneephros (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 ES or iPS cell colonies. The 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. Generation of functional erythrocytes from human ES or iPS cell-derived definitive hematopoiesis

Feng Ma, Yasuhiro Ebihara[1], Shinji Mochi-

zuki, Sachiyo Hanada, Emiko Matsuzaka, Yuji Zaike[2], Hiromitsu Nakauchi[3], Kohichiro Tsuji

A critical issue for utilization of hESC or hiPSC in possible clinical use is whether they can derive terminally mature progenies with the normal function. To solve this, we examined hESC or hiPSC-derived erythroid cells in coculture with mFLSC or AGM cells. By the coculture, large quantity of hESC or hiPSC-derived erythroid progenitors allowed us to analyze the development of erythropoiesis at a clone level and to investigate their function as oxygen carrier. The results showed that the globin expression in the erythroid cells in individual clones changed in a time-dependent manner. In particular, embryonic ε globin positive erythrocytes decreased, while adult-type β globin positive cells increased to almost 100% in all single clones we examined, indicating they had already been fated to definitive hematopoiesis. Enucleated erythrocytes also appeared in the clonal erythroid progenies. A comparison analysis showed that hESC-derived erythroid cells took a similar pathway in differentiation to human cord blood $CD34^+$ progenitor-derived erythrocytes when traced by glycophorin A, CD 71 and CD81. Furthermore, these hESC-derived erythroid cells could function as oxygen carrier, and had a sufficient glucose-6-phosphate dehydrogenase activity. The present study provided an experimental model to explore early development of human erythropoiesis, hemoglobin switching, erythroid pathogenesis, and to discover drugs for hereditary diseases in erythrocyte development. The quantitative production and their functional maturation indicate that hPSC-derived erythrocytes can be a novel potential source for therapeutic transfusion.

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

Yasuhiro Ebihara[1], Feng Ma, Shinji Mochizuki, Sachiyo Hanada, Emiko Matsuzaka, Yuji Zaike[2], Hiromitsu Nakauchi[3], Kohichiro Tsuji

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 CD 45−, CD34−, CD14−, CD105+, CD166+, CD 31−, and SEA-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.

4. Differential production of connective tissue-type and mucosal mast cells from hESC for anti-allergy drug screening

Feng Ma, Yang Wenyu, Yanzheng Gu, Yasuhiro Ebihara[1], Shinji Mochizuki, Sachiyo Hanada , Emiko Matsuzaka , Hiromitsu Nakauchi[3], Kohichiro Tsuji

MC function as effector cells in allergy and atopic disease. Therefore, anti-allergy drugs have been established to diminish MC function. However, since the acquisition of an abundance of human MC (hMC) is difficult because of no culture method producing massive hMC, most anti-allergy drugs targeted animal MC. Thus, efficient discovery of effective anti-allergy drugs needs to establish the culture system of massive hMC. Then, hESC are considered as a potential cell source for hMC. In human, two types of MC have been characterized; connective tissue-type and mucosal MC (CTMC and MMC, respectively). CTMC contain tryptase, chymase, MC carboxypeptidase and cathepsin G in their secretory granules, are predominantly located in normal skin and in intestinal submucosa, and involve in atopic dermatitis. MMC contain tryptase in their secretory granules, but lack the other proteases, are the main type of MC in normal alveolar wall and in small intestinal mucosa, and involve in allergic rhinitis or bronchial asthma. Although MC can be generated from human adult $CD34^+$ HPC *in vitro*, these MC are mainly MMC. So far, there lacks an evidence for the direct derivation of CTMC from adult HPC. We achieved successful production of hESC-derived $CD34^+$ HPC, using coculture with

AGMS-3 cells for 1-2 weeks. In suspension culture favoring MC differentiation within 3weeks, hESC-derived progenitors generated mature MC that shared a chymase / tryptase double positive phenotype and strongly expressed c-Kit, similar to human skin derived CTMC. On the other hand, hESC-derived multipotential hematopoietic progenitors obtained in clonal culture developed into MC for a longer time (over 5 weeks) and only expressed tryptase, with no or few chymase, similar to human $CD34^+$ cell-derived MMC. Since the current culture system of hESC can produce differentially a large number of CTMC and MMC, our study may highlight a new understanding for MC development and finally benefit the screening for anto-allergy drugs.

5. Generation of mature eosinophils from human pluripotent stem cells

Feng Ma, Yang Wenyu, Yanzheng Gu, Yasuhiro Ebihara[1], Shinji Mochizuki, Sachiyo Hanada , Emiko Matsuzaka , Hiromitsu Nakauchi[3], Kohichiro Tsuji

Eosinophils are multifunctional leukocytes implicated in the pathogenesis of numerous inflammatory processes. As the major effectors, eosinophils function in a variety of biological responses, allergic diseases and helminth infections. It is generally accepted human eosinophils develop through a pathway initially sharing common feature with basophils. However, there lacks a clear chart for early development of human eosinophils, such as during embryonic or fetal stages. We established an efficient method for producing eosinophils from hESC and hiPSC. By a two-step induction, we first generated multipotential HPC by co-culturing hPSC with AGMS-3 cells for 2 weeks. Then, total co-culture cells were transferred into suspension culture favoring eosinophil development with addition of IL-3 and other factors (stem cell factor, interleukin-6, thrombopoietin, Flt-3 ligand) . The maturation of hPSC-derived eosinophils was shown in a time-dependent manner, first co-expressing eosinophil-and basophil-specific markers [eosinophil peroxidase (EPO), and 2D7, respectively], then the portion of eosinophil markers gradually increased while that of basophil markers decreased, typically mimicking the development of eosinophils from human adult hematopoietic progenitors. By flowcytometric analysis, an eosinophil-specific surface marker, Siglec-8, was also expressed on these hESC/iPSC-derived eosinophils in a time-dependent manner, paralleling to those with EPO. The expression of eosinophil-specific granule cationic proteins (EPO, MBP, ECP, EDN) and IL-5 receptor mRNA was also detected by RT-PCR. Furthermore, transmission electron microscopy (TEM) observation confirmed the eosinophil property. Eosinophils derived from hiPSCs hold similar characteristics as those from hESCs. Our study provides an experimental model for exploring early genesis of eosinophils, especially in uncovering the mechanisms controlling the development of the initial innate immune system of human being in normal and diseased individuals.

6. Hematopoiesis of human induced pluripotent stem cells derived from patients with Down syndrome

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Trisomy 21, genetic hallmark of Down syndrome, is the most frequent human chromosomal abnormality. Infants and children with Down Syndrome (DS) are known to have some hematological disorders with an increased risk of developing leukemia. Ten to 20% of newborn with DS are diagnosed as neonatal preleukemic status, transient myeloproliferative disorder (TMD), and approximately 30% of TMD patients are predisposed to acute megakaryoblastic leukemia (AMKL). Recently, acquired mutations in the N-terminal activation domain of the *GATA1* gene, leading to expression of a shorter GATA1 isoform (GATA1s), have been reported in AMKL and TMD, but neither patients nor mice with germline mutations leading to expression of GATA1s developed AMKL and TMD in the absent of trisomy 21. These findings suggested that trisomy 21 itself directly contributes to the development of AMKL and TMD. However, the role of trisomy 21 in hematopoiesis, particularly in the human fetus remains poorly understood. To better understand the effects of trisomy 21 on hematopoiesis in embryonic stage and leukemogenesis, we employed hiPSC derived from patients with DS (DS-hiPSC). Six DS-hiPSC and 5 hiPSC lines (control) from healthy donors were all created from skin fibroblasts and reprogrammed by the defined 3 or 4 reprogramming factors (OCT3/4, KLF4, and SOX2, or c-MYC in addition to the 3 factors, respectively). We generated blood cells from DS-hiPSC and

controls with coculture system using AGMS-3 cells. The cells from hiPSC were harvested at day 11 or 12 of coculture and analyzed the presence of hematopoietic markers and the potentials of hematopoietic colony formation. In the experiments using hiPSC reprogrammed by 3 factors, human CD34 expression in harvested cells from DS-hiPSC or controls were detected 10.06±4.35% and 3.04%, respectively. We next examined the hematopoietic colony formation. Both myeloid and erythroid colonies were detected. Number of colonies formed from DS-hiPSC was 43.7±11.1 to 74.3±11.2 per an iPS cell colony, which was approximately 2 to 3.5 folds the number of control. Similar results were obtained in the experiments using hiPSC reprogrammed by 4 factors. These results indicated that hiPSC derived from patients with DS could differentiate into multiple hematopoietic cell lineages and the differentiation into hematopoietic lineage was promoted in DS patients.

7. Suppressed neutrophil development in hematopoiesis of induced pluripotent stem cells derived from a severe congenital neutropenia patient with ELA2 mutation

Takafumi Hiramoto, Yasuhiro Ebihara[1], Shinji Mochizuki, Shohei Yamamoto[1], Masao Nagasaki[6], Yoichi Furukawa[4], Hiromitsu Nakauchi[3], Masao Kobayashi[7], Kohichiro Tsuji: [6]Functional Genomics, Human Genome Center, [7]Department of Pediatrics, Hiroshima University Graduate School of Biomedical Sciences.

Severe congenital neutropenia (SCN) is a rare disorder characterized by severe neutropenia present at birth, an arrest of neutrophilic differentiation at the promyelocyte or myelocyte stage, and a propensity to develop acute myeloid leukemia and myelodysplasia. Mutations of the ELA2 gene encoding neutrophil elastase have been identified in the majority cases of SCN, but the mechanisms which disrupt neutrophil development in SCN with ELA2 mutation have been unclear. To understand the mechanisms, we established three hiPSC clones from bone marrow stromal cells of a patient having heterozygous mutation in ELA2 gene at exon 5, 707 region by transfection with retrovirus vector which expressed human OCT3/4, SOX2, KLF4, and c-MYC (SCN-iPSC). We then examined the hematopoietic differentiation of SCN-iPSC and control iPSC which were generated from healthy donors by the same method to SCN-iPS cells, using coculture system with AGMS-3 cells. The cocultured cells were harvested at day 12, and $CD34^+$ cells were separated. Hematopoietic colony assay was performed using these $CD34^+$ cells. Although number and size of erythroid and mixed-lineage colonies derived from SCN-iPS cells were almost similar to control, those of myeloid colonies derived from SCN-iPSC were significantly less and smaller than control. In particular, we could detect few number of neutrophil colonies from SCN-iPSC. Since SCN patients need granulocyte colony-stimulating factor (G-CSF) treatment to increase peripheral neutrophils, we conducted the hematopoietic colony assay with G-CSF alone to examine the sensitivity of granulopoiesis derived from SCN-iPSC and control iPSC to G-CSF. Myeloid colony formation reached a plateau at 1 to 10 ng/mL of G-CSF in control iPS cells, while the number and size of myeloid colonies gradually increased at up to 1000 ng/mL in SCN-iPS cells, but did not attain the control level. In suspension culture with myeloid differentiation-oriented cytokines including 10 ng/mL of G-CSF, $CD34^+$ cells from control iPS cell increased 23.3-fold for 2 weeks, and mature neutrophils predominantly occupied in the cultured cells. By contrast, $CD34^+$ cells from SCN-iPS cells gradually decreased, and few neutrophils, but mainly monocytic cells were contained in the culture. We also carried out microarray analysis using CD34+ cells stimulated by myeloid differentiation-oriented cytokines for 2 days to identify the genes which led to impaired granulopoiesis in SCN-iPS cells. As a result, LEF-1, C/EBP alpha, Cyclin D1 and BCL2 were downregulated in the cultured cells from SCN-iPS cells compared with those from control iPS cells. These results demonstrated that the development of neutrophils was selectively impaired in the hematopoiesis derived from SCN-iPSC, and that the stimulation of higher concentration of G-CSF compensated the impaired development of neutrophils to some extent, indicating SCN-iPSC can provide a useful tool to understand pathogenesis of SCN with ELA2 mutation.

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We are conducting clinical stem cell transplantation, especially using unrelated cord blood as a promising alternative donor in IMSUT research hospital. We are also engaged in the clinical and basic research for promotion of transplantation as well as regenerative medicine.

(1) Hematopoietic Stem Cell Transplantation (HSCT)
Our facility is a main hub of hematopoietic stem cell transplantation (HSCT) centers in Japan. In close association with Department of Hematology/Oncology in the IMSUT research hospital, as many as 600 cases of allogeneic HSCT have been performed and HSCT-related complications including acute/ chronic GVHD and opportunistic infection have been treated until 2010. Recent years unrelated cord blood has turned to be our major stem cell source in HSCT. Since 1998 we have performed more than 250 cases of cord blood Transplantation (CBT) in adults and demonstrated outstanding clinical results among domestic and overseas HSCT centers. During such a transition of our stem cell source, immunological reconstitution from the CB graft, optimal use of immunosuppressive agents as well as viral infection/ reactivation are becoming our main theme to be elucidated, and we are now approaching these issues in collaboration with other divisions in the center.

(2) iPS cell and hematopoietic stem cell (HSC) research
Recent development of induced pluripotent stem (iPS) cells has suggested the possible application of reprogrammed somatic cells to individualized therapy for intractable disorders. We are trying to generate iPS cells using lentiviral vector and tetracycline-inducible gene expression system for introducing and expressing 3 or 4 factors required for generation of iPS cells with relatively homogeneous genetic background. We are also challenging to reprogram mature blood cells into HSC according to the similar strategy used for iPS cells.

1. Matched HLA Haplotype Contributes to Reduce Sever Acute GVHD with Conserving GVL Effect in HLA-Mismatched Cord Blood Transplantation.

Takahashi S, Ooi J, Kato S, Kawakita T, Tojo A

We studied the clinical outcomes of 170 consecutive adult patients who received unrelated CBT between August 1998 and January 2011 in the institute of medical Science, University of Tokyo. Patients received previous allogeneic transplants were excluded from this study. All

patients received myeloablative regimens including 12 Gy of total body irradiation, cyclosporine plus short term methotraxate for GVHD prophylaxis and almost same supportive care by the institutional protocol. By low-resolution typing method for HLA-A, -B and -DR loci, 6 patients received matched grafts, 57 received 1 antigen-mismatched and 107 received 2 antigens-mismatched grafts in the graft-versus-host (GvH) direction. We have determined the HLA haplotype based on common haplotypes in Japanese population referred from the 11th International Histocompatibility Workshop and other previous reports. We evaluated the impact of haplotype matching on cumulative incidences of hematopoietic recovery, of GVHD, of relapse and of non-relapse mortality (NRM) using the Pepe and Mori's test. Estimates of overall and disease-free survivals were calculated using the Kaplan-Meier method and analyzed by the log-rank test. Thirty-three among all 170 pairs were defined as the haplotype-matched pairs sharing same haplotypes in both grafts and recipients. The age, sex, cytomegalovirus serological status, diagnosis, risk of the disease at the transplant, numbers of total nucleated cells and $CD34^+$ cells at the cryopreserved were not significantly different between both groups with and without matched haplotypes. Engraftment of platelet after CBT tended to be earlier in haplotype-matched group compared with control group among the 1 antigen-mismatched pairs in the host-versus-graft direction (median: 38 days versus 44 days) and among the 2 antigens-mismatched pairs (median: 38 days versus 42 days), but those were not significant. The cumulative incidences of grades III and IV acute GVHD in patients with haplotype-matched (7%) were significantly lower than non-matched group (9%) among 2 antigens-mismatched pairs in the GvH direction ($P=0.033$). Notably, cumulative incidences of relapse tended to be lower in haplotype-matched patients among this group (3 years cumulative incidences were 7% in haplotype-matched patients versus 21% in non-matched patients, $P=0.086$). The haplotype matching effects were not observed in survival rates, cumulative incidences of NRM among any HLA-mismatched pairs. Those data suggest that untyped variation carried on the HLA haplotytpe might be better to be matched. The haplotype matching seemed to effect on lower risk of sever acute GVHD, on the other hand, graft-versus-leukemia effect was conserved in the setting of HLA-mismatched CBT. Second myeloablative allogeneic stem cell transplantation (SCT) using cord blood for leukemia relapsed after initial allogeneic SCT.

2. Unrelated cord blood transplantation (CBT) after myeloablative conditioning in adults with advanced myelodysplastic syndromes.

Sato A, Ooi J, Takahashi S, Tsukada N, Kato S, Kawakita T, Yagyu T, Tojo A

We analyzed the disease-specific outcomes of adult patients with advanced myelodysplastic syndrome (MDS) treated with cord blood transplantation (CBT) after myeloablative conditioning. Between August 1998 and June 2009, 33 adult patients with advanced MDS were treated with unrelated CBT. The diagnoses at transplantation included refractory anemia with excess blasts ($n=7$) and MDS-related secondary AML (sAML) ($n=26$). All patients received four fractionated 12 Gy TBI and chemotherapy as myeloablative conditioning. The median age was 42 years, the median weight was 55 kg and the median number of cryopreserved nucleated cells was $2.51\times10(7)$ cells per kg. The cumulative incidence of neutrophil recovery at day 50 was 91%. Neutrophil recovery was significantly faster in sAML patients ($P=0.04$). The cumulative incidence of plt recovery at day 200 was 88%. Plt recovery was significantly faster in CMV seronegative patients ($P<0.001$). The cumulative incidence of grade II-IV acute GVHD (aGVHD) and extensive-type chronic GVHD was 67 and 34%, respectively. Degree of HLA mismatch had a significant impact on the incidence of grade II-IV aGVHD ($P=0.021$). TRM and relapse at 5-years was 14 and 16%, respectively. The probability of EFS at 5 years was 70%. No factor was associated with TRM, relapse and EFS. These results suggest that adult advanced MDS patients without suitable related or unrelated BM donors should be considered as candidates for CBT.

3. Second myeloablative allogeneic stem cell transplantation (SCT) using cord blood for leukemia relapsed after initial allogeneic SCT.

Ooi J, Takahashi S, Tsukada N, Kato S, Sato A, Uchimaru K, Tojo A

There are many reports of second allogeneic stem cell transplantation (allo-SCT) using cord blood (CB) for graft failure after initial allo-SCT. However, the efficacy of second allo-SCT using CB for patients with leukemia relapsed after initial allo-SCT is unknown. We report the results of second allo-SCT using CB in seven adult patients with leukemia relapsed after initial allo-SCT. All patients received a myeloablative con-

ditioning regimen including oral busulfan 16 mg /kg, intravenously fludarabine 100mg/m(2) and cyclophosphamide 120 mg/kg. All but one patient had myeloid reconstitution and four patients remain alive at between 4 and 40 months after second SCT. We conclude that second myeloablative allo-SCT using CB may be feasible in selected patients with the relatively younger age, less organ damage and longer time interval between first and second allo-SCT.

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

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

Chronic myeloid leukemia (CML) is a clonal myeloproliferative disorder generally believed to originate from a hematopoietic stem cell carrying the BCR-ABL fusion gene, which generally encodes 210kD and 190kD constitutively active tyrosine kinases termed as p210 and p190, respectively. In spite of the putative stem cell origin and the competence for differentiation toward mature B cells, there is a longstanding consensus that CML never involves the T cell lineage at least in chronic phase. To gain insight into this apparent conflict, we used *in vitro* T cell differentiation model from murine pluripotent stem cells (PSCs) as well as hematopoietic stem cells (HSCs). C57BL/6 MEFs were reprogrammed using a polycistronic lentiviral Tet-On vector encoding human Oct4, Sox2 and Klf4, which were tandemly linked via porcine teschovirus-1 2A peptides, together with another lentiviral vector expressing rtTA driven by the EF-1α promoter. Almost all the vector sequences including the transgenes were deleted by adenovirus-mediated transduction of Cre recombinase after derivation of iPSCs, and only remnant 291-bp LTRs containing a single loxP site remained in the genome. A clone of MEF-iPSCs were retrovirally transduced with p190ΔccER, a ligand-controllable p190-estrogen receptor fusion protein, whose tyrosine kinase activity absolutely depends on 4-hydroxytamoxyfen (4-HT). For T cell lineage differentiation, p190ΔccER-MEF-iPSCs were recovered from a feeder-free culture supplemented with LIF and plated onto a subconfluent OP9-DL1 monolayer in the presence of Flt3 ligand and IL7 with or without 0.5 μM 4-HT. After 3 weeks of culture, iPSC-derived blood cells were collected and subjected to FACS analysis for their lineage confirmation. About 70% of lymphocyte-like cells from the 4-HT(−) culture expressed CD3, but only 20% of counterparts from the 4-HT(+) culture expressed CD3, suggesting impaied T cell development by Bcr-Abl. Next, c-Kit$^+$Sca1$^+$Lin$^-$ (KSL) bone marrow cells were prepared by FACS from 8-weeks old C57BL/6 mice treated with 5-FU. KSL cells were similarly transduced with p190 ΔccER and were subjected to the OP9-DL1 coculture system with or without 0.5 μM 4-HT. After 2 weeks of culture, 95% of lymphocytes from the 4-HT(−) culture revealed CD3$^+$TCRβ$^+$ phenotype, but only 30% of those were double positive in the presence of 4-HT. In addition, 90 % of lymphocytes from the 4-HT(−) culture progressed to the DN2 stage with c-Kit-CD44$^+$ CD25$^+$ phenotype, whereas 50% of those from the 4-HT(−) culture arrested at the DN1 stage showing c-Kit$^+$CD44$^+$CD25$^-$. Since IL7 plays a central role at the stage from DN1 to DN2 of progenitor T cells, Bcr-Abl is suggested to impair T cell development possibly through interfering with the IL7 signal. The precise mechanism underlying impaired T lymphopoiesis by Bcr-Abl is under investigation.

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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 stem cells like ES cells, iPS cells, and hematopoietic stem 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 reprogramming, maintenance of pluripotency, and differentiation, and eventually to develop new strategies for regenerative medicine.

1. Screening of surface antigens of iPS cells using a retrovirus-mediated signal transduction method SST-REX.

Toshikhiko Oki, Jiro Kitaura, Masunori Kajikawa[1], and Toshio Kitamura: [1]ACTGen, Komagane, Nagano.

We previously developed a retrovirus-mediated signal sequence trap method SST-REX as a screening method for surface and secreted proteins. We searched surface antigens of cancer cells or immune cells. Here we used SST-REX for iPS to identify iPS-specific surface antigens, a surface antigen "catalog" of iPS cells, and attempted to develop iPS-specific antibodies. So far, we have identified 40 iPS cell antigens, found that at least 3 of them were expressed rather specifically in iPS cells and ES cells, and developed specific antibodies to these 3 antigens and investigated expressions of these antigens in iPS cells. We also investigated the effects of transduction of these antigens on iPS induction, and transduction of one of the antigen enhanced reprogramming process, though the precise mechanisms remain to be investigated.

2. 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 with

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)) not present on RasGRP4. In conclusion, 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.

3. Development of new retroviral vectors.

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

We 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. We developed new vectors like, 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

4. 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 metaphase, MgcRacGAP plays some roles in the segregation of chromosomes probably as a Cdc42-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 cell-cycle dependent mechanism: increase in S/G2/M phase and decrease in early G1 phase, suggesting that MgcRacGAP may play some roles in G1 check point. The ubiquitin-dependent degradation of MgcRacGAP is one of the mechanisms that account for its decrease in G1 phase. Using the proteome analysis and retroviral transduction, we identified the 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.

5. Pepp2 as a candidate oncogene for gastric cancer and leukemia.

Fumi Shibata-Minoshima, Toshihiko Oki, Fumio Nakahara, Jiro Kitaura, Junya Fukuoka, and Toshio Kitamura

We have searched for potential oncogenes by screening cDNA libraries derived from gastric cancer cell lines, pancreatic cancer cell lines, and glioma cell lines, using retrovirus-mediated expression cloning. Several genes were identified including *PIM-1, PIM-2, PIM-3, GADD45B* and Reproductive HOmeobox genes on the X chromosome gene F2 (*RHOXF2*) as candidate oncognes (Fumi Shibata-Minoshima et al. International Journal of Oncology 2011). Although no mutation in these genes was found, these molecules were highly expressed in cancer cell lines, suggesting that they play important roles in cell transformation. Among them, we focused on a transcriptional repressor RHOXF2. Transduction of RHOXF2 rendered HF6 cells factor-independent, while knockdown of RHOXF2 inhibited growth of a gastric cancer cell line HGC 27 in which RHOXF2 is highly expressed. In addition, RHOXF2-transfuced HF6 cells quickly induced leukemia when transplanted to sublethally irradiated mice. Moreover, RHOXF2 is highly expressed in some of leukemia cell lines and a variety of human cancer samples including colon cancers and lung cancers. Thus, these results indicated that RHOXF2 is involved in carcinogenesis.

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

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