Center for Stem Cell Biology and Regenerative Medicine

Division of Stem Cell Therapy
幹細胞治療部門

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Stem cells are generally defined as clonogenic cells capable of both self-renewal and multilineage differentiation. Because of these unique properties, stem cells offer the novel and exciting possibility of organ reconstitution in place of transplanted or artificial organs in the treatment of organ failure. In addition, stem cells are considered as ideal target cells for gene/cell therapy. The goal of this laboratory is to provide new insights into stem cell biology as well as approaches to therapeutic intervention for various intractable diseases.

1.

a) Definitive proof for direct reprogramming of hematopoietic cells to pluripotency

Motohito Okabe, Makoto Otsu, Ahn Dong Hyuck, Toshihiro Kobayashi, Yohei Morita, Yukiko Wakiyama, Masafumi Onodera, Koji Eto, Hideo Ema, & Hiromitsu Nakauchi

Generation of induced pluripotent stem cells (iPSCs) generally utilizes fibroblastic cells, but direct reprogramming of other cell types may prove useful in both research and clinical settings. Here we show that murine marrow hematopoietic cells can be directly reprogrammed to pluripotency by retroviral transduction of iPSC factors. Established clones showed typical characteristics of iPSCs including ability to contribute to chimerism in mice. Most importantly, generation of iPSC clones was feasible from marrow progenitors that had been reconstituted from a single highly purified hematopoietic stem cell, thus providing definitive proof of direct hematopoietic cell reprogramming to pluripotency. These results will pave the way for further use of hematopoietic cells for iPSC generation, while allowing easier and less invasive procedures than those currently used for establishment of human iPSCs.

b) A threshold in expression levels of gp91^{phox} transgene limits the degree of functional correction in granulocytes after gene therapy for X-CGD

Yasuo Takeuchi, Makoto Otsu, Masafumi Onodera, Hiromitsu Nakauchi

X-linked chronic granulomatous disease (X-CGD) is a primary immunodeficiency characterized by functional defects in phagocytes. Although stem cell-gene therapy is considered a promising therapy option, successful long-term reconstitution of gene-corrected granulocytes has not been established. Using gp91^{phox} mutant mice as a model of X-CGD, we sought to scrutinize the functional reconstituion in granulocytes after retroviral-mediated gene transfer into hematopoietic stem/progenitor cells (HSPCs). We utilized a new fluorescent dye, APF, to assess production of specific reactive oxygen species

(sROS) in granulocytes, as it could reliably enable a quantitative measurement of sROS.

Bone marrow HSPCs (c-Kit+, lineage-) from X-CGD mice were transduced with a retroviral vector harboring murine $gp91^{phox}$ cDNA together with Kusabira Orange (KuO) gene. These successfully transduced cells (~100% efficiency) were transplanted into X-CGD mice after receiving TBI. Levels of sROS production in conjunction with KuO fluorescence intensities were assessed in Gra-1+ granulocytes of recipient mice by multi-color flow cytometry analyses.

Interestingly, these reconstituted Gra-1+ cells were clearly separated into 2 fractions: KuO^{bright} APF-positive and KuO^{dull} APF-negative populations. We thought two possibilities to explain this result: 1) $gp91^{phox}$ gene was not expressed at all in KuO^{dull} populations, or 2) there was a threshold in expression levels of $gp91^{phox}$ for functional correction in X-CGD granulocytes, which was $not^{reached}$ in KuO^{dull} populations. Although preliminary, our PCR and RT-PCR analyses on sorted granulocyte populations so far favored the above second possibility, as they demonstrated the presence of $gp91^{phox}$ transgene and also its expression in KuO^{dull} APF-negative populations.

These results will have implications into future development of a durable gene therapy strategy for X-CGD by maximizing its efficacy in functional reconstitution, detailed analyses are ongoing.

c) Hematopoietic stem cell engraftment in unconditioned hosts: Niche space is not a limiting factor

Sachie Suzuki, Makoto Otsu, Hiromitsu Nakauchi

Background: Hematopoietic stem cell (HSC) transplantation (HSCT) represents a curative treatment for various disorders including hematopoietic malignancies and primary immunodeficiency diseases. Most HSCT requires conditioning regimens such as total body irradiation and/or chemotherapy to ensure engraftment of HSCs by emptying recipients' marrow niches. This conditioning is toxic, and many attempts have been made to reduce its toxicity. Previous studies showed that transplantation of either total bone marrow (BM) cells (Blood 81: 2566) or purified HSCs (J Exp Med 203: 73) could lead to visible long-term chimerism in nonmyeloablated recipients when infused at extraordinarily high doses; ~2×10^8 and ~4,000 cells for total BM and HSCs, respectively. These data strongly suggest that large numbers of HSCs are necessary to colonize the small niche space unoccupied by host HSCs in the unconditioned hosts. We postulated, however, that niche space is not a limiting factor in unconditioned host bone marrow. Instead, transplanted HSCs simply cannot out-compete the great numerical excess of pre-functioning / pre-existing host HSCs. To address this issue, we sought to establish a novel transplantation model that would allow detection of hidden HSC engraftment in unconditioned hosts.

Methods: This transplantation model requires 2 steps of cell infusions. First, limited numbers of test HSCs are infused into unconditioned hosts. Absence of donor cell chimerism is then confirmed long-term after transplantation. Second, induction cells are infused in order to stimulate donor cell chimerism by selectively reducing host HSCs without disturbing donor HSC repopulation. To enable this, we utilized a parent-to-F1 transplantation model exploiting allogeneic T cells. We used Ly5.1-C57BL/6 (B6-5.1) × Ly5.2-DBA2 F1 (BDF1-5.1/5.2) mice as recipients. Test donor HSCs (CD34negative/low, c-Kit+, Sca-1+, Lineage-negative BM cells) were from B6-5.1 mice. This combination represents a transplantation model with no rejection mediated by immune barriers. Splenic T cells from Ly5.1/Ly5.2 F1 B6 (B6-5.1/5.2) mice were used as induction cells. B6-5.1/5.2 T cells were expected to eliminate host BDF1 HSCs selectively by graft versus host reaction (GVHR) while not impairing possible B6-5.1 HSC repopulation. B6-5.2 rescue BM cells were also transplanted to ensure survival of host BDF1 mice. In most cases, GVHR eventually subsided in the process of gradual reconstitution of B6 donor hematopoiesis. Hematopoietic contribution by B6-5.1 HSCs was subsequently assessed in recipients by multi-color flow cytometry analyses.

Results: We first prepared 113 BDF1-5.1/5.2 mice, which received purified B6-5.1 HSCs (10, 25, 50 cells) without conditioning. As expected, none of them showed engraftment as evidenced by analysis of donor chimerism in peripheral blood 8-16 weeks post transplant. We then infused B6-5.1/5.2 T cells (2×10^7 cells) into each recipient. Most mice (n=98) survived and thus were available for long-term analyses. Interestingly, we detected emergence of donor B6-5.1 hematopoiesis in 13 out of 98 (~13.3%) BDF1-5.1/5.2 mice after T cell infusion. This late-appearing (or -onset) donor cell chimerism was observed even in the recipients (n=5) of as few as 10 donor HSCs. Donor cell chimerism was multi-lineage in most cases and, once established, it was stable long-term, with one mouse showing as high as ~25% donor cells.

Conclusions: We have demonstrated that as

few as 10 HSCs can engraft in unconditioned hosts. These results support our notion that the unconditioned bone marrow has sufficient niche space for transplanted HSCs. Myeloablation is useful for immune suppression and in giving the transplanted HSCs a selective advantage, but not in creating extra niche space for them. The large numbers of HSCs that are believed to be required for engraftment are actually required for competition against host HSCs that are far greater in number: With smaller transplanted HSC numbers, engraftment occurs, but is veiled.

2.

a) Establishment of mouse PMF model by the introduction of constitutive STAT5a into purified hematopoietic stem cells.

Takafumi Shimizu, Shin Kaneko, Akira Nakagawa, Shoichi Iriguchi, Akihiko Ito, and Hiromitsu Nakauchi

Polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofiblosis (PMF) are pathologically related and now classified under myeloproliferative neoplasm (MPN). The somatic activating mutation in the JAK2 tyrosine kinase, JAK2V617F, is now broadly recognized as a mutation responsible for MPN. Recent studies revealed that PV phenotype can be generated in homozygous JAK2V617F transgenic mice, while ET or atypical CML-like marked leukothrombocytosis with mild myelofibrosis can be observed in heterozygous JAK2V617F mice. These results indicate that expression levels of JAK2V617F may influence PV and ET phenotypes. On the other hand, typical PMF phenotype has not been generated by the introduction of JAK2V617F. According to the WHO criteria, PMF could be defined as "spent phase of hematopoiesis" with fibrosis formation followed by increased bone marrow cellularity as consequences of granulocytic proliferation and megakaryocyte changes with ineffective hematopoiesis. In this study, we focused on STAT5a, a direct downstream molecule of JAK2, because we previously reported that upon transplantation, purified CD34- lineage- sca-1+ c-Kit+ (CD 34-KSL) hematopoietic stem cells (HSCs) transduced with constitutive active form of STAT5A acted as MPN initiating cells causing granulocytosis without erythrocytosis/thrombocytosis. Based on these observations, we attempted to make PMF model through mimicking typical PMF dynamics; hyper proliferation of HSCs by the introduction of constitutive active STAT5a and following early HSC exhaustion.

Transplantation of STAT5a (1*6) transduced HSCs resulted in generation of 57 MPN mice (total 83 mice), while no MPN mouse was obtained by STAT5a (1*6) transduced HPCs (total 12 mice). Pathological analysis revealed that majority (70%) of MPN mice had PMF phenotype as defined by leukoerythroblastosis and dacryocytosis without leukothrombocytosis. These mice with PMF phenotype showed marked splenomegaly with extramedullary hematopoiesis, and granulocytic proliferation with megakaryocyte change. In BM, granulocytic proliferation advanced to severe myelofibrosis and osteomyelosclerosis in very short period of time (4 to 8 weeks). Those mice died of hemorrhage induced by pancytopenia within a few months, much faster than the mice with JAK2V617F based PV/ET models. Immunohistological analysis revealed that dominance of Gr-1 / Mac-1 positive granulocytes and CD41 positive small megakaryocytes strongly expressing TGF-beta, a putative inducer of fibroblastosis in BM of PMF mice.

By transplanting STAT5a(1*6) transduced HSCs, we were able to develop mice with phenotype closely resembling human PMF. Because PMF is rare disease, this animal model should be useful for understanding etiology of PMF, for evaluating existing treatment, and for developing therapeutics targeting STAT5a or its downstream pathway.

b) Monoclonal guidance of T lineage lymphocytes from human induced pluripotent stem cells originating from a single peripheral T lymphocyte

Toshinobu Nishimura, Shin Kaneko, Haruo Gotoh, Naoya Takayama, Takafumi Shimizu, Sou Nakamura, Ai Tachikawa-Kawana, Satoshi Takahashi, Nobukazu Watanabe, Koji Eto, and Hiromitsu Nakauchi

There has been much effort to develop clinically relevant methods for using antigen- or disease-specific TCR expressing T lymphocytes purified and expanded in vitro. To date, however, such T-cells have not been proven effective because they proceed to exhaustion during ex vivo processing; losing the potential for both long term survival and proliferation. To overcome the problem of exhaustion, we focused on the potential of induced pluripotent stem cells (iPSCs) which have a capacity for indefinite self-renewal while maintaining pluripotency. This feature expects us to induce an unlimited number of T lymphocytes showing reactivity to specific antigens when iPSCs were established from antigen-specific T-cells.

Peripheral T lymphocytes were isolated from healthy volunteers, reprogramming factors were transduced into fresh or thawed T lymphocytes. Standard ES-like colonies were observed and they exhibited ES-like characters. The TCR gene rearrangements remained in these T-cell-derived iPS (T-iPS) colonies were determined, and shown to be monoclonal in all T-iPS lines.

The reliable commitment from T-iPSCs into T lineage cells by murine stromal cell line (OP9-DL1) based co-culture was evidenced by expression of TCRα/β heterodimer and cell surface markers such as CD3. Genetic analyses confirmed that the induced T lineage cells expressed TCRs those had engraved in the pre-differentiated T-iPSCs genomes.

These data suggest that further optimization of these processes for T-iPS generation and disease-specific monoclonal T lineage cell expansion might lead to the development of infinitely and repeatedly suppliable disease- and patient-specific T-cell therapy.

3.

a) Analyses of cell surface proteins on hepatic stem/progenitor cells in mouse fetal liver

Sei Kakinuma, Akihide Kamiya

Hepatic stem/progenitor cells exhibit active proliferative ability and bipotency for hepatic and cholangiocytic lineages. However, phenotypes of such cells are not fully elucidated. We analyzed profile of cell surface proteins on murine fetal hepatic stem/progenitor cells. In vitro analysis by use of FACS-based colony formation assay indicated that hepatic stem/progenitor cells were enriched in CD13+CD45-Ter 119- fraction in mouse mid-gestational fetal liver. In vivo transplantation assay showed that primary CD13+CD45-Ter119- cells in fetal liver were able to engraft as hepatocytes and to repopulate regenerating liver. Profiling of surface proteins revealed that CD13+CD45-Ter119- cells in fetal liver expressed a lot of cell surface markers in a uniform pattern, including several already-known markers. Our data suggest homogeneity of cell surface molecules on primary fetal hepatic stem/progenitor cell fraction.

b) Prospective and clonal analyses of hepatic stem cells during fetal and postnatal liver development

Akihide Kamiya, Ken Okada, Kei-ichi Ito

The existence of stem cells in normal postnatal livers has been described. However, clonal isolation and analysis of these cells has never been possible due to lack of specific surface markers. Here, we identified CD13 and CD133 as markers expressed on the stem cell population in postnatal liver and established an efficient single-cell culture system to analyze them clonally. Expansion and reclone-sorting of postnatal-liver stem cells confirmed that these cells have the capacity for bi-potent differentiation and for self-renewal activity. After postnatal -liver stem cells were cultured for a long time, some of these cells differentiated to oval-like cells which expressed albumin, cytokeratin 19, and oval-cell surface markers. Thus, cells purified from normal postnatal livers in this study are sources of oval cells, candidate progenitor cells proliferated in injured-livers. CD13 and CD 133 were expressed on stem cells derived from both fetal and postnatal liver, whereas CD24 and CD49f were expressed only on postnatal-liver stem cells. These markers are useful in tracking stem cell components during liver development. Our primary culture system of clone-sorted hepatic stem cells should contribute to understanding of homeostasis and regeneration in normal as well as diseased liver.

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Center for Stem Cell Biology and Regenerative Medicine

Laboratory of Diagnostic Medicine

幹細胞治療研究センター
病態解析分野

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 purpose is to conduct clinical research and develop post-transplant monitoring systems to improve the safety of stem cell therapies. Through collaborations with hospitals and research groups performing hematopoietic stem cell and organ transplantations in Japan, we have developed several problem-based clinical studies to tackle the issues of graft failure, acute GVHD, the relapse of leukemia, and the recurrence of hepatitis after liver transplantation. Additionally, we use basic science methods to investigate themes from our clinical studies and develop new diagnostic techniques and therapies.

1. Clinical studies through collaborations with hospitals and research groups

a) Analysis of pathophysiology after myeloablative cord blood transplantation

Nobukazu Watanabe, Satoshi Takahashi[1], Naobumi Matsuno, Stephanie C. Napier, Jun Ooi[1], Nobuhiro Tsukada[1], Toshiro Kawakita[1], Seiko Kato[1], Aki Sato[1], Arinobu Tojo[1], Hiromitsu Nakauchi: [1]Research Hospital, IMSUT

Although umbilical cord blood is increasingly being used as an alternative donor source to treat hematological malignancies, cord blood transplantation (CBT) is frequently complicated by graft failure, acute GVHD, and the relapse of primary diseases. We are collaborating with the medical staff of the Research Hospital at IMSUT to gain a better understanding of the mechanisms behind these complications. We are approaching this project with three goals: Diagnosis of graft failure and relapse of leukemia using a FACS-based method of chimerism analysis (HLA-Flow method), regulation of immunosuppressants by Th1 cytokine assays, and analysis of cytokine-induced endothelial cell injury by detection of microparticles.

b) Prediction of the onset of Adult T Cell Leukemia (ATL) from human T-lymphotropic virus type 1 (HTLV-1) asymptomatic carriers

Kaoru Uchimaru[1], Tian Yamin[2], Seiichiro Kobayashi[2], Nobuhiro Ohno[1], Yuri Isobe[1], Mayuko Tsuda[1], Hiroshi Zaike[3], Nobukazu Watanabe, and Arinobu Tojo[1,2]: [1]Research Hospital, [2]Department of Molecular Therapy, [3]Department of Laboratory Medicine, IMSUT

Among the one million human T-lymphotropic virus type 1 (HTLV-1) carriers in Japan, approximately one thousand progress to Adult T Cell Leukemia (ATL) every year. Through collaborations with the Research Hospital and two laboratories at IMSUT, we are analyzing and comparing peripheral blood from HTLV-1 carri-

ers and patients with ATL to find a predictable phenotypic change of immune cells just before ATL onset.

c) Investigation of pre-engraftment immune reaction and graft failure after reduced intensity cord blood transplantation

Naobumi Matsuno, Nobukazu Watanabe, Shuichi Taniguchi[1]: [1]Department of Hematology, Toranomon Hospital

Reduced intensity cord blood transplantation is being widely performed due to the increase of senior patients with CBT-treatable diseases. This method of transplantation, however, causes a strong immune reaction with resulting graft failure. To help elucidate the mechanisms of these conditions, we are analyzing some immunological parameters of recipients, especially mixed chimerism, na?ve and memory phenotype, and expression levels of IL-7Rα.

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

Stephanie C. Napier, Nobukazu Watanabe, Akinobu Takaki[1], Takahito Yagi[2]: [1]Department of Gastroenterology and Hepatology, [2]Department of Gastroenterological Surgery, Transplant and Surgical Oncology, Okayama University Graduate School of Medicine and Dentistry

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 cause future infections and malignancies. To understand the mechanism underlying these pathologic conditions, we are investigating the following: Flow cytometry-based method of chimerism analysis (HLA-Flow method), detection of regulatory T cells and allospecific T cells, and identification of HCV-specific $CD8^+$ T cells using tetramers. We have just started a collaborative study with Okayama University to clarify the complicated liver pathophysiology involved before and after transplantation.

2. Generation of allele-specific anti-HLA monoclonal antibodies

Stephanie C. Napier, Satoshi Yamazaki[1], Nobukazu Watanabe, and Hiromitsu Nakauchi[1]: [1] JST-ERATO, IMSUT.

The difficulty in generating allele-specific anti-human leukocyte antigen (HLA) monoclonal antibodies (ASHmAb) 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-B51 transgenic mice against non-HLA-B51 HLA tetramers. ASHmAb 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 Section

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The STEM CELL BANK was established by the support of the Project for Realization of Regenerative Medicine, starting in April, 2009. The STEM CELL BANK belongs to the Center for Stem Cell Biology and Regenerative Medicine, The Institute of Medical Science. The Stem Cell Bank conducts (i) optimization and standardization of a protocol to establish disease-specific iPS cell lines for broad range of medical and pharmacological studies, and (ii) supply of preserved iPS cells. Additionally the members of this section are engaging in basic research on the generation of blood cells such as platelets in vitro from human ES cells / human iPS cells. The research program aims at the development of safe and stable blood supply for transfusion independently of blood donation and of gene therapy using established hematopoietic stem cells derived from human iPS cells with an appropriate validation. Throughout other research programs we have demonstrated the novel findings as follows:

1) c-MYC is a critical regulator for efficient generation of platelets showing normal function from human induced pluripotent stem cells

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The treatment of patients requiring repeated transfusion can be hampered by the unavailability of human leukocyte antigen-matched platelets. Human induced pluripotent stem cells (hiPSCs) are a potentially abundant source of such platelets, but how best to select iPSC clones is not resolved. Using an *in vitro* culture system yielding a "hematopoietic niche" that concentrates hematopoietic progenitors, we showed *c-MYC* to have beneficial effects on platelet generation from hiPSCs, though its effect on thrombopoiesis in mouse models is controversial. We found that an appropriate level of *c-MYC* reactivation but not other reprogramming factors in hiPSC clones during hematopoietic differentiation contributes to enhanced megakaryopoiesis and increases platelet yield. *In vivo* imaging revealed that iPSC-derived platelets contribute to thrombus formation following laser-induced vessel wall injury, indicating normal hemostatic function. Collectively, *c-MYC* levels determine the efficiency of thrombopoiesis from iPSCs, and

may be the key to clinically useful platelet production from selected iPSC clones.

2) Lnk/Sh2b3 regulates integrin αIIbβ3 outside-in signaling in platelets leading to stabilization of developing thrombus *in vivo*

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The nature of the cellular events underlying thrombus formation and platelet kinetics remain unclear because of the absence of a modality for analysis. Lnk/Sh2b3 is an adapter protein that inhibits thrombopoietin-mediated signaling. As a result, megakaryocyte and platelet counts are elevated in $Lnk^{-/-}$ mice. Here we describe Lnk's unanticipated role in stabilizing thrombus formation and clarify its activities in platelets, which are transduced through integrin αIIbβ3-mediated outside-in signaling. We used genetic depletion of Lnk and BM transplantation to equalize platelet counts in wild-type and $Lnk^{-/-}$ mice. By then combining $FeCl_3$- or laser-induced injury and our *in vivo* imaging system that enables observation of single platelet behavior and multiple steps in thrombus formation, we determined that Lnk is an essential contributor to the stabilization of developing thrombi within vessels. $Lnk^{-/-}$ platelets exhibited a reduced ability to fully spread on fibrinogen and mediate clot retraction, reduced tyrosine phosphorylation of the β3 integrin subunit, and reduced binding of Fyn to αIIbβ3. These results provide new insight into the mechanism of αIIbβ3-based outside-in signaling, which appears to be coordinated in platelets by Lnk, Fyn and integrin. They also suggest that modulators of outside-in signaling could represent new therapeutic targets for prevention of cardiovascular events.

3) The actin polymerization regulator WAVE2 is required for early bone marrow repopulation by hematopoietic stem cells

Takunori Ogaeri, Koji Eto, Makoto Otsu, Hideo Ema, and Hiromitsu Nakauchi: Division of Stem Cell Therapy and Stem Cell Bank

The Rho GTPase family members play essential roles in hematopoiesis. Of these, Rac1 is thought to be required for the appropriate spatial localization of hematopoietic stem and/or progenitor cells (HSPCs) within the bone marrow (BM), whereas Rac2 likely plays a role in BM retention of HSPCs. To elucidate molecular mechanisms underlying Rac-mediated functions in hematopoietic stem cells (HSCs), we studied WAVEs, the specific effectors downstream of the Rac GTPases in actin polymerization. We here show that $CD34^{-/low}c\text{-}Kit^{+}Sca\text{-}1^{+}lineage^{-}$ HSCs (34^{-}KSL HSCs) express WAVE2 but neither WAVE1 nor WAVE3. Since WAVE2 knockout mice are embryonic-lethal, we utilized HSCs in which expression of WAVE2 was reduced by small interfering RNA. We found that knockdown (KD) of WAVE2 in HSCs affected neither *in vitro* colony formation nor cell proliferation but did impair *in vivo* long-term reconstitution. Interestingly, WAVE2 KD HSCs exhibited unaltered homing, but showed poor BM repopulation detected as early as day 5 after transplantation. Mechanistic studies in WAVE2 KD HSCs revealed modest, but significant impairment in both cobblestone-like area-forming on stromal layers and actin polymerization upon integrin ligation by fibronectin. These results suggest that WAVE2-mediated actin polymerization, potentially downstream of Rac1, plays an important role in intramarrow mobilization and proliferation of HSCs, which are believed to be crucial steps for long-term marrow reconstitution after transplantation.

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Center for Stem Cell Biology and Regenerative Medicine

Laboratory of Developmental Stem Cell Biology
幹細胞治療研究センター
幹細胞探索分野

Project Associate Professor Hideo Ema M.D., Ph.D. | 特任准教授 医学博士 依馬秀夫

The mission of this project is to explore basic principles in stem cell biology that can be translated into stem-cell therapy. Hematopoietic stem cells have already been used in transplantation medicine such as bone marrow and cord blood transplantation, but are expectedly applied to a more variety of clinical settings. Mouse HSCs have been an excellent stem cell model because of the existence of established methods for their functional identification. We have attempted to manipulate HSCs on demand with understanding of regulatory mechanisms for self-renewal and differentiation in mouse HSCs.

1. *Ex vivo* enhancement of repopulating and self-renewal potentials in hematopoietic stem cells by a minimal combination of cytokines

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Ex vivo manipulation of tissue-specific stem cells on demand is a major interest in stem cell biology and therapy. Various culture conditions have been described for *ex vivo* expansion of hematopoietic stem cells (HSCs). However, the lack of quantitative assays which permit rather easy measurement of self-renewal potential in HSCs prevents us to compare these reported conditions. In this study, we devised serial competitive repopulation by introducing the concept of secondary repopulating units (RU) in distinction to primary RU which has been originally defined by David Harrison. Primary RU was used to assess short- and long-term repopulating activities in HSCs. Secondary RU was used to assess self-renewal potential in HSCs. Using this method, we found that one week serum-free bulk culture of highly purified mouse HSCs with the combination of stem cell factor (SCF) and thrombopoietin (TPO) markedly increases their short-term and long-term repopulating activities as well as self-renewal potential. The simple cultures enabled a limited number of HSCs alone to rescue lethally irradiated mice, followed by a full level of long-term reconstitution. We also found that interleukin-12 additively enhanced the increases of repopulating and self-renewal potentials in HSC cultures by SCF+TPO. We previously made observations that HSCs undergo *in vitro* asymmetric self-renewal division with SCF+TPO in clonal studies. Symmetric self-renewal division almost certainly should have occurred in this study. This discrepancy is best explained by the probabilities different between asymmetric and symmetric self-renewal in HSCs. HSCs are extremely heterogeneous in terms of self-renewal potential and only rare HSCs with great self-renewal potential are presumably able to undergo symmetric self-renewal in vitro. In addition, cell-cell interaction also might have been involved in culture of pooled HSCs. This study implies that the simplest combinations of cytokines should be re-

assessed as the standard conditions for study of molecular mechanisms underlying for HSC self-renewal and for potential *ex vivo* expansion of human HSCs.

2. G_0 phase length in hematopoietic stem cells differs between bone marrow and spleen niches

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Hematopoietic stem cells (HSCs) reside in special microenvironments, termed niches, in the bone marrow (BM) of adult mice. In concept, the number of stem cells is determined depending on the physical space available as niches, and the fates of stem cells are regulated by the niches. Endosteal and perivascular regions have been proposed for niche sites in the BM, and osteoblasts and vascular endothelial cells are considered to be the major components in endosteal and perivascular niches, respectively. Other types of cells might be involved in organization of the BM niches. Apart from niches' cellular components, and from the niches' microlocalization, we considered the niche in this study as a functional entity for HSC regulation and focused on analysis of its function.

The spleen is another hematopoietic organ in adult mice. During embryonic development, HSCs migrate from the liver and possibly also from the placenta into the spleen around embryonic day 14 as well as into the BM around embryonic day 17. Thereafter, HSCs reside in both spleen and BM throughout the life of a mouse. The spleen serves as an active hematopoietic organ in lethally irradiated mice for a while after transplantation with BM cells. Spleen colony-forming assays depend on this transiently active hematopoiesis in the spleen. The spleen is a major site of extramedullary hematopoiesis in pathological conditions such as myeloproliferative diseases. These facts imply the presence of unique HSC niches in the spleen. Because there are no osteoblasts in the spleen, the regulation of HSCs in the spleen possibly differs from that in the BM. In order to verify this hypothesis, we characterized HSCs in the spleen as compared with those in the BM.

We compared long-term repopulating activity and frequency of long-term repopulating cells (LTRCs) between BM and spleen from the same mice. We then purified HSCs in the spleen similarly to those in the BM. Using purified HSCs, BM and spleen HSCs were quantitatively and clonally compared. Cell cycling was also compared between BM and spleen HSCs. To distinguish spleen HSCs from circulating HSCs, pairs of parabiotic mice were analyzed. To our knowledge, this is the first study to evaluate HSCs in the spleen quantitatively in comparison with those in the BM. We found that repopulating and self-renewal potentials are similar on a clonal basis between BM and spleen HSCs. Analysis of parabiotic mice suggested that most HSCs are long-term residents in each organ. Cell cycle analysis revealed that spleen HSCs cycle more frequently than do BM HSCs, resulting in, on average, G_0 phase length in BM HSCs nearly twice as long as that in spleen HSCs. These data indicate that HSC hibernation is locally regulated in each hematopoietic organ. One of the major roles of the niche may be the control of periodic transition between G_0 and G_1 phases in HSCs.

3. DNA methyltransferase Dnmt3b but not Dnmt3a is essential for the development of mouse hematopoietic stem cells

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DNA methylation is an essential epigenetic modification required for normal development. The DNA methyltransferases Dnmt1, Dnmt3a, and Dnmt3b catalyze cytosine methylation in genomic DNA. In principle, Dnmt3a and Dnmt3 b exhibit de novo methyltransferase activity, whereas Dnmt1 acts as a maintenance methyltransferase. DNA methylation patterns are established during embryogenesis and gametogenesis, and are maintained in somatic cells. Embryogenesis is severely impaired in Dnmt1- or Dnmt3b-deficient mice. Dnmt3a is required for the establishment of both maternal and paternal imprints. Spermatogenesis is impaired in Dnmt3a-deficient mice.

Hematopoietic stem cells (HSCs) can clonally give rise to all mature blood lineages for a lifetime by their self-renewal and multilineage differentiation capacities. In theory, one HSC with a certain pattern in genomic DNA methylation give rise to many blood cells with a great variety of patterns. We examined whether de novo

methylation is required for their differentiation processes to mature blood cells. We found that neither Dnmt3a nor Dnmt3b is essential for HSCs to differentiate into multilineages. In marked contrast, it has recently been reported that HSC self-renewal and mulilineage differentiation totally depend the activity of Dnmt1. These data together suggest that de novo methylation plays a crucial role during the development of HSCs, but after DNA methylation is established in HSCs, both self-renewal and differentiation take place in HSCs and their progeny as long as DNA methylation levels are maintained by Dnmt1.

To verify this hypothesis, we focused on analysis of fetal liver where the HSC pool expands drastically during development. Dnmt3 $a^{-/-}$ or Dnmt3$b^{-/-}$ fetal liver HSCs were compared with wild type fetal liver HSCs by *in vitro* as well as *in vivo* assays. Interestingly, we found that HSCs require Dnmt3b but not Dnmt3 a for their development, unlike germline stem cells which require Dnm3a but not Dnmt3b for their development. Similarly to adult HSCs, neither Dnmt3a nor Dnmt3b was essential for developing HSCs to differentiate into multilineages. We once reported that either Dnmt3a or Dnmt3b is essential for self-renewal in adult HSCs. Detailed analysis of Dnmt3a/Dnmt3b conditional knockout mice in this study confirmed that either one plays a role to maintain self-renewal potential in adult HSCs. This study now provides a simple view that de novo DNA transmethylase is required for acquisition of potentials in developing HSCs while maintenance DNA transmethylase is required for maintenance of potential in adult HSCs.

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Donation Laboratories and Research Units

Laboratory of Stem Cell Regulation
文部科学省　再生医療の実現化プロジェクト
幹細胞制御領域

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The major goal of our laboratory is to understand how stem cells are regulated and how we can apply this knowledge for stem cell-based regenerative medicine. Stem cells can differentiate into tissue-specific cells 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. Over the last year we mainly focused on understanding the mechanism how the fibrinolytic pathway regulates myeloid-cell dependent neoangiogenesis during tissue regeneration (1) and examined the role of the fibrinolytic system for lymphoma growth (2).

1) Tissue type plasminogen activator regulates myeloid-cell dependent neoangiogenesis during tissue regeneration

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Ischemia of the heart, brain and limbs is a leading cause of morbidity and mortality worldwide. Treatment with tissue type plasminogen activator (tPA) can dissolve blood clots and can ameliorate the clinical outcome in ischemic diseases. But the underlying mechanism by which tPA improves ischemic tissue regeneration is not well understood. Bone marrow (BM)-derived myeloid cells facilitate angiogenesis during tissue regeneration. Here we report that a serpin-resistant form of tPA by activating the extracellular proteases matrix metalloproteinase-9 and plasmin expands the myeloid cell pool and mobilizes $CD45^+$ $CD11b^+$ pro-angiogenic, myeloid

cells, a process dependent on vascular endothelial growth factor-A (VEGF-A) and Kit ligand signaling. tPA improves the incorporation of CD $11b^+$ cells into ischemic tissues, and increases expression of neoangiogenesis-related genes including VEGF-A. Remarkably, transplantation of BM-derived tPA-mobilized $CD11b^+$ cells and VEGFR-1^+ cells, but not carrier-mobilized cells or CD11b- cells, accelerates neovascularization and ischemic tissue regeneration. Inhibition of VEGF-signaling suppresses tPA-induced neovascularization in a model of hindlimb ischemia. Thus, tPA mobilizes $CD11b^+$ cells from the BM and increases systemic and local (cellular) VEGF-A, which can locally promote angiogenesis during ischemic recovery. tPA might be useful to induce therapeutic revascularization in the growing field of regenerative medicine.

2) The fibrinolytic system regulates myeloid cell recruitment and lymphoma growth via MMP-9.

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Hematopoietic cells such as myeloid cells that are present in the tumor microenvironment promote tumorigenesis by stimulating tumor cell growth and metastasis. The mechanism by which these cells are recruited into tumor tissues_of patients with lymphoproliferative disorders is incompletely understood. Proteases such as plasminogen (Plg)/plasmin (Plm) and matrix metalloproteinase (MMPs) play a role in lymphocyte biology and have been implicated in cell migration. Here, we provide evidence that Plg synergizes with MMP-9/gelatinase B during lymphoma growth. Genetic Plg deficiency and drug-mediated plasmin blockade delayed lymphoma growth and diminished $CD11b^+$ $F4/80^+$ myeloid cell infiltration into lymphoma tissues, processes dependent on the presence of MMP-9. Genetic deletion and drug-induced ablation of Plg in lymphoma-bearing mice resulted in reduced plasma levels of the growth factors vascular endothelial growth-A (VEGF-A) and Kit ligand (KitL), both of which are known to_enhance myeloid cell proliferation. Collectively, the data presented demonstrate a previously undescribed role of Plm in lymphoproliferative disorders whereby Plm modulates the proteolytic and growth factor tumor microenvironment by enhancing myeloid cell infiltration. Thus, targeting the Plg pathway may be an attractive approach for the regulation of lymphoma growth.

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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

Associate Professor Kohichiro Tsuji

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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 embryonic stem (ES) cells and induced pluripotent stem (iPS) cells 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 ES cells

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ES cells (ESC) are pluripotent cells derived from the inner cell mass of preimplantation embryos. Since ESC have the ability to be maintained in culture indefinitely as undifferentiated cells, yet they are capable of forming more differentiated cell types, human ES cells (hESC) are expected as a novel source of human transplantable cells. We then planed to produce HSC for HSCT and functional blood cells for transfusion medicine from hESC. This study was started on December 20, 2003 with the permission by the ethical committee of the Japanese Government. We then developed a novel method for the efficient production of hematopoietic progenitors from hESC by co-culture with stromal cells derived from murine fetal liver (mFLSC) at 14 to 15 days post coitus (dpc), in which embryonic hematopoiesis dramatically expands at midgestation. In the co-culture, various hematopoietic progenitors were generated, and this hematopoietic activity was concentrated in cobblestone-like (CS) cells within differentiated hESC colonies. The CS cells expressed CD34 and retained a potential for endothelial cells. They also contained hematopoietic colony-forming cells, especially erythroid and multilineage colony-forming cells at high frequency. The multipotential hematopoietic progenitors 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 ESC nor potentials to differentiate into endoderm and ectoderm at a clonal level. The developed co-culture system of hESC can provide a novel source for hematopoietic and blood cells applicable to cellular therapies and drug screenings.

2. Generation of functional erythrocytes from hESC-derived definitive hematopoiesis

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

A critical issue for utilization of hESC in possible clinical use is whether they can derive terminally mature progenies with the normal function. To solve this, we examined hESC-derived erythroid cells in coculture with mFLSC. By the coculture, large quantity of hESC-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, CD71 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 hESC-derived erythrocytes can be a novel potential source for therapeutic transfusion.

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

Feng Ma, Yang Wenyu, Yasuhiro Ebihara[1], Sachiyo Hanada, 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 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 $CD34^+$ hematopoietic progenitor cells *in vitro*, these MC are mainly MMC. So far, there lacks an evidence for the direct derivation of CTMC from adult hematopoietic progenitors.

We achieved successful production of hESC-derived $CD34^+$ hematopoietic progenitors, using co-culture with mFLSC 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 anti-allergy drugs.

4. Derivation of multipotential hematopoietic progenitors and mature blood cells from human induced pluripotent stem cells

Feng Ma, Natsumi Nishihama, Yang Wenyu, Yasuhiro Ebihara[1], Sachiyo Hanada, Daisuke Tomizawa, Koji Eto[4], Hiromitsu Nakauchi[3] and Kohichiro Tsuji: [4]Division of Stem Cell Bank, Center for Stem Cell Biology and Regenerative Medicine

The establishments of hESC and hiPSC have constructed a firm base for regenerative medicine. As mentioned above, we developed a culture system for efficient production of hESC-derived multipotential hematopoietic progenitors and functionally mature erythrocytes, thus providing an experimental model to explore early events in human erythropoiesis. We then applied a similar culture system to induce hiPSC to differentiate into hematopoietic progenies. hiPSC (253G1, 253G5, 201B6 and 201B7, kindly provided from Dr. Yamanaka, Kyoto University) were used in our experiments. To induce differentiation to hematopoietic cells, undifferentiated hiPSC were cocultured with a mouse feeder stromal cell line derived from

AGM region (AGMS-3). On different times, cocultured cells were harvested by 0.05% trypsin/EDTA solution and re-culture in a semisolid culture to produce hematopoietic colonies. All hiPSC lines were capable of generating hematopoietic cells in clonal culture. Nine tenths of the clonal erythroid cells expressed definitive β-globin, which is comparable to hESC-derived ones. Furthermore, hiPSC-derived blood cells (granulocytes, mast cells and megakaryocytes) expressed various mature markers, indicating their fully functional maturation. Thus, we have established a culture system to induce hiPSCs to multipotential hematopoietic progenitors and mature blood cells. Our study may be used as an experimental model and finally help in clinical cures for hereditary blood diseases.

5. hESC-derived mesenchymal stem cells capable of efficiently maintaining hESC and hiPSC under animal serum-free conditions

Yasuhiro Ebihara[1], Feng Ma, Sachiyo Hanada, Daisuke Tomizawa, Haruo Onoda[2], Koji Eto[4], Naoki Oyaizu[2], Hiromitsu Nakauchi[3], Kohichiro Tsuji,

hESC and hiPSC have the ability to differentiate into all cells types in the body and hold great promise for regenerative medicine. However, the culture maintaining undifferentiated hESC and hiPSC depends on animal feeder cells and/or serum in most cases. Such a culture system is a huge obstacle for clinical applications of these stem cells because of xenogeneic pathogen contamination. To solve this issue, we developed a novel culture method for maintaining undifferentiated hESC and hiPSC using mesenchymal stem cells (MSC) as feeder cells and platlet lysate (PL) instead of animal serum. When undifferentiated hESC (line H1) cultured on murine embryonic fibroblast (MEF) feeder cells were recultured on gelatin-coated culture dishes with PL-containing media in the absence of MEF feeder cells. Cells were passaged several times with PL containing media, and then stromal cells were induced after 6 to 8 weeks. The stromal cells were spindle-like shaped, revealed a phenotype of CD45-, CD34-, CD14-, CD105+, CD166+, CD31-, and SEA-4-, and had the ability to differentiate into mesenchymal tissues such as bone, cartilage and fat *in vitro*, indicating these cells were MSC. MEF feeder cells and undifferentiated hESC were undetectable in the hESC-derived MSC by reverse transcription polymerase chain reaction analysis.

In the coculture with the hESC-derived MSC in the presence of PL-containing media, undifferentiated hESC (line H1) were maintained at least for four weeks. The cocultured hESC expressed specific markers for undifferentiated ESC, such as Oct-3/4, Sox-2, and Nanog, and formed teratoma containing ectodermal, endodermal, and mesodermal tissues in the transplantation into non-obese diabetic/severe combined immunodeficient mice. These hESC-derived MSC also have the ability to keep another hESC (line khES-1, kindly provided by Dr. Nakatsuji, Kyoto University) and hiPSC (line 235 G1, kindly provided by Dr. Yamanaka, Kyoto University) undifferentiated state. In addition, khES-1 cells generated MSC with the ability same to H1 cell-derived MSC. Interestingly, hESC or hiPSC isolated by passing through 100 micro-meter filter were capable of forming undifferentiated stem cell colonies in the coculture with hESC-derived MSC. These results indicate that hESC-derived MSC are able to be substituted for MEF feeder cells in the absence of animal serum but in the presence of PL in the maintenance of hESC and hiPSC, and efficiently support the proliferation of undifferentiated stem cells, even from single hESC or hiPSC. The current culture system can be useful for the clinical application of hESC and hiPSC.

6. Establishment of disease-specific iPS cells and analysis of their differentiation into diseased tissue cells

Feng Ma, Natsumi Nishihama, Yang Wenyu, Yasuhiro Ebihara[1], Sachiyo Hanada, Daisuke Tomizawa, Koji Eto[4], Hiromitsu Nakauchi[3] and Kohichiro Tsuji

Tissue culture of immortal cell strains from diseased patients is a useful resource for medical research but has been largely limited tumor cell lines or transformed derivatives of native tissues. Therefore, direct reprogramming promises to be a facile source of patient-derived cells, and the disease-specific iPS cells would be valuable for medical research. Such iPS cells capable of differentiation into various tissues affected under each condition could provide new insights into disease pathophysiology by permitting analysis in human system, under controlled conditions *in vitro*, using a large number of genetically modifiable cells, and in a manner specific to the genetic lesion in each whether known or unknown. In addition, by comparison of normal and pathologic tissue formation, and by assessment of the reparative effects of drug treatment *in vitro*, disease-specific iPS cells offer an opportunity to recapitulate pathologic human tissue formation *in vitro*, and a new technology platform for drug screening. We are now establishing iPS cells specific for congenital bone

marrow failure syndrome such as Fanconi anemia or Kostmann syndrom, infantile leukemia, Down syndrome, neurofibromatosis type 1 and allergic diseases such as atopic dermatitis, bronchial asthma and allergic rhinitis.

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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 2009. Recent years unrelated cord blood has turned to be our major stem cell source in HSCT. Since 1998 we have performed more than 300 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. 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 condition-

ing. 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 55kg and the median number of cryopreserved nucleated cells was 2.51×10(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.

2. Unrelated CBT after myeloablative conditioning in adults with acute myeloid leukemia

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

We analyzed the disease-specific outcomes of adult acute myelogenous leukemia (AML) patients treated with unrelated cord blood transplantation (CBT) after myeloablative conditioning. Between August 1998 and February 2008, 77 adult patients with AML were treated with unrelated CBT. All patients received 4 fractionated 12 Gy total body irradiation (TBI) and chemotherapy as myeloablative conditioning. The median age was 45 years, the median weight was 55kg, the median number of nucleated cells was 2.44×10(7)/kg, and the median number of CD34-positive cells was 1.00×10(5)/kg. All patients received a single and HLA mismatched cord blood unit. The cumulative incidence of neutrophil recovery at day 50 and platelet recovery at day 200 was 94.8% and 91.7%, respectively. A higher CD34-positive cell dose was associated with faster hematopoietic recovery. The cumulative incidence of grade III to IV acute graft-versus-host disease (aGVHD) and extensive-type chronic GVHD (cGVHD) was 25.1% and 28.6%, respectively. With a median follow-up of 78 months, the probability of event -free survival (EFS) at 5 years was 62.8%. The 5-year cumulative incidence of treatment related-mortality (TRM) and relapse was 9.7%, 25.8%, respectively. In multivariate analyses, the risk factor identified for event free survival (EFS) was disease status and cytogenetics. These results suggest that unrelated CBT after myeloablative conditioning could be safely and effectively used for adult patients with AML.

3. Retrospective comparative study of myeloablative unrelated CBT for acute leukemia between older patients (50～55) and younger patients (＜50)

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

Increasing recipient age is a well-known risk factor for graft-versus-host disease (GVHD) and treatment-related mortality (TRM) and has a negative impact on allogeneic hematopoietic stem cell transplantation. Since the incidence of severe GVHD after cord blood transplantation (CBT) is lower than that after transplants using bone marrow or mobilized peripheral blood grafts from adult cells, we should expect better outcomes from CBT in older patients. To evaluate the feasibility and efficacy of myeloablative unrelated CBT in patients aged between 50 and 55 years, we performed a retrospective comparison of 100 patients with acute leukemia who received cord blood grafts at our institution. Nineteen older patients (median age, 52; range, 50-55) and 81 younger patients (median, 36; range, 16-49) received a myeloablative conditioning regimen including 12 Gy of total body irradiation and chemotherapy. GVHD prophylaxis included cyclosporine with (n＝96) or without (n ＝4) methotrexate. There were no significant differences in the incidences of grades II to IV acute GVHD, extensive-type chronic GVHD, TRM, and the probability of overall and disease-free survival between these groups. These results suggest that, in patients with acute leukemia, myeloablative CBT might be as safe and effective in patients aged between 50 and 55 years as in younger patients.

4. 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 conditioning regimen including oral busulfan 16mg /kg, intravenously fludarabine 100mg/m(2) and cyclophosphamide 120mg/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.

5. Myeloablative CBT in adults with ALL.

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

We analyzed the disease-specific outcomes of adult ALL treated with cord blood transplantation (CBT) after myeloablative conditioning. Between October 2000 and November 2007, 27 adult patients with ALL were treated with unrelated CBT. All patients received four fractionated 12 Gy TBI and chemotherapy as myeloablative conditioning. The median age was 36 years, the median weight was 57kg and the median number of nucleated cells was 2.47×10^7/kg. All patients received a single and HLA-mismatched cord blood unit. The cumulative incidence of neutrophil recovery at day 30 and platelet recovery at day 200 was 92.6 and 92.3%, respectively. With a median follow-up of 47 months, the probability of EFS at 5 years was 57.2%. The 5-year cumulative incidence of TRM and relapse was 3.7 and 27.4%, respectively. These results suggest that unrelated CBT after myeloablative conditioning could be safely and effectively used for adult patients with ALL.

6. Drug-inducible direct reprogramming of somatic cells to pluripotency

Bidisha C, Izawa K, Tojo A

The major concerns about the present iPS technology include not only low induction efficiency of iPS cells but also genomic integration of viral vectors, causing unexpected secondary events. To resolve these unfavorable issues, we intend to use a single, polycistronic lentiviral vectors encoding 3 reprogramming factors without c-Myc (3F; Oct4, Klf4 and Sox2). In this vector, porcine teschovirus-1 2A sequences that trigger ribosome skipping were inserted between human 3F cDNAs, and a loxP site was placed in the truncated 3′-LTR to remove almost all the vector elements from established iPS cells. We also prepared the two types of reprogramming system using constitutive EF1α promoter and inducible tetracycline operator (TetO) -controlled minimal CMV promoter, respectively. We already succeeded in establishment of several lines of murine embryonic fibroblast-derived IPS cells and demonstrated their ES cell-like features *in vitro* and *in vivo*. We are now planning to generate patient- and disease-specific iPS cell lines for developing disease modeling in the hematological field.

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Project Research Associate	Toshihiko Oki, M.D., D.M.Sc.		特任助教	医学博士	沖　　俊　彦

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, surface antigen "catalog" of iPS cells, and attempted to develop iPS-specific antibodies. So far, we have identified 40 iPS cell antigens, and found that at least 3 of them were expressed rather specifically in iPS cells and ES cells. while developing specific antibodies to these 3 antigens, we are also investigating the effects of these molecules in generation of iPS cells to study the roles of iPS-specific antigens in the processes of reprogramming or maintenance of ES/iPS cells.

2. Applications of STAT3 inhibitors to stem cells

Toshikhiko Oki, Jiro Kitaura, Fumi Shibata, Akiho Tuchiya, Toshiyuki Kawashima, and Toshio Kitamura:

LIF-STAT3 signaling is one of the most important signals in ES/iPS cells, especially in murine ES cells in which LIF is the only maintenance factor for the present. We established a screening method for inhibiting IL-6 signal, and identified two small compounds as STAT3 inhibitors. We also identified several constitutively active STAT3 mutants in our study of IL-6 signaling. In this project, we utilize these reagents to investigate the mechanisms of reprogramming of somatic cells, maintenance and differentiation of ES/iPS cells and eventually to develop the tools to control these processes.

3. Development of new retroviral vectors.

Toshikhiko Oki, Jiro Kitaura, Fumi Shibata, and Toshio Kitamura:

We developed an effective retroviral transduc-

tion system consisted of vectors named as pMXs, pMYs, pMZs and pMCs and packaging cells named as PLAT-E, PLAT-A, and PLAT-F. We also attempt to develop 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 systems, and vectors for miroRNA or shRNA. We utilize these vectors in studying stem cell biology and also in developing the innovative tools for regenerative medicine.

4. Co-ordinated 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 using an MgcRacGAP-GFP fusion protein that expression of MgcRacGAP increases in the early G1 phase in parallel with Geminin, suggesting that MgcRacGAP may play some roles in G1 check point. In summary, our results implicate MgcRacGAP in coordination of cell cycle progression and cell fate determination.

5. 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 are now in the process of analyzing molecular basis of RJSI-1 and 2 inhibition of STAT proteins, and evaluating its effects in a tumor-burden model. In addition, we have started collaboration with companies to modify these compounds for optimization, thus eventually to develop anti-cancer drugs.

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