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. Generation of Functional Pancreas via Intra- or Inter-specific Blastocyst Complementation Using Pluripotent Stem Cells

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Since the complex interactions among cells and tissues required for organogenesis during development are difficult to recapitulate *in vitro*, current stem cell therapy mainly targets diseases that can be treated by cell transplantation, such as Parkinson's disease or diabetes mellitus. One of the ultimate goals of regenerative medicine, however, is regeneration of a solid organ using stem cells for organ replacement. To address this issue, we hypothesized first that a niche for organogenesis is vacant in post-blastocyst mutant mouse embryos genetically precluded from development of a certain organ, and second that injected pluripotent stem cell (iPSC)-derived cells would colonize this niche, compensate for the developmental defect, and form a donor-induced organ *in vivo*. We tested this hypothesis using *Sall1-/-* blastocysts for kidney and *Pdx1-/-* blastocysts for pancreas. In both models, defective cells were totally replaced and kidneys and pancreas were formed almost entirely by injected iPSC-derived cells. Chimeric mice of *pdx1-/-* genotype survived to adulthood without any sign of diabetes. Furthermore, upon transplantation into diabetic iPSC donor mice, islets prepared from the generated pancreas could normalize glucose levels long-term without eliciting rejection. As blastocyst complementation for human organogenesis requires xenogenic approaches, we injected mouse or rat PSCs into rat

or mouse BLs respectively, generating inter-specific chimeras and thus confirming that PSCs can contribute to xenogenic development between mouse and rat. We further injected rat wild-type PSCs into *Pdx1*$^{-/-}$ mouse blastocysts, generating normally functioning rat pancreas in *Pdx1*$^{-/-}$ mice. These data constitute proof of principle for inter-specific blastocyst complementation and for generation *in vivo* of organs derived from donor PSCs using a xenogenic environment. Generation of organs using rat iPSCs and blastocyst complementation *in vivo* provides a new strategy for understanding organogenesis and a novel approach for organ supply.

2. Irradiated bone marrow environment impairs pre-engraftment hematopoietic stem cells by exposure to tumor necrosis factor a

Sachie Suzuki, Makoto Otsu, Hiromitsu Nakauchi

Hematopoietic stem cell transplantation (HSCT) represents a curative treatment for various disorders. Most HSCT requires cytoreductive conditioning such as total body irradiation (TBI) and/or chemotherapy to ensure engraftment of HSCs by emptying recipients' marrow niches. Our preliminary experiments, however, revealed that TBI could induce local inflammation within marrow environment. Of note is that conditioning regimens widely used in the current HSCT settings are compatible with HSC exposure to inflammatory storm in post-irradiation bone marrow (BM). Since certain inflammatory cytokines have been shown to affect HSC functions in in vitro, we sought to investigate what effects irradiated BM environment would have on transplanted donor HSCs using murine transplantation models.

We first tested whether infusion of HSCs at varying timing post TBI would affect transplantation outcomes and HSC functions. To this end, fifty HSCs ($CD34^-KSL$ cells) obtained from Ly5.1-B6 mice were transplanted into lethally irradiated Ly5.2-B6 mice with 1×10^6 competitor Ly5.1/5.2-B6 BM cells at day 0, 3, or 5 post irradiation. No mice survived in the group that received transplants 5 days post irradiation, indicating insufficient HSC engraftment and hematopoietic reconstitution. Although hematopoiesis reconstituted in long term survivors was comparable between another two groups (d0 and d3), we found significant difference in donor HSC ability when tested in a competitive repopulation assay using Ly5.1-KSL cells sorted from primary recipients: Test HSCs obtained from mice transplanted 3 days after TBI showed poor secondary reconstitution ability, suggesting alteration of pre-engraftment HSC functions depending on transplantation protocols.

We then tested whether in vivo short-term exposure of HSCs to irradiated BM environment would have negative effects on HSC functions. Test HSCs (400 cells) from Ly5.1-B6 mice were transplanted into Ly5.2-B6 primary recipients at varying time points (day 0, 1, 2, or 3) after TBI. Approximately 24 h later, BM samples were subjected to a competitive repopulation assay to assess secondary reconstitution ability in test HSCs that homed to irradiated BM environment. Consequently, test HSCs that were exposed to day 2 BM post TBI showed marked impairment in their long-term reconstitution ability. When "BM-homed" HSCs were enumerated 24 h after infusion, we found modestly impaired HSC homing to day 2 BM compared to day 0 BM, indicating negative effects on HSCs transplanted during a peak phase of inflammatory storm. Among several inflammatory cytokines tested, only TNF-α inhibited colony formation in a dose-dependent manner in liquid culture using purified HSCs. When in vivo HSC exposure to irradiated BM environment was tested using TNF-α KO mice as primary recipients, significant recovery of HSC ability was observed, indicating a major role for this inflammatory cytokine in the HSC-inhibitory effect within the irradiated host tissues. We finally sought to test if blocking of TNF-α signaling in HSCs at a peak phase of inflammation could lead to better transplantation outcomes. We utilized the peptide previously shown to block TNFR signaling, and confirmed that pre-incubation of HSCs with this molecule did suppress TNF-α-induced reactive oxygen species production in HSCs. Studies are ongoing to assess beneficial effects of this "protection" measure on HSCs upon transplantation into irradiated recipients.

We demonstrated that inflammatory response in BM environment 2-3 days after irradiation had a negative effect on donor HSCs regarding both homing efficiency and secondary reconstitution ability. These findings provide important implications for developing the measures that enable HSCs to escape from this inhibitory effect to achieve far more improvement in clinical HSCT outcomes.

3. Role of CXCR4 Signaling in Hematopoietic Stem Cell Repopulation

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

The major dilemma of clinical hematopoietic stem cell (HSC) transplantation is the low level of engraftment. Here we focus on one chemokine receptor, CXCR4, which plays an essential role for HSCs not only in migration and homing but also in repopulation. Meanwhile, it has been reported that overexpression of CXCR4 on human $CD34^+$ progenitor cells increases their response to stromal-derived factor 1 (SDF-1) leading to higher repopulation of NOD/SCID mice. Besides improvement of engraftment, however, it is also important to maintain HSC self-renewal potency in order to achieve better long term transplantation outcomes. To elucidate the precise role of CXCR4 signaling in HSCs, we purify and use a $CD34^-$c-$Kit^+Sca\text{-}1^+$Lineage $maker^-$ ($CD34^-$KSL) population as stem cell sources. Overexpression of wild-type (wt)- and C-terminal truncated type (ΔC)-CXCR4 in HSCs was achieved and confirmed by flow cytometry analysis, which showed correlation of surface CXCR4 expression with EGFP intensity, a maker of transferred gene. We here used ΔC-CXCR4 as a gain-of function mutant form, not as a loss-of-function control, for which we could expect intensified signaling events according to the previous reports. Competitive transplantation results demonstrated enhanced in vivo long term repopulation potency in HSCs overexpressing ΔC-CXCR4. When characterized further in other assays, CXCR4-mutated HSCs also showed enhancement both in their in vivo repopulation and Cobblestone-Area Forming abilities. All these CXCR4-mediated effects were abolished in HSCs that had been deleted of CXCR4, supporting central roles of this receptor at the crucial steps in hematopoietic reconstitution after HSC transplantation. Experiments are in progress to test whether transient augmentation of CXCR4 signaling can also lead to enhancement of self-renewal potential in HSCs.

4. Proliferation of early fetal hepatic stem/progenitor cells requires interaction with mesenchymal cells and inhibition of ROCK activity.

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Hepatic stem/progenitor cells (hepatoblasts) have potential to proliferate and differentiate into both hepatocytes and cholangiocytes. In this study, we established a culture system for early-fetal liver hepatoblasts. Single $CD13^+Dlk^+$ cells from E11.5 and E13.5 fetal livers formed individual large colonies on collagen type-I coated dish. In contrast, $CD13^+Dlk^+$ cells from E9.5 and E10.5 fetal livers could form few colonis in the same culture condition. Single purified cells from E9.5 and E10.5 livers formed individual large colonies in the co-culture system with mouse embryonic fibroblast (MEF) cells as feeders. These colonies consisted of both albumin-positive hepatocytic cells and cytokeratin 19 (CK19)-positive cholangiocytic cells, indicating that E9.5 $CD13^+Dlk^+$ cells have the ability of hepatoblasts. In addition, inhibition of Rock or non-muscle Myosin-II (the downstream component of Rock) was necessary for effective expansion of E9.5 $CD13^+Dlk^+$ cells, but not for that of E 13.5 cells. While expression of genes relating to differentiation and maturation of hepatoblasts increased during fetal liver development, expression of cytokeratin19 and Sox17 were the highest at E9.5 and decreased dramatically thereafter in sorted $CD13^+Dlk^+$ cells. In this study, we show that CD13 and Dlk are cell surface markers of hepatoblasts during early- to mid-fetal liver devleopment. Purified cells from E9.5 mouse fetal livers can proliferate clonally in the co-culture sytem with MEF and inhibition of Rock-MyosinII pathway significantly induced expansion of these cells.

5. Proliferation of hepatic stem/progenitor cells by spatio-temporally expressed genes during development.

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

We have recently developed a novel co-culture system of hepatic stem/progenitor cells (hepatoblasts) with Mouse Embryonic Fibroblasts (MEFs). In this co-culture system, hepatoblasts form large colonies consisted of Albumin+ hepatocytes and Cytokeratin19+ cholangiocytes. However, these colonies stopped proliferating after replating, suggesting that this co-culture system could not maintain the self-renewal activity of hepatoblasts. In this research, we focused on nuclear factors that were highly expressed in fetal hepatoblasts and sought for genes that were required for the self-renewal of hepatoblasts through functional screening. Among the candidate factors, we found a novel gene that enhances the proliferation of hepatoblasts. Overexpression of Cbx8 induces colony formation of hepatoblasts in a long-term culture. Since Cbx8 was a member of the Polycomb Repressor Complex 1 (PRC1), it allowed us to

speculate that it functions through a similar mechanism as Bmi-1, which are involved in the maintenance of various types of stem cells. Hepatoblasts are main cells expressing Cbx8 in fetal livers, thus Cbx8 may play important roles in liver development.

7. Generation of monoclonal TCR-expressing human T-lineage lymphocytes from induced pluripotent stem cells of single peripheral T-lymphocyte origin

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Adoptive immunotherapy is a promising approach to cancer immunotherapy that circumvents some of the limitations of active immunotherapy. However, its effectiveness is often hampered by exhaustion of antigen-specific T cells. To address this issue, we utilized induced pluripotent stem (iPS) cells generated from human peripheral T lymphocyte (T-iPS cells). Rearrangement of T-cell receptor (TCR) genes was maintained in these T-iPS cells which, because T-iPS cells have unlimited self-renewal capacity, could be expanded *ex vivo*. These T-iPS cells were then re-differentiated into $CD3^{+}TCR\alpha\beta^{+}$ functional T-lineage cells *in vitro* with higher efficiency than was possible for embryonic stem cells, iPS cells derived from fibroblasts, or cord blood cells. Complete monoclonality of productive TCR transcripts, transcribed from the genome inherited from the original progenitor T cell, was observed in re-differentiated T-lineage cells: No signature of *de novo* TCR gene rearrangement could be identified. The technology described opens a way for effective T-cell therapy with antigen-specific monoclonal TCR.

8. STAT5A over-expressing hematopoietic stem cells in primary myelofibrosis mouse induce by-stander exhaustion of non-transduced normal hematopoietic stem cells

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Acquired somatic JAK2 mutation, JAK2V617F, is broadly recognized as a responsible mutation for Myeloproliferative neoplasms (MPNs). Recent studies indicate that expression levels of JAK2V617F may influence disease type selection such essential thrombocythemia (ET), polycythemia vera (PV) and primary myelofibrosis (PMF). Typical PMF animal model, however, had not been generated by JAK2V617F transgenic or total bone marrow (BM) transduction approaches. In the last annual report, we have reported successful generation of PMF mouse model by transplantation of STAT5a(1*6); constitutive active STAT5a, a direct downstream molecule of JAK2, expressing highly purified CD34-KSL HSCs into lethally irradiated recipient mice. So far, analyses of the model in detail revealed following features of PMF.

First, TGF-β expression by increasing atypical MgKs causes substantial proliferation of fibroblasts and increase of reticulum/collagen fibers in BM. Second, STAT5a(1*6) expression level may influence disease type selection and progression by modifying differentiation tendency to MgKs, and it could be usefully predicted by measuring STAT5A expression level in PB as surrogate biomarker of MPNs. Third, not like the other MPNs, 2ndary BM transplantation of PMF mice doesn't reproduce PMF in a new recipient. Reasons are clarified as spent phase hematopoiesis by STAT5A(1*6) transduced HSC and loss of hematopoietic function of STAT5A (1*6) negative normal HSC associated with fibrosis.

9. Prevention of teratomagenesis in iPSC-based therapies with Nanog promoter driving HSV-TK / GCV mediated "pluripotent cell-suicide" system

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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, could have some latent risks of teratomagenesis by contamination of undifferentiated iPSCs in the graft, *de novo* tumorigenesis from transplanted differentiated cells by insertional mutagenesis, and dysfunction of the graft. Aiming at realizing safer iPS cell-based therapies by reducing at least the first risk, we've been trying to develop a cell "suicide" inducible system which enables undifferentiated

cells to be eliminated by apoptosis both *in vitro* and *in vivo*. At the beginning of experiment, unexpected high risk of teratomagenesis in autologous transplantation setting was revealed by inoculation assay of 1×10^6 C57BL/6 mouse tail tip fibroblast (B6-TTF) derived iPSCs into testis of B6 mouse. Teratoma-formation rate was significantly higher in autologous setting than allogeneic setting, moreover, equally high to immunodeficient setting using Balb/c-nude mice as recipients. We therefore applied herpes simplex virus thymidine kinase (HSV-TK) gene and prodrug ganciclovir (GCV) mediated "suicide" system to autologous iPSC-based therapies to control teratomagenesis. A lentiviral vector harboring HSV-TK under murine *Nanog* promoter was established and transduced to B6-TTF iPSCs. Transduced iPSCs (Nanogp-TK/iPSCs) were cloned by Flow cytometry. Nanog promoter driving HSV-TK induces GCV dependent cell death to Nanog expressing iPSCs by synthesizing DNA analogues made from GCV. As expected, some clones showed remarkable apoptotic sensitivity to GCV *in vitro*, while embryoid bodies from those clones didn't. Besides, inoculation assay of 1×10^6 B6 TTF Nanogp-TK/iPSCs to B6 mice with continuous GCV administration showed complete prophylaxis of teratomagenesis. These results suggest the significance of "suicide" system as a safety switch preventing teratomagenesis in iPSC-based therapies.

10. Suicide gene modified central memory T lymphocyte infusion therapy against relapsed leukemia after allogeneic stem cell transplantation.-An amended protocol of TK-DLI gene therapy.

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Peripheral T lymphocytes exert their proliferative potential and effector functions by recognizing antigens on tumor cells and play a central role in tumor immunity, and therefore, a substantial number of clinical protocols have been proposed using manipulated and *ex vivo* expanded T lymphocytes (June CH, *J Clin Invest*, 2007). To date, however, such highly expanded T lymphocytes have not been proven fully effective in treating diseases, because, at least in part, those cells are usually differentiated terminally and lose potentials of long-term survival, proliferation, and effector functions (Krevanoff CA, *Immunol. Rev.*, 2006).

To avoid such inefficiency caused by an ordinary *ex vivo* expansion protocol with OKT-3 (anti-CD3 antibody) stimulation alone, we have established a new *ex vivo* T lymphocyte manipulation protocol with anti-CD3/anti-CD28 antibody-conjugated magnetic beads and common gamma chain cytokines. This method allows us to preserve genetically modified, alloreactive, and self-renewing central memory T lymphocytes (Kaneko S, *Blood*, 2009). According to favorable results obtained from a set of humanized animal experiments, a cell processing protocol of TK-DLI gene therapy, which is conducted as a clinical trial against relapsed leukemia in Tsukuba University Hospital, has been changed to the new one. By the end of 2009, the internal review board and the Ministry of Health, Labour, and Welfare approved the amended TK-DLI clinical protocol for other 5 relapsed leukemia patients. The protocol is now open for the registration.We expect a better clinical outcome with the new protocol than the former one (1 complete remission in 5 relapsed leukemia patients).

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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 post-transplant monitoring systems to improve the safety of hematopoietic stem cell and organ transplantations. Through collaborations with hospitals 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. We also started a new project with IMSUT hospital to investigate the cell biology of Adult T Cell Leukemia (ATL) cells. 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

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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 two goals: Diagnosis of graft failure and relapse of leukemia using a FACS-based method of chimerism analysis (HLA-Flow method), and regulation of immunosuppressants by Th1 cytokine assays.

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

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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 Hos-

pital and two laboratories at IMSUT, we are analyzing and comparing peripheral blood from HTLV-1 carriers and patients with ATL to find a predictable phenotypic change of immune cells just before ATL onset in order to begin more effective treatment.

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

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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 lineage-specific mixed chimerism, naïve and memory phenotype and expression levels of IL-7Rα in T cells.

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 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.

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

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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-A2, A24, and B51 transgenic mice against non-HLA-A2, A24, and 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

幹細胞治療研究センター
ステムセルバンク

Project Associate Professor Koji Eto, M.D., Ph.D. ｜特任准教授 医学博士 江 藤 浩 之

The STEM CELL BANK, starting in 2009, conducts primarily the program linked with 'the Project for Realization of Regenerative Medicine', supported by the Ministry of Education, Culture, Sports, Science and Technology-Japan. We carry out (i) optimization and standardization of a protocol to establish disease-specific induced pluripotent stem (iPS) cells for broad range of medical and pharmacological studies, and (ii) generation and supply of various types of disease-specific- and healthy donor-iPS cells. We also participate in our own projects of generation of platelets, erythrocytes, and hematopoietic stem cells from human iPS cells. The research program aims at the development of safe and stable blood supply for transfusion independent of blood donation, and strategy of gene therapy using established hematopoietic stem cells derived from human iPS cells with an appropriate validation. In 2010, we additionally performed the projects to elucidate molecular mechanisms underlying the adhesion-dependent bone marrow niche system by murine hematopoietic stem cells, or to virtually observe inflammation-related thrombus formation at single cell level in a capillary of mice model using novel in vivo imaging system.

1) Transient activation of c-MYC expression is critical for efficient platelet generation from human induced pluripotent stem cells

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Human induced pluripotent stem cells (hiPSCs) are a potentially abundant source of blood cells, but how best to select iPSC clones suitable for this purpose from among the many clones that can be simultaneously established from an identical source is not clear. Using an *in vitro* culture system yielding a hematopoietic niche that concentrates hematopoietic progenitors, here we show that the pattern of *c-MYC* reactivation after reprogramming influences platelet generation from hiPSCs. During differentiation, reduction of *c-MYC* expression following initial re-activation of c-MYC expression in selected hiPSC clones was associated with more efficient *in vitro* generation of $CD41a^{+}CD42b^{+}$

platelets. This effect was recapitulated in virus integration-free hiPSCs using a doxycycline-controlled c-MYC expression vector. *In vivo* imaging revealed that these $CD42b^+$ platelets were present in thrombi following laser-induced vessel wall injury. In contrast, sustained and excessive *c-MYC* expression in megakaryocytes was accompanied by increased p14 (*ARF*) and p16 (*INK4A*) expression, decreased *GATA1* expression, and impaired production of functional platelets. These findings suggest that the pattern of *c-MYC* expression, particularly its later decline, is key to producing functional platelets from selected iPSC clones.

2) Integrin αvβ3 ligation regulates thrombopoietin-mediated reconstitution potential of mouse hematopoietic stem cells

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Throughout life, one's blood supply depends upon sustained division of hematopoietic stem cells (HSCs) for self-renewal and differentiation. Within the bone marrow microenvironment, an adhesion-dependent or -independent niche system regulates HSC functions. Here, we show the novel adhesion-dependent mechanism via integrin β3 signaling for HSC maintenance. Specific ligation of β3 integrin on HSCs with antibody or extracellular matrix protein prevented loss of long-term repopulating (LTR) activity during *ex vivo* culture. The actions required the activation of αvβ3 integrin dependent upon thrombopoietin (TPO), an essential cytokine for dormant HSCs. Subsequent outside-in signaling via phosphorylation at Tyr747 in the β3 subunit cytoplasmic domain was indispensable for TPO-dependent LTR activity of HSCs in vivo, which accompanied with enhanced expression of Vps72, Mll1 and Runx1, known as critical factors in maintaining HSC activity. Thus, our findings demonstrate the mechanistic link between β3 integrin and TPO, which may contribute to maintenance of LTR activity in vivo and during ex vivo expansion.

3) Proinflammatory cytokines play key roles in thrombus formation composed of discoid platelets on intact endothelium in mice

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Inflammation is now known to play a central role in the pathogenesis of cardiovascular diseases, and to contribute to thrombotic responses. The direct contribution made by inflammation to thrombosis *in vivo* remains unclear, however. To clarify the multicellular process underlying thrombus development, we improved a direct visualization technique of thrombus formation in the mesenteric capillaries of living mice; to characterize the rapid kinetics of the initiation and progression of thrombus formation; and to assess the contribution of proinflammatory cytokines. Using this technique, we were able to induce thrombus formation without apparent endothelial cell (EC) disruption, which is completely different from previous thrombosis models, and determine how discoid platelets aggregate into thrombi on the intact EC. In our model, thrombus formation is initiated by reactive oxygen species (ROS) produced, and associated with the inflammatory cytokines TNF-alpha as well as and IL-1. Kinetic analysis revealed that initial platelet attachment onto intact EC is mediated through the TNF-alpha/TNF-R1 axis, where initial binding of endothelial von Willebrand factor with GPIb-alpha and subsequent integrin signaling is indispensable. Collectively our in vivo imaging shed light on the underlying molecular mechanism linking thrombosis and inflammation.

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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
幹細胞探索領域

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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 expansion of regular and latent hematopoietic stem cells

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Ex vivo manipulation of hematopoietic stem cells (HSCs) on demand is a major interest in stem cell biology and therapy. This year we have reported the existence of latent HSCs defined as ones which exhibit little repopulating activity in primary recipient mice after transplantation but progressively exhibit a higher level of repopulation after secondary transplantation. Such HSCs comprise 2～3% of HSCs in adult mouse bone marrow. We have used serial competitive repopulation originally designed by us to detect latent HSCs as well as regular HSCs. On the other hand, we have long sought culture conditions which permit to expand HSCs *in vitro*. We have recently found that the combination of stem cell factor (SCF) and thrombopoietin (TPO) and that of SCF and interleukin-12 (IL-12) can significantly expand HSCs in serum-free culture. Since intracellular signaling of TPO differs from that of IL-12, at least in part, it was expected that the combination of all these three cytokines would give more HSC expansion. In effect, the greatest level of increase in long-term repopulation activity in our hands has so far been obtained under this culture condition.

We moreover noticed that the combination of SCF and TPO primarily led to high levels of repopulating activity in primary and secondary recipient mice, but such an activity significantly decreased after tertiary transplantation. We thought that HSCs might have been exhausted after robust self-renewal divisions in primary and secondary recipient mice. In contrast, the combination of SCF and IL-12 primarily led to moderate levels of repopulation in primary and secondary recipient mice, but to our surprise, increasing levels of repopulation were observed in tertiary and even quarternary recipient mice. These data suggest that HSCs expanded by TPO largely differ from those expanded by IL-12. We assumed at this moment that TPO mainly acts on regular HSCs whereas IL-12 mainly acts on latent HSCs upon in vitro induction of self-

renewal in HSCs. We are going to verify this hypothesis. Nonetheless, the combination of SCF, TPO, and IL-12 holds promising applications in *ex vivo* expansion of human HSCs.

2. Differential effect of Wnt3a on hematopoietic stem cells in fetal liver and adult bone marrow

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Wnt3a was reported to act together with stem cell factor on hematopoietic stem cells (HSCs) as a self-renewal-inducing factor (Reya *et al.*, Nature 423: 409, 2003). Since this is the only self-renewal factor for HSCs formally reported, to verify their claim is extremely important. We had spent lots of time in an attempt to reproduce their data with no success at all. In the mean time, fetal liver HSCs from Wnt3a-deficient mice were reported to be impaired in reconstitution of secondary recipient mice but not to be apparently impaired in that of primary recipient mice (Luis et al., Blood 113: 546, 2009). It is possible that HSCs are stimulated by Wnt3a in a certain point of developmental stage, and as a result self-renewal potential is induced or enhanced. To address this issue, HSCs were purified from fetal liver of E13.5 embryos, cultured in the presence or absence of Wnt3a, and transplanted into lethally irradiated mice along with competitor cells.

Similarly to adult bone marrow (BM) HSCs, fetal liver HSCs showed a significant increase in long-term repopulating activity after culture with stem cell factor (SCF) and thrombopoietin (TPO) for 7 days. Long-term repopulating activity was maintained in recipient mice after secondary transplantation. These data thus suggest that the combination of SCF+TPO is effective in enhancing self-renewal potential in both fetal liver and BM HSCs. Interestingly, Wnt3a strengthened the effect of SCF+TPO and led to further increase in self-renewal potential in fetal liver HSCs but not in BM HSCs. Since Wnt3a expression level remains very low throughout mouse development from an embryo to an aged mouse, it is difficult to compare its protein levels among various tissues. But, it is possible that Wnt3a protein is more abundant in fetal liver than in adult BM. Alternatively, frizzled receptor members differ between fetal and adult HSCs. Epigenetic differences between these HSCs may explain why Wnt3a effects only on fetal liver HSCs, but not adult BM HSCs. We still need to verify these possibilities. However, it is conceivable that Wnt3a acts on HSCs in a developmental stage-specific manner.

3. Enhancement of self-renewal potential in hematopoietic stem cells by overexpression of candidate genes

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Manipulation of gene expression in hematopoietic stem cells (HSCs) is certainly one of the ways to expand HSCs. Overexpression of HoxB4 is a good example to show how this strategy works well. HoxB4-expressed HSCs significantly more expand *in vivo* as well as *in vitro* than do wild type ones. Interestingly, embryonic stem cells (ESCs) give rise to HSCs *in vitro* by overexpressing HoxB4 although we have noticed that repopulating activity of ESC-derived HSCs is not so sufficient as that of adult bone marrow HSCs.

Leukemogenesis by HoxB4 overexpression alone has not been reported in a mouse model. Myeloproliferative neoplasm, however, remains as a caveat because constitutive foreign gene expression may result in more or less abnormal proliferation in HSCs. Even if this system cannot be readily applied to human HSCs, we are able to have an excellent tool to address the issue of how to modify HSCs for their efficient and unlimited *ex vivo* expansion.

Sauvageau *et al.* have recently published the list of candidate genes of which overexpression leads to *ex vivo* expansion of HSCs (Cell 137: 369, 2009). To reproduce their data and to have good positive controls to compare with our candidate genes, some of their genes in addition to *HoxB4* were included in this study. Our candidate genes mainly came from our gene expression array data. A total of 20 candidate genes were initially analyzed in this study. Candidate genes were cloned into a GCDNsam retroviral vector containing internal ribosomal entry followed by EGFP. Fifty $CD34^{-}Kit^{+}Sca\text{-}1^{+}Lineage^{-}$ cells, highly enriched in HSCs, were pre-incubated with stem cell factor and thrombopoietin for 24 hours, and infected with retrovirus for the following 24 hours. Transduced cells were post-incubated for 48 hours during which medium change was frequently performed to remove excess of virus, and then transplanted in each of 5 lethally irradiated mice together with 2×10^{5} bone marrow competitor cells. Secondary transplantation was performed 20 or more weeks after transplantation to ensure enhancement of self-renewal potential.

First of all, we found that mouse *HoxB4* works better than human *HOXB4* for expansion of mouse HSCs although human *HOXB4* had been often used in a mouse model by many researchers. As expected, *HoxB4*-transduced HSCs showed significantly higher levels of long-term repopulation as compared with those achieved by freshly isolated HSCs or GFP alone (empty vector)-transduced HSCs. We were interested in genes which increase repopulation activity greater than or equivalent to does *HoxB4*. Genes from Sauvageau's list including *Ski* increased repopulation levels, but all were inferior to *HoxB4*. One of our candidate genes, which encodes a signal molecule showed better reconstitution levels than did HoxB4. We are currently focusing on this gene. Of great interest is whether or not the combination with HoxB4 induces more expansion of HSCs without transformation into myeloid proliferative neoplasm.

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Donation Laboratories and Research Units

Laboratory of Stem Cell Regulation, Center for Stem Cell Biology and Regenerative Medicine

幹細胞制御領域

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The major goal of our laboratory is to understand the role of adult stem cells in tissue regeneration and cancer biology and develop novel therapies for diseases as clinical applications. Stem cells from adult tissues have the unique capacity to repair damaged tissue, a process controlled in part by the microenvironment. Additionally, bone marrow derived cells, which are supplied from adult tissue stem cells play key roles in formations of diseases, inflammation and immunological responses. Proteases like matrix metalloproteinases (MMPs), as part of the cell microenvironment act as processing (ectodomain shedding) 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. Recently evidence show that the fibrinolytic system (composed of various serine proteases) can regulate MMP activity. We unrevealed the mechanism how the fibrinolytic system regulates hematopoietic cell recruitment through MMP activation to promote tissue regeneration in the ischemic niche (1), to promote lymphoid cell growth in the cancer microenvironment (niche) (2) thereby altering abnormal angiogenesis and tumor growth. In addition we showed that the same fibrinolytic factors alter the progression of immunological or inflammatory diseases through the processing of biological factors (3). Moreover, we examined the role of membrane type MMP for hematopoesis and tissue regeneration (4).

1. Fibrinolytic system regulates myeloid-cell dependent neoangiogenesis during tissue regeneration depend on matrix metalloproteinase activation

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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. Additionally, we found besides VEGF, other angiogenesis initiation factor-Egfl7 promote mobilization of BM derived cells.

2. Plasmin inhibitor reduces lymphoid tumor growth by suppressing matrixmetallproteinase-9 dependent CD11b+/F4/80+ myeloid cell recruitment.

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Activation of the fibrinolytic system during lymphoma (cancer) 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, 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 Plg deficiency and drug-mediated plasmin blockade delayed lymphoma growth and diminished MMP-9 dependent CD11b+F4/80+ myeloid cell infiltration into lymphoma tissues. A neutralizing 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 plasmin in lymphoproliferative disorders and provide strong evidence that specific blockade of Plg represents a promising approach for the regulation of lymphoma growth.

3. Fibrinolytic factors regulate the progression of immunological diseases through the processing of biological modulators.

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Inflammatory cytokines released upon cell damage can cause excessive tissue destruction in immunological and inflammatory disease progression like sepsis, initiate diseases, systemic inflammatory diseases and as side effects of tissue transplantation, representatively, graft-versus-host disease (GVHD). Certain key cytokines are processed by activation of matrix metalloproteinases (MMPs) during disease progression. Many kinds of MMP inhibitors have been expected for the treatment of GVHD in the past, but its widespread use is hampered by severe and serious side effects. Others and we showed that the fibrinolytic factors, namely plasmin, modulate the activation of several MMPs. Therefore we hypothesized that the fibrinolytic system might be a novel molecular target in therapies for immunological diseases. We observed increased thrombosis and fibrinolytic system activation (fibrinogen, plasmin-α_2 antiplasmin inhibitor complex and tissue-plasminogen activator, plasminogen activator inhibitor complex in plasma concentrations) during the development of acute GVHD in mice and in humans. Drug-induced blockade of plasmin activity during acute GVHD development improved survival rate without serious side effects in mice. Of importance, plasmin inhibitor treatment blocked the processing of key cytokines for GVHD progression *in vitro* and *in vivo*. These data have major implications for transplantation medicine, as pharmacological inhibition of plasmin seems to lead to the development of tolerance without the need for intensive immunosuppression.

4. Membrane type of matrix metalloproteinase plays a critical role in the modulation of hematopoiesis.

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Membrane type 1-MMP (MT1-MMP) can activate MMP-9 in the process of mutual activation of MMP. It has already known that bone marrow (BM) myeloablation due to irradiation or anti-cancer drug administration induces MT1-MMP expression, but the role of MT1-MMP in hematopoiesis is not well understood. We examined MT1-MMP deficient mice (MT1-MMP−/−) 12 days after birth. MT1-MMP−/− were suffering from pancytopenia, and showed reduced numbers of bone marrow mononuclear cells (BMMCs), splenocytes and number of hematopoietic progenitor cells in the BM, although the number of hematopoietic stem cells (HSCs) in BMMCs was no significantly different. BM cytospins from MT1-MMP−/− mice showed mild erythropoietic disturbance and severer impairment of myelopoiesis. Kit-ligand (KitL) is a hematopoietic factor affecting all hematopoietic lineages. Interestingly, KitL levels were significantly lower in MT1-MMP−/− mice than in wild type (WT) counterparts. MT1-MMP knockdown by shRNA or/and siRNA impaired KitL expression and secretion in transfected stroma cells compared to Mock controls, demonstrating that reduced KitL plasma levels were due to impaired release/production and not due to reduced numbers of stromal cells in MT1-MMP−/−. Similarly, impaired proliferation and differentiation of MT1-MMP−/− BMMCs *in vitro* could be restored by addition of exogenous sKitL. Others and we reported that BM ablation induces the production of CXCL12, also known as stromal cell derived factor-1 (SDF-1), which plays a key role in stem cell homing and B-cell lymphopoiesis. Reduced SDF-1 expression was observed in BMMCs of MT1-MMP−/− mice and genetic knockdown of MT1-MMP resulted in lower SDF-1 expression both on a transcriptional and protein level in stromal cells. BMMCs of MT1-MMP−/− showed a decrease in the percentage of mature B cells compare to controls. Knocking down of MT1-MMP in stromal cell reduced the number of adherent hematopoietic cells, but addition of rec. SDF-1 could reverse the phenotype. These results suggested stromal-derived MT1-MMP was functionally important to maintain HSC function in long-term cultures of WT HSCs. Thus, MT1-MMP is a critical modulator of hematopoiesis, as it alters the growth factor production of niche cells.

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Center for Stem Cell Biology and Regenerative Medicine

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

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Our major goal is to cure patients suffering from life-threatening diseases by the treatment with processing of various stem cells. Currently our efforts are directed toward the establishment of novel therapies using human pluripotent stem cells (hPSC), such as embryonic stem (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 pluripotent stem cells

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ES cells are pluripotent cells derived from the inner cell mass of preimplantation embryos, and iPS cells 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 (HSCT) 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 human ES and iPS cells by co-culture with stromal cells derived from murine fetal liver (mFLSC) at 14 to 15 days post coitus (dpc) or AGM-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 ES or iPS cell 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 Mochizuki, Sachiyo Hanada, Sahoko Matsuzaka, Yuji Zaike[2], Hiromitsu Nakauchi[3] Kohichiro Tsuji

A critical issue for utilization of human ES or iPS cells in possible clinical use is whether they can derive terminally mature progenies with the normal function. To solve this, we examined human ES and iPS cells-derived erythroid cells in coculture with mFLSC or AGM cells. By the coculture, large quantity of human ES or iPS cell-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 human ES or iPS cell-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, Sahoko 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 human ES or iPS cells, to differentiate into mature blood cells without animal serum and cells. To achieve this, we first induced hPSC to differentiate into mesenchymal stem cells (MSC). When human ES or iPS cells cultured on murine embryonic fibroblast (MEF) feeder cells were recultured on gelatin-coated culture dishes with platelet lysate (PL)-containing media in the absence of MEF feeder cells. Cells were passaged several times with PL containing media, and then MSC were induced after 6 to 8 weeks. The MSC were spindle-like shaped, revealed a phenotype of CD45−, CD34−, CD14−, CD105+, CD166+, CD31−, and 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, Sahoko 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 nor-

mal 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 mFLSC or AGM-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, Sahoko 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 human ES and iPS cells. By a two-step induction, we first generated multipotential HPC by co-culturing hPSC with AGM-3 cells for 2 weeks. Then, total coculture 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 (EPO+ cells from 56.4% at day 7 to 94.4% at day 21, while 2D7+ cells from 62.8% to 25.7%, respectively), 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 (from 10.8% at day 7 to 91.3 % at day 21), 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. The function of hES/hiPSC-derived eosinophils is being under investigation. 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 (Wechsler *et al*., 2002; Mundschau *et al*., 2003), 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 human iPS cells derived from patients with DS (DS-hiPSC). Six DS-hiPS and 5 hiPS cell lines (control) from healthy donors, which we used here, 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 AGM-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 (p-value$<$0.05). Similar results were obtained in the experiments using human iPS cells reprogrammed by 4 factors. These results indicated that human iPS cells derived from patients with DS could differentiate into multiple hematopoietic cell lineages and the differentiation into hematopoietic lineage was promoted in DS patients.

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Center for Stem Cell Biology and Regenerative Medicine

Division of Stem Cell Transplantation
幹細胞移植分野

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We are conducting clinical stem cell transplantation, especially using 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 200 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 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 x 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. The impact of steroid use as a GVHD treatment or prophylaxis within 100 days after CBT

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

The incidence of severe graft-versus-host disease (GVHD) in cord blood transplantation (CBT) is generally low, but still exists. In our institute, we use cyclosporine (CsA) and short term methotrexate (MTX) as GVHD prophylaxis and minimally use steroid to avoid infection or infection-related complications. In this study, we retrospectively analyzed the clinical data to clarify the impact of steroid use to the outcome of CBT. PATIENTS: We have performed 140 CBT after myeloablative conditioning using CsA with short term MTX as GVHD prophylaxis for adults at IMSUT between August 1998 and October 2008. The median age was 39 (range, 16-55) years and the median number of cryopreserved nucleated CB cells was 2.38 (range, 1.21-5.69) $x10^7$/kg. Although 82 of 140 patients (59%) suffered from grade II-IV aGVHD, only 31% patients received steroid after CBT. Steroid was used in 17 patients (12%) as a treatment for mainly GVHD and the dosage of prednisolone in the treatment group were 2 mg/kg (n=7), 1 mg/kg (n=8), and 0.5mg/kg (n=2). Twenty-six patients (19%) changed CsA to steroid because of intolerability (20: renal dysfunction, 4: encephalopathy, 2: others) and received 1mg/kg (n=4) or 0.5mg/kg (n=22) (alternative group). Overall survival in 5 years were 78% in the non-steroid use group, 71% in the treatment group, however 45% in the patients with alternative steroid use. The intolerability of CsA within 100 days after CBT seems to be a significant poor factor. We should modify the procedures including post-transplant immune modulation in such patients.

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 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.

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 57 kg 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 or 4 reprogramming factors (Oct4, Klf4 and Sox2 with or without c-Myc). In this vector, porcine teschovirus-1 2A sequences that trigger ribosome skipping were inserted between human 3F or 4F cDNAs, and a loxP site was placed in the truncated 3′-LTR to remove almost all the vector elements including the transgenes after derivation of 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. For the latter system, another lentiviral vector expressing reverse tetracycline transactivator (rtTA) driven by the EF-1α promoter was combined. We already succeeded in establishment of several lines of C57BL/6 mouse embryonic fibroblast-derived IPS cells, and pluripotency of these cells were confirmed by their expression of embryonic stem cell signatures as well as formation of teratomas containing all three germ layer-derived tissues in NOD-SCID mice. Furthermore, Adenovirus-mediated expression of Cre recombinase successfully excised the lentiviral vector components and left only remnant 291-bp SIN LTRs containing a single loxP site. 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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Center for Stem Cell Biology and Regenerative Medicine

Division of Stem Cell Signaling
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Our major interest is to elucidate the mechanisms of pluripotency, self-renewal and the control of cell division and differentiation of 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, to 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 for surface antigens of cancer cells or immune cells. Here we used SST-REX 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, 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, although the precise mechanisms remain to be investigated.

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 and one of these mutants enhanced reprogramming fibroblast. We are also interested in 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 Shibarta, 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 systems, and 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 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. We also found that several mechanisms, such as transcription, protein degradation, and microRNA involve these processes. In summary, our results implicate MgcRacGAP in coordination of cell cycle progression and cell fate determination.

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 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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