Center for Stem Cell Biology and Regenerative Medicine

Division of Stem Cell Therapy
幹細胞治療部門

Professor	Hiromitsu Nakauchi, M.D., Ph.D	教　授	医学博士	中　内　啓　光
Assistant Professor	Koji Eto, M.D., Ph.D.	助　教	医学博士	江　藤　浩　之
Project Assistant Professor	Nobukazu Watanabe, M.D., Ph.D	特任助教	医学博士	渡　辺　信　和
Assistant Professor	Makoto Otsu, M.D., Ph.D.	助　教	医学博士	大　津　　真
Assistant Professor	Akihide Kamiya, Ph.D.	助　教	理学博士	紙　谷　聡　英
Project Assistant Professor	Shin Kaneko, M.D., Ph.D.	特任助教	医学博士	金　子　　新

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. TGF-β as a candidate bone marrow niche signal to induce hematopoietic stem cell hibernation

Satoshi Yamazaki, Atsushi Iwama[1], Shin-ichiro Takayanagi, Koji Eto, Hideo Ema, and Hiromitsu Nakauchi: [1]Department of Cellular and Molecular Medicine, Graduate School of Medicine, Chiba University

Dormancy or hibernation of HSCs, which is indispensable for HSC maintenance, is known to occur solely in the particular bone marrow (BM) microenvironment known as the HSC niche. Most of the HSCs are in the G0 phase in the BM niche. However, HSCs are recruited into the cell cycle at long intervals, on average every one to two months. Thus, the capacity to enter and to leave a hibernation-like state is one of the properties of "stemness". The so-called 'stromal cells' in the HSC BM niche, including osteoblasts, fibroblasts, adipocytes, and endothelial cells, produce a number of secreted and membrane-bound growth factors. Several signaling pathways have been characterized that keep HSCs in hibernation or undifferentiated states. These include the Ang-1-Tie-2 signal, the Notch ligand-Notch signal, the N-cadherin homotypic signal, and the transforming growth factor-β (TGF-β) signal. However, the precise molecular mechanisms underlying HSC hibernation remain largely elusive. We have recently shown that freshly isolated CD34-KSL hematopoietic stem cells (HSCs) in a hibernation state exhibit inhibited lipid raft clustering. Lipid raft clustering induced by cytokines is essential for HSCs to augment cytokine signals to the level enough to reenter the cell cycle. Here we screened candidate niche signals that inhibit lipid raft clustering, and identified that TGF-β efficiently inhibits cytokine-mediated lipid raft clustering and induces HSC hibernation *ex vivo*. Smad2 and Smad3, the signaling molecules directly downstream from and activated by TGF-β receptors were specifically activated in CD34-KSL HSCs in a hibernation state, but not in cycling CD34+ KSL progenitors. These data uncover a critical role for TGF-β as a candidate niche signal in the

control of HSC hibernation and provide TGF-β as a novel tool for *ex vivo* modeling of the HSC niche.

2. Establishment of an *in vitro* Culture System for Hematopoiesis from Embryonic Stem cells

a) Generation of functional platelets from human embryonic stem cells *in vitro* via ES-sacs, VEGF-promoted structures that concentrate hematopoietic progenitors

Takayama N, Nishikii H, Usui J, Tsukui H, Sawaguchi A[1], Hiroyama T[2], Eto K, Nakauchi H.: [1]University of Miyazaki; [2]RIKEN Bio Resource Center

Human embryonic stem cells (hESCs) could potentially represent an alternative source for blood transfusion therapies and a promising tool for studying the ontogeny of hematopoiesis. When we cultured hESCs on either C3H10T1/2 or OP-9 cells to facilitate hematopoiesis, we found that exogenous administration of vascular endothelial growth factor promoted the emergence of sac-like structures, which we named "embryonic stem cell-derived sacs" (ES-sacs). These ES-sacs consisted of multiple cysts demarcated by cellular monolayers that retained some of the properties of endothelial cells. The spherical cells inside ES-sacs expressed primarily CD 34, along with VE-cadherin, CD31, CD41a and CD45, and were able to form hematopoietic colonies in semisolid culture and to differentiate into mature megakaryocytes by day 24 in the presence of thrombopoietin. Apparently, ES-sacs provide a suitable environment for hematopoietic progenitors. Relatively large numbers of mature megakaryocytes could be induced from the hematopoietic progenitors within ES-sacs, which were then able to release platelets that displayed integrin aIIbb3 activation and spreading in response to ADP or thrombin. This novel protocol thus provides a means of generating platelets from hESCs, which could serve as the basis for efficient production of platelets for clinical transfusion and studies of thrombopoiesis.

(b) Metalloproteinase regulation improves *in vitro* generation of efficacious platelets from mouse embryonic stem cells

Nishikii H, Eto K, Tamura N[1], Hattori K, Heissig B, Kanaji T[2], Sawaguchi A[3], Goto S[1], Ware J[4], Nakauchi H.: [1]Tokai University; [2]Kurume University; [3]University of Miyazaki; [4]University of Arkansas

Embryonic stem cells (ESCs) could potentially compensate for the lack of blood platelets available for use in transfusions. Here, we describe a new method for generating mouse ESC-derived platelets that can contribute to hemostasis *in vivo*. Flow cytometric sorting of cells from embryoid bodies on day 6 demonstrated that c-Kit$^+$ integrin aIIb (aIIb)$^+$ cells, but not CD31$^+$ cells or vascular endothelial-cadherin$^+$ cells, are capable of megakaryopoiesis and the release of platelet-like structures by day 12. aIIbb3-expressing ESC-derived platelets (ESPs) exhibited ectodomain shedding of glycoprotein (GP) Iba, GPV, and GPVI, but not aIIbb3 or GPIbb. ESPs showed impaired aIIbb3 activation and integrin-mediated actin reorganization, critical events for normal platelet function. However, the administration of metalloproteinase inhibitors, GM6001 or TAPI-1, during differentiation increased the expression of GPIba improving both thrombogenesis *in vitro* and post-transfusion recovery *in vivo*. Thus, the regulation of metalloproteinases in culture could be useful for obtaining high-quality, efficacious ESPs, as an alternative platelet source for transfusions.

3. Mechanisms of thrombopoiesis: how do megakaryocytes generate platelets?

a) Growth and maturation of megakaryocytes is regulated by Lnk/Sh2b3 adaptor protein through crosstalk between cytokine- and integrin-mediated signals

Takizawa H[1], Eto K, Yoshikawa A[1], Nakauchi H, Takatsu K[2], Takaki S[1].: [1]Research Institute, International Medical Center of Japan; [2]University of Toyama

Various cytokines and growth factors control the differentiation and maturation of megakaryocytes (MKs). However, the mechanism regulating platelet release from MKs is not well understood. Here, we investigated a role of Lnk/Sh2b3, an intracellular adaptor protein, in megakaryopoiesis. Number of MK progenitor in bone marrow (BM) of wild-type or *Lnk*$^{-/-}$ mice and their sensitivity to thrombopoietin (TPO) were determined in colony-forming unit assay. Using BM-derived wild-type or *Lnk*$^{-/-}$ MKs stimulated with TPO, activation of the signaling molecules was biochemically analyzed and effect of integrin stimulation on TPO signals was studied by addition of vascular cell adhesion molecule (VCAM-1). Platelet production from MKs in the presence of VCAM-1 was counted by flow cytometry and their morphological change was observed by time-lapse microscopy. *Lnk*$^{-/-}$ mice showed elevated platelets and ma-

ture MKs due to enhanced sensitivity of progenitors to TPO. Erk1/2 phosphorylation induced by TPO was augmented and prolonged in $Lnk^{-/-}$ MKs while activation of signal transducers and activators of transcription (Stat)3, Stat5, and Akt was normal. Wild-type MKs, but not in $Lnk^{-/-}$ MKs on VCAM-1 showed reduced Stat5 phosphorylation and mitogen-activated protein kinases activation upon stimulation with TPO. Additionally, the presence of VCAM in culture accelerated spontaneous platelet release from mature wild-type MKs, but not from $Lnk^{-/-}$ MKs. Results suggest that contact of MKs with adhesion molecules via integrins might contribute to platelet release, which is under Lnk-mediated regulation of Stat-5 activation and show that Lnk functions in responses controlled by cell adhesion and in crosstalk between integrin- and cytokine-mediated signaling.

b) Calyculin A retraction of mature megakaryocytes proplatelets from embryonic stem cells

Tamaru S[1], Kitajima K[2], Nakano T[2], Eto K, Yazaki A[1], Kobayashi T[1], Matsumoto T[1], Wada H[1], Katayama N[1], Nishikawa M[1].: [1]Mie University; [2]Osaka University

Platelets are produced by megakaryocytes (MKs) through proplatelet formation (PPF), or cytoplasmic extensions, in vitro. Through the use of video-enhanced light microscopy, as well as localization of cytoskeletal proteins by confocal microscopy, the reaction of fully mature MK proplatelets, derived from murine embryonic stem cells, to various agents was studied. Calyculin A (protein phosphatase 1/2A inhibitor) treatment induced proplatelet retraction. In MKs with PPF, the expression of actin, myosin IIA, monophosphorylated myosin light chain (MLC-P1), and diphosphorylated myosin light chain (MLC-P2) was diffusely located. Following calyculin A treatment, actin was diffusely localized in retracted MKs and was expressed particularly in the periphery. MLC-P1 was also localized primarily in the periphery; however, MLC-P2 was expressed mostly in the inner area of proplatelets. Protein phosphatase inhibitors may result in increased hyperphosphorylation of localized MLC, which could alter the balance of actomyosin force in a cell, and therefore induce proplatelets retraction.

4. Development of stem cell-based cell and gene therapy strategies towards clinical trials

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) Development of a safe and effective stem cell transplantation strategy by the use of antibody-based minimum intensity preconditioning and delayed infusion of suicide-gene transduced donor T lymphocytes in the mouse model of X-linked chronic granulomatous disease (X-CGD)

Yasuo Takeuchi, Makoto Otsu, Akihiro Kume, Sachie Suzuki, Shin Kaneko, Motohito Okabe, Emiko Takeuchi , Masafumi Onodera , Hiromitsu Nakauchi

Allogeneic hematopoietic stem cell transplantation (Allo HSCT) is useful for treatment of life-threatening, non-malignant diseases. The procedures, however, inevitably involve substantial risks including 1) conditioning toxicity, 2) graft rejection, 3) graft versus host disease (GVHD). To develop a safe and effective transplantation strategy for clinical application, we have designed a combinatorial method consisting of anti-CD40L monoclonal antibody (α-CD40L mAb)-based minimum conditioning and delayed infusion of suicide-gene transduced donor T lymphocytes in murine HSCT models. We used C57BL/6 x C3H F1 mice as donors and C57BL/6 x DBA2 F1 mice as recipients to mimic haploidentical allo-HSCT. Low dose irradiation (3 Gy) on day -1 followed by α-CD40L mAb injection

(2 mg) and donor bone marrow cell infusion (2x 10^7) on day 0, reproducibly led to induction of stable mixed chimerism (45.2 $^{+}$/$_{-}$12.5 % B cell donor chimerism at 12 wks post-BMT) in treated mice (12 of 12). Next, we injected donor T cells (1x10^7) into mixed chimeric mice. They showed subsequent enhancement of donor cell chimerism, also having exhibited some symptoms of GVHD. We are now in the process of introducing the herpes simplex virus-thymidine kinase (HSV-TK)/GCV system into donor T lymphocytes as a fail-safe measure. Although still preliminary, these results likely indicate the possible development of a safe and effective HSCT strategy that utilizes; 1) harmless α-CD40L mAb -based chimerism establishment, and 2) secure induction of full-donor chimerism with the use of donor T cells equipped with the fail-safe suicide system. Finally, we are now attempting this procedure for the X-CGD mouse model, which is characterized by functional defects in phagocytes. We now propose this transplantation strategy as a promising treatment option applicable for many intractable disorders including X -CGD.

c) A novel strategy of hematopoietic stem cell transplantation for severe combined immunodeficiency diseases

Sachie Suzuki, Makoto Otsu, Hiromitsu Nakauchi

Severe combined immunodeficiency disease (SCID) such as X-linked severe combined immunodeficiency disease, adenosine deaminase (ADA) deficiency, and recombinase-activating gene (RAG) deficiency is a group of life-limiting diseases caused by congenital defects of responsible genes. SCID is often curable by hematopoietic stem cell transplantation (HST). HST, however, is accompanied with significant risks for treatment-related mortality due to graft failure, infection, graft versus host disease (GVHD) and other complications. Although stem cell-gene therapy, a newly emerging treatment option, has become reality for these diseases, HST will remain as a standard treatment if inherent risks can be further reduced. We will report In the meeting the development of a novel HST strategy that we named "donor lymphocyte infusion (DLI)-based conditioning Stem cell Transplantation (DLIST)". As a model of allogeneic HST for human diseases, we used Rag2-deficiency mice (C57BL/6, $H2^b$) as recipients and C3H/He mice ($H2^k$) as donors. Recipients were transplanted C3H bone marrow (BM) cells either; without any preconditioning treatment (G-1), with sub-lethal dose irradiation (G-2), or days 11-12 after donor T lymphocyte infusion without irradiation (G-3: DLIST group) and then tested for hematological and immunological reconstitution. As expected, mice in G-1 showed only minimal engraftment of donor cells whereas G-2 animals developed robust donor cell-hematopoiesis and immunity. Interestingly, DLIST-recipients showed long-term repopulation of donor myeloid cells, indicating that donor HSCs stably engrafted in recipients despite the absence myeloablative preconditioning. Reconstitution of T and B cell immunity was also evidenced in DLIST-recipients. Furthermore, donor T lymphocytes that had emerged after DLIST showed acquisition of immunological tolerance for recipient cells, which eventually allowed for treated mice long-term survival without the evidence of GVHD. These results indicate that pre-infusion of donor T lymphocytes can make "space" in host BM for engraftment of donor HSCs and that developing donor hematopoiesis can reconstitute SCID recipients' immunity by simultaneously suppressing the preceding GVH reactions, thus enabling a DLIST strategy. To further minimize the treatment-related risks, we are currently in the process of introduction of a fail-safe system into donor lymphocytes, which is based on the use of the herpes simplex virus-thymidine kinase (HSV-TK) gene and ganciclovir (GCV).

5. Sall4 Regulates Cell Fate Decision in Fetal Hepatic Stem/Progenitor Cells

Tsunekazu Oikawa, Akihide Kamiya, Sei Kakinuma , Mikio Zeniya[1] , Ryuichi Nishinakamura[3] , Hisao TAJIRI[2] , and Hiromitsu Nakauchi: [1]Division of Gastroenterology and Hepatology, Department of Internal Medicine, Jikei University School of Medicine, Tokyo, Japan; [2]Department of Gastroenterology, Jikei University Graduate School of Medicine, Tokyo, Japan; [3]Division of Integrative Cell Biology, Institute of Molecular Embryology and Genetics, Kumamoto University, Kumamoto, Japan

Fetal hepatic stem/progenitor cells, called hepatoblasts, differentiate into both hepatocytes and cholangiocytes. The molecular mechanisms regulating this lineage segmentation process remain unknown. Sall4 has been shown to be among the regulators of organogenesis, embryogenesis, maintenance of pluripotency, and early embryonic cell-fate decisions in embryonic stem cells. The expression and functional roles of Sall 4 during liver development have not been elucidated. We here provide their first description in hepatoblasts. To investigate functions of Sall4 in

fetal liver development, $Dlk^+CD45^-Ter119^-$ hepatoblasts derived from embryonic day 14 mouse livers were purified and in vitro gain and loss of function analyses and in vivo transplantation analyses were performed using retrovirus- or lentivirus-mediated gene transfer. We demonstrated that Sall4 was expressed in fetal hepatoblasts but not adult hepatocytes. The expression level of Sall4 gradually fell during liver development. Overexpression of Sall4 in hepatoblasts significantly inhibited maturation induced by oncostatin M and extracellular matrix in vitro, as evidenced by morphological changes and suppression of hepatic maturation marker gene expression. When bile duct-like structures were induced by collagen-gel embedded culture, overexpression of Sall4 markedly augmented size and number of cytokeratin19^+-branching structures. Knockdown of Sall4 inhibited formation of these branching structures. With in vivo transplantation, Sall4 enhanced differentiation of cytokeratin19^+-bile ducts derived from transplanted hepatoblasts. These results suggest that Sall4 plays a crucial role in controlling the lineage commitment of hepatoblasts, not only inhibiting their differentiation into hepatocytes, but also driving their differentiation toward cholangiocytes.

6. Analyses of Cell Surface Molecules on Hepatic Stem/Progenitor Cells in Mouse Fetal Liver

Sei Kakinuma, Haruhiko Ohta, Akihide Kamiya, Yuji Yamazaki, Tsunekazu Oikawa, Ken Okada, Hiromitsu Nakauchi

Hepatic stem/progenitor cells possess active proliferative ability and the capacity for differentiation into hepatic and cholangiocytic lineages. Our group and others have shown that a prospectively defined population in mid-gestational fetal liver contains hepatic stem/progenitor cells. However, the phenotypes of such cells are incompletely elucidated. We analyzed the profile of cell-surface molecules on primary hepatic stem/progenitor cells. Expression of cell surface molecules on primary hepatic stem/progenitor cells in mouse mid-gestational fetal liver was analyzed using flow cytometric multicolor analyses and colony-formation assays. The potential of the cells for liver repopulation was examined by transplantation assay. We found that CD13 (aminopeptidase N) was detected on the cells of previously reported (Dlk/Pref-1^+) hepatic stem / progenitor fraction. Colony-formation assays revealed that the $CD13^+$ fraction, compared with the Dlk^+ fraction, of non-hematopoietic cells in fetal liver was enriched in hepatic stem/progenitor cells. Transplantation assay showed that such fraction exhibited repopulating potential in regenerating liver. Moreover, flow cytometric analysis for over 90 antigens demonstrated enrichment of hepatic stem/progenitor cells using several positive selection markers, including (hitherto unknown) CD13, CD73, CD106, and CD133. Our data indicated that CD13 is a positive selection marker for hepatic stem/progenitor cells in mid-gestational fetal liver.

7. Idiopathic Myelofibrosis Generated by The Transplantation of STAT5A(1*6) Transduced Hematopoietic Stem Cells to Irradiated Mice

Takafumi SHIMIZU, Shin KANEKO, and Hiromitsu NAKAUCHI

We previously reported that myeloproliferative disease (MPD) can be generated in transplanted mice not by abnormal hematopoietic progenitor cells, but by abnormal hematopoietic stem cells (HSC) genetically modified with STAT5A(1*6), constitutive active form of STAT5 A (Kato et al, J Exp Med 2005). It indicated that the activation of STAT5 pathway in purified HSC allows them to transform into long-lasting MPD stem cells that can provide extreme number of progeny cells working as MPD effector cells. To know the detail of pathogenesis caused by STAT5A hyper-activating MPD stem cells, we followed up the hematopoiesis and pathological findings of STAT5(1*6) transduced HSC transplanted mice, then we found that these mice developed into severe myelofibrosis within a few month with showing neither hyper erythrocytosis nor hyper thrombocytosis. These mice also showed increase of dysplastic megakaryocytes and decrease of erythroblasts in bone marrow, and splenomegary with leukoerythroblastosis. Those symptoms are quite similar to idiopathic myelofibrosis (IMF) patient. Moreover, the bone marrow transplantation from myelofibrotic mice also caused myelofibrosis in secondary transplanted mouse, indicating that STAT5 A(1*6) transduced HSC transplanted to primary recipient had contained MPD/IMF stem cells which can reproduce same disease phenotype in secondary transplanted mice.

We have developed an animal model of rare MPD, idiopathic myelofibrosis, by cancer stem cell transplantation which are generated by retroviral transduction of STAT5A(1*6) to HSC. The disease model explains the contribution of abnormal activation of JAK-STAT pathway in HSC to initiation and progression of IMF, resembling to the case of polycythemia vera and

essential thrombocytosis.

8. Three-year clinical follow-up of 5 patients treated with suicide gene modified donor lymphocyte infusion therapy in Japan

S. Kaneko[1,2], Y. Okoshi[2], M. Otsu[1], N. Nemoto[2], K. Suzukawa[2], Y. Hasegawa[2], H. Kojima[2], T. Fukushima[2], R. Sumazaki[2], Y. Harada[2], H. Sakamaki[3], M. Tsuchida[4], S. Kato[5], C. Bonini[6], C. Bordignon[6], M. Onodera[7], T. Nagasawa[8], S. Chiba[2], H. Nakauchi[1] on behalf of the Tsukuba TK-DLI Gene Tharapy Group: [1]Dept. Stem Cell Therapy, IMSUT; [2]Tsukuba University Hospital; [3]Komagome Tokyo Metropolitan Hospital; [4]Ibaraki prefectural children's Hospital; [5]Tokai University; [6]Scientific Institute San Raffaele, Milano, Italy; [7]National Center for Child Health and Development; [8]Tsukuba Kinen Hospital

Suicide gene, herpes virus thymidine kinase gene (*hsv-tk*), modified donor T lymphocyte infusion (TK-DLI) is one of the promising approach to cure high risk hematological malignancy patients treated by T cell depleted haploidentical hematopoietic stem cell transplantation. Despite the success of T lymphocyte based gene therapy in haplo SCT setting to reduce relapse unrelated mortality with control of graft-versus-host disease is becoming clear, there remains some difficulties to be solved for inducing complete remission to patients suffered from relapsed leukemia after SCT. The solutions would be 1) very early detection of leukemia relapse and enough reduction of leukemia burden before TK-DLI, 2) make, 'niche', a space for TK-T lymphocytes, and 3) maintain TK-T lymphocytes during ex vivo manipulation to have proliferative and survival potentials.

A phase I/II clinical trial of TK-DLI have been performed in the Tsukuba University Hospital to 5 patients (2 AML, 2 ALL, and 1 MDS) since 2002. Actually the five patients for relapsed leukemia had one or more problems as listed above, and it resulted in only temporal and insufficient clinical response in 4 of 5 patients. Only one MDS patient survives more than 3 years after TK-DLI without any sign of relapse. Different from the other patients in treatment failure, He was in hematological remission at the time of TK-DLI and had been in immunosuppressive status of 6 month after SCT, suggests that he had been in ideal clinical conditions for TK-DLI.

According to the result, we are optimizing clinical and manipulation protocols. They include 1) detection of molecular relapse and immediate action of TK-DLI, 2) a lymphodepletion conditioning with Fludarabin and Cyclophosphamide before TK-DLI, and 3) the usage of CD3/CD28 stimulation+IL-2, instead of OKT3+high concentrated IL-2, to obtain suicide gene transduced central memory TK-T lymphocytes.

9. Analysis of immune reconstitution after cord blood transplantation

a) Clinical study for recipients after cord blood transplantation

Nobukazu Watanabe, Satoshi Takahashi[1], Masayuki Ishige, Yumiko Ishii, Jun Ooi[1], Akira Tomonari[1], Nobuhiro Tsukada[1], Takaaki Konuma[1], Seiko Kato[1], Aki Sato[1], Arinobu Tojo[1], Hiromitsu Nakauchi: [1]Research Hospital, IMSUT

Although umbilical cord blood has been increasingly used as an alternative donor source to treat hematological malignancies, cord blood transplantation (CBT) is frequently complicated by graft failure and relapse of primary diseases. We collaborate with medical staff in Research Hospital, IMSUT to understand the pathogenesis of graft failure and relapse of leukemia. Our clinical researches are as follows; study of patho-physiology after HLA-mismatched transplantation using FACS-based method of chimerism analysis (HLA-Flow method), the influences of HLA mismatch on induction of cytomegalovirus-specific T cells, and regulation of immunosuppressant by Th1 cytokine assays.

b) Generation of allele-specific anti-HLA monoclonal antibodies

Satoshi Yamazaki[1,3], Nao Suzuki, Tsuneyoshi Saito[1], Yumiko Ishii, Masafumi Takiguchi[2], Nobukazu Watanabe , and Hiromitsu Nakauchi[3]: [1]ReproCELL Inc., [2]Center for AIDS Research , Kumamoto University , [3]JST-ERATO.

That generation of allele-specific anti-human leukocyte antigen (HLA) monoclonal antibodies (ASHmAb) is very difficult is well known. This is thought to be due to the unique epitope structure, an assemblage of amino acid residues that lie separately in the amino acid sequence of human HLA, and to its low antigenicity compared with that of common epitopes recognized as xenogeneic determinants by mice. We develop a rapid and efficient strategy to generate ASHmAb. Different from usual immunization methods is that we suppressed 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 by this strategy will be useful for HLA typing and for clinical diagnosis, such as flow cytometry-based chimerism analysis for early detection of graft failure and relapse of leukemia after HLA-mismatched hematopoietic stem cell transplantation.

Publications

Sogo T, Kawahara M, Ueda H, Otsu M, Onodera M, Nakauchi H, Nagamune T. T cell growth control using hapten-specific antibody /interleukin-2 receptor chimera. *Cytokine.* 2009 *In Press*

Kakinuma S, Nakauchi H, Watanabe M. Hepatic stem/progenitor cells and stem-cell transplantation for the treatment of liver disease. *J Gastroenterol.* 44: 167-72, 2009

Yamazaki S, Suzuki N, Saito T, Ishii Y, Takiguchi M, Nakauchi H, Watanabe N. A rapid and efficient strategy to generate allele-specific anti-HLA monoclonal antibodies. *J. Immunol. Method*. 343: 56-60, 2009

Ogaeri T. Eto K. Otsu M. Ema H. Nakauchi H. The actin polymerization regulator WAVE2 is required for early bone marrowrepopulation by hematopoietic stem cells. *Stem Cells*. 2009 *In press*.

Oikawa T, Kamiya A, Kakinuma S, Zeniya M, Nishinakaumura R, Tajiri H, Nakauchi H, Sall 4 Regulates Cell Fate Decision in Fetal Hepatic Stem/Progenitor Cells. *Gastroenterology*. 136: 1000-11, 2009

Yashiro Y, Bannai H, Yabiku T', Minowa T, Miyano M, Osawa M, Iwama A, Nakauchi H, Transcriptional Profiling of Hematopoietic Stem Cells by High-Throughput Sequencing. *Int. J Hematology*. *Int. Hematol*. 89: 24-33, 2009

Hyun I, Lindvall O, Ahrlund-Richter L, Cattaneo E, Cavazzana-Calvo M, Cossu G, De Luca M, Fox IJ, Gerstle C, Goldstein RA, Hermerén G, High KA, Kim HO, Lee HP, Levy-Lahad E, LiL, LoB, Marshak DR, McNab A, Munsie M, Nakauchi H, Rao M, Rooke HM, Valles CS, Srivastava A, Sugarman J, Taylor PL, Veiga A, Wong AL, Zoloth L, Daley GQ. New ISSCR guidelines underscore major principles for responsible translational stem cell research. *Cell Stem Cell*. 3: 607-9, 2008

Suzuki A, Sekiya S, Onishi M, Oshima N, Kiyonari H, Nakauchi H, Taniguchi H. Flow cytometric isolation and clonal identification of self-renewing bipotent hepatic progenitor cells in adult mouse liver. *Hepatology*. 48: 1964-78, 2008

Chiba T, Miyagi S, Saraya A, Aoki R, Seki A, Morita Y, Yonemitsu Y, Yokosuka O, Taniguchi H, Nakauchi H, Iwama A. The polycomb gene product BMI1 contributes to the maintenance of tumor-initiating side population cells in hepatocellular carcinoma. *Cancer Res*. 68: 7742-9, 2008

Agata H, Kagami H, Watanabe N, Ueda M. Effect of ischemic culture conditions on the survival and differentiation of porcine dental pulp-derived cells. *Differentiation*, 76: 981-993, 2008.

Ma F, Ebihara Y, Umeda K, Sakai H, Hanada S, Zhang H, Zaike Y, Tsuchida E, Nakahata T, Nakauchi H, Tsuji K. Generation of functional erythrocytes from human embryonic stem cell-derived definitive hematopoiesis. *Proc Natl Acad Sci USA*. 105: 13087-92, 2008

Sanuki S, Hamanaka S, Kaneko S, Otsu M, Karasawa S, Miyawaki A, Nakauchi H, Nagasawa T, Onodera M. A new red fluorescent protein that allows efficient making of murine hematopoietic stem cells. *THE JOURNAL OF GENE MEDICINE*. 10: 965-71, 2008

Yamazaki S, Iwawa A, Takayanagi SI, Eto K, Ema H, Nakauchi H. TGF-β as a candidate bone marrow niche signal to induce hematopoietic stem cell hibernation. *Blood*. 113: 1250-1256, 2008

Kamiya A, Kakinuma S, Onodera M, Miyajima A, Nakauchi H. Prospero-related homeobox 1 and liver receptor homolog 1 coordinately regulate long-term proliferation of murine fetal hepatoblasts. *Hepatology*. 48: 252-64, 2008

Nishikii H, Eto K, Tamura N, Hattori1 K, Heissig B, Kanaji T, Sawaguchi A, Goto S, Ware J, Nakauchi H. Metalloproteinase regulation improves *in vitro* generation of efficacious platelets from mouse embryonic stem cells. *J Exp Med* P. 205: 1917-27, 2008

Watanabe N, Takahashi S, Ishige M, Ishii Y, Ooi J, Tomonari A, Tsukada N, Konuma T, Kato S, Sato A, Tojo A, Nakauchi H. Recipient-derived cells after cord blood transplantation: dynamics elucidated by multicolor FACS, reflecting graft failure and relapse. *Biol Blood Marrow Transplant*. 14: 693-701, 2008

Takizawa H, Eto K, Yoshikawa A, Nakauchi H, Takatsu K, Takaki S. Growth and maturation of megakaryocytes is regulated by Lnk/Sh2b3 adaptor protein through crosstalk between cytokine- and integrin-mediated signals. *Exp Hematol*. 36: 897-906, 2008

Takayama N, Nishikii H, Usui J, Tsukui H,

Sawaguchi A, Hiroyama T, Eto K, Nakauchi H. Generation of functional platelets from human embryonic stem cells in vitro via ES-sacs, VEGF-promoted structures that concentrate hematopoietic progenitors. *Blood*. 1: 5298-306, 2008

Nabekura T, Nagasawa T, Nakauchi H, Onodera M. An immunotherapy approach with dendritic cells genetically modified to express the tumor-associated antigen, HER2. *Cancer Immunol Immunother*. 57: 611-22, 2008

Seita J, Asakawa M, Ooehara J, Takayanagi SI, Morita Y, Watanabe N, Fujita K, Kudo M, Mizuguchi J, Ema H, Nakauchi H, Yoshimoto Y. Interleukin-27 directly induces differentiation in hematopoietic stem cells. *Blood*. 111: 1903-12, 2008

Center for Stem Cell Biology and Regenerative Medicine

Laboratory of Stem Cell Regulation

幹細胞治療研究センター
幹細胞制御分野

Project Associate Professor Koichi Hattori, M.D., Ph.D.
Project Assistant Professor Beate Heissig, M.D., Ph.D.

特任准教授 医学博士 服 部 浩 一
特任助教 医学博士 ハイジッヒ・ベアーテ

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 potential use of metalloproteinase inhibitors for the in vitro generation of embryonic cell-derived platelets (2).

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

Makiko Ohki, Yuichi Ohki, Haruyo Akiyama, Makoto Ishihara, Chiemi Nishida, Yoshihiko Tashiro, Hiromitsu Komiyama[1], Leif R. Lund[2], Atsumi Nitta[3] Kiyofumi Yamada[3], Zhenping Zhu[4], Hideoki Ogawa[1], Hideo Yagita[1], Ko Okumura[1], Zena Werb[5], Beate Heissig, Koichi Hattori: [1]Atopy (Allergy) Research Center, Dep. Cardiology, Dep. of Surgery, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan, [2]Finsen Laboratory, Rigshospitalet, Strandboulevarden 49, 2100 Copenhagen, Denmark, [3]Nagoya University Graduate School of Medicine, 65 Tsuruma-cho, Showa-ku, Nagoya, Aichi, 466-8560, Japan, [4]ImClone Systems, NY, [5]Department of Anatomy, University of California, San Francisco, 513 Parnassus Avenue, San Francisco, CA 94143-0452, USA.

Ischemia of the heart, brain and limbs is a leading cause of morbidity and mortality worldwide. Bone-marrow (BM)-derived cells facilitate angiogenesis during tissue regeneration and in cancer, but the molecular mechanisms of this facilitation are incompletely understood. We have previously shown that proteases, including matrix metalloproteinases (MMP) can regulate hematopoiesis by modulating kit ligand release. Here we report that the classical fibrinolytic factor tissue-type plasminogen activator (tPA), by activating the extracellular protease plasmin and MMP-9 mobilized BM-derived hematopoietic $CD45^+/CD11b^+$ myeloid cells, a process dependent on KitL and VEGF signaling. tPA improved cell incorporation into ischemic tissues in the later phase of ischemic recovery, tPA induced mobilization of a $CD45^+/CD11b^+$ cell population, and increased expression of neoangiogenesis-related genes including vascular endothelial growth factor (VEGF) receptors. Remarkably, transplantation of BM-derived tPA-

mobilized CD11b$^+$ cells, and VEGFR1$^+$ cells, but not carrier-mobilized cells or CD11b- cells accelerated neovascularisation in part by augmenting the angiogenic factor VEGF-A. Inhibition of VEGF signaling suppressed tPA-induced neovascularisation in a model of hindlimb ischemia. Thus, tPA modulates mobilization of CD11 b$^+$ cells from the BM during ischemic recovery and promotes angiogenesis locally. tPA might be useful to induce therapeutic neovascularisation in the growing field of regenerative medicine.

2) Metalloproteinase regulation improves in vitro generation of efficacious platelets from mouse embryonic stem cells.

Hidekazu Nishikii, Koji Eto, Noriko Tamura[1], Koichi Hattori, Beate Heissig, Taisuke Kanaji[2], Akira Sawaguchi[3], Shinya Goto[1], Jerry Ware[4], Hiromitsu Nakauchi: [1]Division of Cardiology, Tokai University School of Medicine, Isehara 259-1193, Japan, [2]Division of Hematology/Oncology, Kurume University School of Medicine, Fukuoka 830-0011, Japan, [3]Department of Anatomy, University of Miyazaki Faculty of Medicine, Miyazaki 889-1692, Japan, [4]Department of Physiology & Biophysics, University of Arkansas for Medical Sciences, Little Rock, AR 72205, USA

Embryonic stem cells (ESCs) could potentially compensate for the lack of blood platelets available for use in transfusions. Here, we describe a new method for generating mouse ESC-derived platelets (ESPs) that can contribute to hemostasis in vivo. Flow cytometric sorting of cells from embryoid bodies on day 6 demonstrated that c-Kit(+) integrin alpha IIb (alpha IIb)(+) cells, but not CD31(+) cells or vascular endothelial cadherin(+) cells, are capable of megakaryopoiesis and the release of platelet-like structures by day 12. alpha IIb beta 3-expressing ESPs exhibited ectodomain shedding of glycoprotein (GP) Ibalpha, GPV, and GPVI, but not alpha IIb beta 3 or GPIb beta. ESPs showed impaired alpha IIb beta 3 activation and integrin-mediated actin reorganization, critical events for normal platelet function. However, the administration of metalloproteinase inhibitors GM6001 or TAPI-1 during differentiation increased the expression of GPIb alpha, improving both thrombogenesis in vitro and posttransfusion recovery in vivo. Thus, the regulation of metalloproteinases in culture could be useful for obtaining high-quality, efficacious ESPs as an alternative platelet source for transfusions.

Publications

1. Nishikii H, Eto K, Tamura N, Hattori K, Heissig B, Kanaji T, Sawaguchi A, Goto S, Ware J, Nakauchi H. Metalloproteinase regulation improves in vitro generation of efficacious platelets from mouse embryonic stem cells. J Exp Med. 205; 1917-27, 2008.
2. Hattori K, Ishihara M, Heissig B. Bone marrow-derived cells contribute to niche formation in cancer progression. Clin Calcium. 18(4): 480-7, 2008.
3. Kerbel RS, Benezra R, Lyden DC, Hattori K, Heissig B, Nolan DJ, Mittal V, Shaked Y, Dias S, Bertolini F, Rafii S. Endothelial progenitor cells are cellular hubs essential for neoangiogenesis of certain aggressive adenocarcinomas and metastatic transition but not adenomas. Proc Natl Acad Sci U S A. 105(34): E54, 2008.
4. 服部浩一，西田知恵美，Heissig Beate：造血幹細胞の体内動態．最新医学 63：2302−2309，2008.
5. 服部浩一，西田知恵美，石原誠人，Heissig Beate：骨髄由来細胞による前転移ニッチの形成機構．The Bone 22：33−37，2008.

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 (HSCs) are capable of both self-renewal and multilineage differentiation. HSCs can clonally produce all blood cells throughout the lifetime of an organism. We have been determined to work on HSCs because these stem cells have already been used in transplantation medicine, and are expectedly applied to a 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 clarify regulatory mechanisms for mouse HSC self-renewal and differentiation.

1. Interleukin-12 in combination with stem cell factor supports self-renewal in and early myelo-lymphoid differentiation from hematopoietic stem cells

Jun Ooehara[1], Azusa Matsubara[1], Shin-ichiro Takayanagi[1], Hiromitsu Nakauchi[1], and Hideo Ema: [1]Division of Stem Cell Therapy, Center for Experimental Medicine, IMUT.

Hematopoietic stem cells (HSCs) have multilineage differentiation potential. As they divide, this potential is restricted in their progeny. However, at which differentiation stages each lineage commitment takes place, and how these commitments take place, are poorly understood. *In vitro* colony assays have been extremely valuable for the study of hematopoiesis, particularly for identification of hematopoietic cytokines and characterization of cytokine networks. Mouse HSCs are able to form *in vitro* colonies in the presence of a combination of cytokines. These colonies mainly consist of morphologically identifiable myeloid cells at various differentiation stages. Whether these colonies also contain B- and T-lymphoid progenitors remains obscure.

The IL-12 family is comprised of heterodimeric cytokines such as IL-12, IL-23, and IL-27. IL-12 and IL-27 are pleiotropic cytokines that not only activate helper T cells and NK cells but also act in hematopoiesis. IL-12, in combination with SCF, Flt3 ligand, or IL-11, has been shown to stimulate hematopoietic progenitors. However, whereas IL-27 has recently been shown to act directly on HSCs to support their differentiation *in vitro*, whether IL-12 directly acts on HSCs and supports their self-renewal and differentiation has never been examined,.

This study was performed with two purposes. The first was to clarify the effect of IL-12 on HSCs. The second was to detect lymphoid differentiation potential in colonies derived from HSCs *in vitro* in the presence of various combinations of cytokines including IL-12. We found that HSCs are able to form colonies in the presence of SCF＋IL-12. Colonies were composed of morphologically rather homogeneous immature cells.

Transplantation experiments revealed that repopulating and self-renewing activity in HSCs

can be increased by serum-free culture with SCF +IL-12. We next attempted to detect myeloid, B-lymphoid, and T-lymphoid differentiation potential in each colony by colony transplantation into individual lethally irradiated mice. In order to detect low levels of repopulation by progenitors, we developed a new competitive repopulating assay that used as competitor cells bone marrow cells which had already been transplanted once. That serial transplantation leads to marked reduction in bone marrow cell repopulation activity is well documented. With this assay, various types of repopulating cells which had not been seen before became detectable. TPO, IL-3, and IL-11, in addition to IL-12, were then tested for their effects on myelo-lymphoid colony formation because these cytokines are known to act directly on HSCs to support their self-renewal as well as their differentiation. Interestingly, while the combinations TPO+SCF, IL-11+SCF, or IL-12+SCF supported myelo-lymphoid colony formation, SCF+IL-3 mainly supported myeloid colony formation, consistent with previous studies. These results suggest that both early myeloid differentiation and early lymphoid differentiation from HSCs are supported by the same set of multiple cytokines.

2. Non-canonical Wnt signal enhances self-renewal potential in hematopoietic stem cells

Jun Ooehara[1], Satoshi Yamazaki[1], Mitsujiro Osawa[1], Akira Sato[2], Akira Kikuchi[2], Hiromitsu Nakauchi[1], and Hideo Ema: [1]Division of Stem Cell Therapy, Center for Stem Cell and Regenerative Medicine, IMSUT, [2]Department of Biochemistry, Graduate School of Science, Hiroshima University, Hiroshima

In order to induce *in vitro* self-renewal in hematopoietic stem cells (HSCs), we first have to make HSCs enter the cell cycle, and we then have to make their daughter cells retain sufficient self-renewal potential. We and other have shown that cytokines can efficiently induce mouse HSC division but mostly resulting in reduction of self-renewal potential in their daughter cells. But, *in vivo* HSCs clonally exhibits multilineage differentiation while their pool is expanded after transplantation, implying that additional external factors, likely provided from HSC niches, should modify the fate of HSCs' daughter cells. Wnt proteins are candidates of such niche factors.

Wnt proteins comprise a large family of cysteine-rich secreted ligands that are essential for a wide variety of developmental and physiological processes. To date 19 members of the Wnt family are known in the mouse and human. By using conditioned media containing Wnt proteins or stromal cell lines expressing them, effects of certain members of the Wnt family on murine and human hematopoiesis were examined. However, their effects on HSCs remained obscure largely because biologically active Wnt proteins to be tested in serum-free culture were not successfully purified. Reya *et al*. and Willart *et al*. were the first to report the effect of purified Wnt3a on murine HSCs. They found that Wnt3a acts like a self-renewal inducing factor on HSCs from normal and Bcl2 transgenic mice. β-catenin signaling was suggested to play a central role in its effect. However, HSCs deficient in β-catenin have shown normal reconstitution activity. β-catenin seems not to be functionally compensated by another member of the family, γ-catenin. Overexpression of an active form of β-catenin in HSCs led to their differentiation block rather than self-renewal. Moreover, it has recently been reported that Wnt5a signaling inhibits Wnt3a signaling, resulting in increased repopulating activity by HSCs. Likewise, whether Wnt3a can induce self-renewal in HSCs has recently become controversial. To resolve this confusing but important matter, we own purified mouse Wnt3a and Wnt5a in lipid- and sugar-modified forms, and tested them for their effects on highly purified mouse HSCs. We found that neither Wnt3a nor Wnt5a acts on HSCs a mitogenic factor, but either one enhances HSC self-renewal potential in a thrombopoietin signal-dependent manner. This study suggests that a certain combination of cytokines and Wnt proteins is useful for *ex vivo* expansion of HSCs, one of the most important but difficult tasks in transplantation medicine.

3. Hematopoietic stem cells in the mouse spleen

Akiko Iseki[1], Yohei Morita[1], Hiromitsu Nakauchi[1], and Hideo Ema: [1]Division of Stem Cell Therapy, Center for Stem Cell and Regenerative Medicine, IMSUT.

The spleen is a hematopoietic organ in mice. During embryonic development, hematopoietic stem cells (HSCs) sequentially migrate into the spleen around embryonic day 14 and the bone marrow (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 are based on this transient hematopoiesis in the spleen. The spleen is a major site of extra-

medullary hematopoiesis in pathological conditions such as myeloproliferative diseases. Osteoblasts have been proposed as one of the stem cell niche components. Because there are no osteoblasts in the spleen, niches in the spleen possibly functions differently from ones in the BM. Our hypothesis is that the regulation of HSCs in the spleen differs from that in the BM. In order to verify this hypothesis, we attempted to characterize HSCs in the spleen as compared with those in the BM.

We compared long-term repopulation activity and frequency of long-term repopulating cells between BM and spleen from the same group of mice. We then attempted to purify HSCs in the spleen similarly to those in the BM. Using purified HSCs, HSCs were compared at the clonal level between spleen and BM. Cell cycle was also compared between BM and spleen HSCs.

Competitive repopulation showed that the repopulating activity per 10^6 BM cells was significantly greater than that per 10^6 spleen cells (about 15-fold). Limiting analysis showed that the frequency of long-term repopulating cells in BM cells was significantly higher than that in spleen cells (about 10-fold). As a result, the mean activity per spleen stem cell was lower than that per BM stem cell. Like BM, CD34-negative, c-Kit-positive, Sca-1-positive, lineage markers-negative ($CD34^-KSL$) cells were highly enriched in HSCs in the spleen. The frequency of $CD34^-KSL$ cells in the spleen was significantly lower than that in the BM. Single-cell transplantation of BM or spleen $CD34^-KSL$ cells revealed that repopulating activities varies in both BM and spleen HSCs. These data indicate that functionally equivalent HSCs exist in the spleen but relatively at a lower frequency. The proportion of pyronin Y-negative G_0 cells among BM $CD34^-KSL$ cells was greater than that among spleen $CD34^-KSL$ cells at any one time. BrdU-uptake analysis showed that spleen $CD34^-KSL$ cells were cycling more frequently than BM $CD34^-KSL$ cells. Parabiosis experiments showed that a portion of spleen HSCs was exchangeable with BM HSCs, but not all of them. These data suggest HSCs are controlled by spleen niches differently from by BM niches.

Publications

Ema, H., and Nakauchi, H. Bloodlines of haematopoietic stem cell research in Japan. Philos Trans R Soc Lond B Biol Sci 363: 2089-2097, 2008.

Seita, J., Asakawa, M., Ooehara, J., Takayanagi, S., Morita, Y., Watanabe, N., Fujita, K., Kudo, M., Mizuguchi, J., Ema, H., Nakauchi, H., and Yoshimoto, T. Interleukin-27 directly induces differentiation in hematopoietic stem cells. Blood 111: 1903-1912, 2008.

Yamazaki, S., Iwama, A., Takayanagi, S., Eto, K., Ema, H., and Nakauchi, H. TGF-beta as a candidate bone marrow niche signal to induce hematopoietic stem cell hibernation. Blood (in press).

Ogaeri, T., Eto, K., Otsu, M., Ema, H., and Nakauchi, H. The actin polymerization regulator WAVE2 is required for early bone marrow repopulation by hematopoietic stem cells. Stem Cells (in press).

Matsumoto, K., Isagawa, T., Nishimura, T., Ogaeri, T., Eto, K., Miyazaki, S., Miyazaki, J.-i., Aburatani, H., Nakauchi, H., and Ema, H. Stepwise development of hematopoietic stem cells from embryonic stem cells. PLoS One (in press).