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Generation of induced pluripotent stem cells derived from primary and secondary myelofibrosis patient samples

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Induced pluripotent stem cells (iPS) derived from disease cells are expected to provide a new experimental material, especially for diseases from which samples are difficult to obtain. In this study, we generated iPS from samples from patients with primary and secondary myelofibrosis. The primary myelofibrosis cells had chromosome 13q deletions, and the secondary myelofibrosis (SMF) cells had JAK2V617F mutations. The myelofibrosis patient cell-derived iPS (MF-iPS) were confirmed as possessing these parental disease-specific genomic markers. The capacity to form three germ layers was confirmed by teratoma assay. By co-culture with specific feeder cells and cytokines, MF-iPS can re-differentiate into blood progenitor cells and finally into megakaryocytes. We found that mRNA levels of interleukin-8, one of the candidate cytokines related to the pathogenesis of myelofibrosis, was elevated predominantly in megakaryocytes derived from MF-iPS. Because megakaryocytes from myelofibrosis clones are considered to produce critical mediators to proliferate fibroblasts in the bone marrow and iPS can provide differentiated cells abundantly, the disease-specific iPS we established should be a good research tool for this intractable disease. © **2014 ISEH - International Society for Experimental Hematology. Published by Elsevier Inc.**

Generation of induced pluripotent stem cells (iPS) from various types of human somatic cells provided promising views for new medical science [1]. After this breakthrough, disease modeling with the use of iPS technology attracted much attention, because iPS technology could provide human disease cell resources and new experimental opportunities that were hardly obtainable in the past [2].

Myelofibrosis is a pre-leukemic neoplastic disease with a generally poor prognosis, although the patients follow a diverse clinical course because of the pathophysiologic heterogeneity of this disease. Indeed, the median life expectancy of myelofibrosis patients is estimated to range from 27 to 135 months depending on the risk group [3]. Although a new therapeutic strategy targeting JAK2 had been tested, its curative effect has been found weaker than expected, compared with the tyrosine kinase inhibitors in practical use for chronic myeloid leukemia patients [4]. Cure is still limited to allogeneic hematopoietic stem cell transplantation, which, however, is associated with a high risk of treatment-related mortality and morbidity and, therefore, is indicated only for young patients without problematic complications [5–8]. Clearly, further investigations are needed to develop novel targeted therapies for myelofibrosis.

The mediators of stimulation of fibroblast proliferation derived from myeloproliferative neoplasms were thought to be cytokines, including basic fibroblast growth factor, platelet-derived growth factor (PDGF) and transforming growth factor β (TGF-β) [9,10]. Other candidate mediators recently reported as related to symptoms of myelofibrosis include hepatocyte growth factor, monokine induced by γ interferon, interleukin (IL)-1 receptor antagonist associated with marked splenomegaly, and IL-8 associated with severe constitutional symptoms [11]. Megakaryocytes, as well as monocytes, derived from neoplasms contain these cytokines and, thus, are one of the suspected main feeders of the responsible mediators [12–14]. However, because of the bone marrow fibrosis characteristic of myelofibrosis,

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primary sufficient hematopoietic samples such as megakaryocytes are hard to obtain. Currently, a certain amount of megakaryocytes from myelofibrosis patients can be obtained only by differentiation of peripheral blood progenitor cells or by the surgical resection of extramedullary lesions [15,16]

In this study, we generated iPS from primary samples of a patient with primary and a patient with secondary myelofibrosis and re-differentiated them into hematopoietic cells including megakaryocytes. These iPS would be a new material for investigating pathogenic mechanisms of myelofibrosis.

Methods

Cells and cell culture

All studies using human cells were reviewed and approved by the institutional review boards of the University of Tokyo. After written informed consent was obtained, bone marrow cells were collected from a patient with primary myelofibrosis and peripheral blood cells from a patient with secondary myelofibrosis. Mobilized peripheral blood cells of a healthy donor for peripheral blood stem cell transplantation were also obtained after informed consent. The characteristics of the patients are listed in Table 1. Both patients were in the intermediate-2 risk group with the same two risk factors—constitutional symptoms and blood blasts >1%—according to the prognostic scoring system of the International Working Group of Myelofibrosis Research and Treatment [3]. We then isolated mononuclear cells from the samples by centrifugation using Lymphoprep (Axis-Shield, Oslo, Norway). We concentrated $CD34^+$ cells using an immunomagnetic separation technique with AUTO MACS (Milteny Biotec, Bergisch-Gladbach, Germany). These cells were cultured with α-MEM containing 20% fetal calf serum, penicillin, streptomycin, 2 mM 2-mercaptoethanol supplemented with 100 ng/mL recombinant human stem cell factor (SCF, Wako Pure Chemical, Osaka, Japan), 10 ng/mL recombinant human thrombopoietin (Kyowa Hakko Kirin, Tokyo, Japan), 100 ng/mL recombinant human FMS-like tyrosine kinase 3 ligand (FL3L, Wako), 10 ng/mL recombinant human IL-3 (Wako), 100 ng/mL recombinant human IL-6 (Wako). Myelofibrosis patient cell-derived iPS (MF-iPS) were maintained in DMEM-F12 medium (Invitrogen, Life Technologies, Carlsbad, CA, USA) supplemented with 20% KnockOut Serum Replacement (KSR, Invitrogen), 0.1 mM 2-mercaptoethanol (Sigma–Aldrich, St. Louis, MO, USA), MEM non-essential amino acids (Invitrogen), penicillin–streptomycin–glutamine (Sigma–Aldrich), and 5 ng/mL recombinant human basic fibroblast growth factor (Peprotech, Rocky Hill, NJ, USA) on mitomycin C (Wako)-treated mouse embryo fibroblast feeder cells.

Mouse C3H10T1/2 cells were purchased from Riken Bio-Resource Center (Tsukuba, Japan) and cultured as previously described.

All cell culture procedures were performed in a humidified 37°C, 5% CO_2 environment.

Table 1. Characteristics of patients

Feature	Secondary myelofibrosis	Primary myelofibrosis
Specific marker	JAK2 V617F mutation	13q deletion
Source of material	Peripheral blood cells	Bone marrow cells
Age	31 y	54 y
Sex	Female	Female
Constitutional symptoms	Yes	Yes
Palpable splenomegaly	Yes	Yes
Palpable hepatomegaly	Yes	Yes
Hemoglobin	13.1 g/dL	10.3 g/dL
White blood cell count	24.1×10^9/L	9.9×10^9/L
Platelets	481×10^9/L	106×10^9/L
Blood blasts	1.0%	1.0%

Production of VSV-G pseudotyped retrovirus for reprogramming

The pMX vectors encoding OCT3/4, SOX2, KLF4 and c-MYC and highly concentrated vesicular stomatitis virus G glycoprotein (VSV-G) pseudotyped retroviral supernatants were prepared as reported previously [2]. With 293GPG cells, a kind gift from Dr. R. C. Mulligan (Children's Hospital Boston, Harvard Medical School, Boston, MA, USA), stable cell lines producing VSV-G pseudotyped retroviral particles containing either the OCT3/4, SOX2, KLF4, or c-MYC gene were established. We centrifuged these for 16 hours at 6000*g* and concentrated the viral supernatant by resuspension in Iscove's modified Dulbecco's medium.

Generation of iPS from primary patient samples

Fibronectin fragment CH296 (RetroNectin, Takara-Bio, Otsu, Japan)-coated plates and virus-bound plates were prepared without centrifugation according to the manufacturer's recommendation. Briefly, the wells were coated with 20 μg/mL RetroNectin and incubated overnight at 4°C. Then, the remaining free sites were blocked with 2% bovine serum albumin for 30 min at room temperature. After removal of bovine serum albumin solution and a phosphate-buffered saline wash, the well was filled with retroviral supernatants and incubated for 4 hours at 37°C. After the viral solution was removed, 1×10^5 $CD34^+$ concentrated patient cells, after stimulation with cytokines as mentioned above, were placed in each well, and the wells were filled with the stimulation culture medium supplemented with cytokines mentioned above. After 24 and 48 hours, viral supernatants were added to the culture medium. After the third infection, we harvested the cells by pipetting and seeded them on mitomycin C-treated mouse embryo fibroblast cells with fresh medium containing half the cytokines for the next 2 days. On day 5, we replaced the medium with the human embryonic stem medium described above containing 0.5 mM valproic acid (Sigma–Aldrich). The medium was changed every other day. After 14 to 21 days, colonies appeared. Morphologically determined human embryonic stem cell-like colonies were picked up and colony-derived cells were seeded separately on new mouse embryo fibroblast feeder cells as single clone-derived iPS should be established. Clones that expanded were used for further analyses.

Fluorescence immunostaining assay

Myelofibrosis iPS were fixed with 3.8% formaldehyde in phosphate-buffered saline and then blocked with 1% bovine serum albumin (Sigma-Aldrich). The antigens were labeled overnight with 1% each of anti-human SSEA-4 conjugated with Alexa480 antibody and anti-human TRA-1-60 conjugated with Alexa555