Generation of induced pluripotent stem cells from primary chronic myelogenous leukemia patient samples

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Induced pluripotent stem cells (iPSCs) can be generated by the expression of defined transcription factors not only from normal tissue, but also from malignant cells. Cancer-derived iPSCs are expected to provide a novel experimental opportunity to establish the disease model. We generated iPSCs from imatinib-sensitive chronic myelogenous leukemia (CML) patient samples. Remarkably, the CML-iPSCs were resistant to imatinib although they consistently expressed BCR-ABL oncoprotein. In CML-iPSCs, the phosphorylation of ERK1/2, AKT, and JNK, which are essential for the maintenance of both BCR-ABL (+) leukemia cells and iPSCs, were unchanged after imatinib treatment, whereas the phosphorylation of signal transducer and activator of transcription (STAT)5 and CRKL was significantly decreased. These results suggest that the signaling for iPSCs maintenance compensates for the inhibition of BCR-ABL. CML-iPSC-derived hematopoietic cells recovered the sensitivity to imatinib although CD34+38−90+45+ immature cells were resistant to imatinib, which recapitulated the pathophysiologic feature of the initial CML. CML-iPSCs provide us with a novel platform to investigate CML pathogenesis on the basis of patient-derived samples.

Introduction

Hematologic malignancies including leukemias are often chemotherapy-resistant, most of which follows an aggressive clinical course.1 Multiple drug therapies are usually required to treat them, although they are occasionally accompanied with many side effects. Thus, the invention of novel targeted therapies based on newly revealed molecular pathogenesis is expected to overcome the current situation.2 However, previous approaches to understanding pathogenesis involve several limitations. Many mouse models of human diseases have been established, but they may not fully recapitulate many aspects of original human diseases.3 Many kinds of cell lines are also available for research. However, they do not cover all diseases, because it is usually difficult to establish a cell line from a primary patient sample. Furthermore, additional gene mutations may be accumulated in cell lines. Theoretically, primary patient samples should be used for research, but the amount of obtained cells may be inadequate for various analyses.

Induced pluripotent stem cells (iPSCs) can be generated from various types of cells by the transduction of defined transcription factors.4-10 In addition to the regenerative medicine,11 iPSCs have been used for studies of the pathogenesis of inherited genetic diseases.12-16 Recently, it was reported that iPSCs were generated not only from normal tissue cells, but also from malignant cells.17-20 In those cases, cancer cells themselves must have been the origins of iPSCs. However, in most published data, established cell lines were used as the source material of cancer cells, including chronic myelogenous leukemia (CML).17 gastrointestinal cancers,18 and melanoma,19 except for the JAK2-V617F mutation (+) polycythemia vera (PV) patient.20 CML is a myeloproliferative neoplasm that originates from hematopoietic stem cells transformed by the BCR-ABL fusion gene. The initial indolent chronic phase (CP) is followed by aggressive stages, the accelerated phase (AP), and the blast crisis (BC), in which immature leukemic cells expand.21 CML is now initially treated with one of several tyrosine kinase inhibitors (TKIs) including imatinib, dasatinib, and nilotinib, which have dramatically improved the long-term survival rate of CML patients up to approximately 90%. However, even TKIs are not able to eradicate the CML clone completely, which is demonstrated by the fact that discontinuation of TKIs in molecular remission CML patients usually leads to the recurrence of the BCL-ABL clone. Therefore, many studies are performed to elucidate the mechanisms of TKI-resistance in CML stem cells and to overcome the resistance.

In this study, we established iPSCs from primary CML patient samples, redifferentiated them into hematopoietic lineage and showed the recapitulation of the pathophysiologic features of the initial disease.


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Methods

Cell and cell culture

Primary samples of CML bone marrow cells were obtained after informed consent. All studies using human cells were reviewed and approved by the institutional review boards (IRBs) of University of Tokyo. Mononuclear cells (MNCs) were isolated by centrifugation through a Ficoll gradient. CD34+ cells were isolated by an immunomagnetic separation technique (auto magnetic-activated cell sorting; MACS). They were cultured with α-minimum essential medium (MEM) containing 20% fetal calf serum (FCS) supplemented with 100 ng/mL stem cell factor (SCF; Wako), 10 ng/mL thrombopoietin (TPO; Wako), 100 ng/mL FL3L (Wako), 10 ng/mL IL3 (Wako), and 100 ng/mL IL6 (Wako).

Normal iPSCs established from cord blood (CB) CD34+ cells or fibroblasts22 and CML-iPSCs were maintained in Dulbecco modified Eagle medium-F12 (Invitrogen) supplemented with 20% knockout serum replacement (KSR; Invitrogen), 0.1mM 2-mercaptoethanol (Sigma-Aldrich), MEM nonessential amino acids (Invitrogen), and 5 ng/mL recombinant human basic fibroblast growth factor (PeproTech) on mitomycin C (MMC)-treated mouse embryoblast (MEF) feeder cells.23 Imatinib (LC Laboratories) was added to the culture medium at the various concentrations (1-10µM). U0126 and LY294004 (LC Laboratories) was added to the culture medium at the various concentrations (1-10 µM).

Microarray analysis

Gene expression analysis was carried out as previously described29 with the use of the Human Genome U133 Plus 2.0 Array (Affymetrix). The hierarchical clustering techniques classify data by similarity and their results are represented by dendrograms. Previously reported data of human embryonic stem (ES) cells (GSM449729) and CML CD34+ cells (GSM366215, 366216, 366221, and 366222) were used to compare the gene expression profile. The microarray data are available on the Gene Expression Omnibus (GEO) database under accession number GSE37982.

RT-PCR and quantitative real-time PCR analysis

After extraction of total RNA with RNAeasy reagents (QIAGEN), reverse transcription was performed with SuperScript III (Invitrogen). Primer
sequences used for the detection of stem cell genes were as previously described.9

Quantitative real-time PCRs (qPCRs) were carried out in the ABI-7000 sequence detection system with SYBR Green PCR Core reagents according to the manufacturer’s instructions (Applied Biosystems). We analyzed expression levels of BCR-ABL fusion transcript as previously described.30 Each assay was performed in triplicate and the results were normalized to GAPDH (glyceraldehyde-3-phosphate dehydrogenase) levels.

PCR primers used for quantitative PCR:

BCR-ABL F TCAGAAGCTTCTCCCTGACATCCGT
BCR-ABL R TCCACTGGCCACAAAATCATACAGT
GAPDH F TGCACCACCAACTGCTTAGC
GAPDH R GGCATGGACTGTGGTCATGAG

Western blotting

Fifty micrograms of cell lysates were subjected to sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis. Antibodies used in immunoblotting were as follows; anti-phospho ERK1/2 (Thr202/Tyr204; Cell Signaling), anti-phospho Akt (Ser473; Cell Signaling), anti-phospho JNK (Thr183/Tyr185; Cell Signaling), anti-phospho-STAT5 (Tyr694; Cell Signaling), and anti-phospho CRKL (Tyr207; Cell Signaling). Enhanced chemiluminescence detection (Amersham) was carried out according to the manufacturer’s recommendations.

Results

Generation of iPSCs from primary CML patient samples

After obtaining informed consent, CD34+ cells were purified from bone marrow mononuclear cells of a CML chronic phase patient. After we stimulated them with cytokines for 2 days, retroviral transduction with the transcription factors OCT3/4, SOX2, KLF4, and MYC was performed. Two days after transduction, we reseeded cells onto MEF cells and cultured them for another 2 days. Then, we replaced the medium with human ES medium supplemented with 5 ng/mL bFGF. To improve the efficiency of the reprogramming, we added VPA,26 a histone deacetylase inhibitor, to the culture (Figure 1). Using a live cell imaging method with Tra-1-60 antibody, bona fide iPSCs were distinguished from deficiently reprogrammed cells.27 As a result, 2 CML-derived iPSCs (CML-iPSCs) were generated, which were derived from independent patients. CML-iPSCs showed the typical morphology as iPSCs (Figure 2A) and expressed the pluripotent markers, such as SSEA-4 and Tra-1-60 (Figure 2B), and the endogenous expression of embryonic stem cell (ESC) characteristic transcripts (OCT3/4, SOX2, KLF4, NANOG, LIN28, and REX1) was confirmed by RT-PCR (Figure 2C). CML-iPSCs also expressed BCR-ABL, which demonstrated that they were truly derived from CML (Figure 2D). Furthermore, fluorescence in situ hybridization with dual color BCR-ABL probes confirmed t(9;22) translocation in CML-iPSCs at the single cell level (supplemental Figure 1A and supplemental Table 1, available on the Blood Web site; see the Supplemental Materials link at the top of the online article). However, although CML-iPSCs expressed BCR-ABL, they were resistant to imatinib (Figure 2E). Teratoma formation capacity was confirmed, demonstrating the pluripotency of CML-iPSCs (supplemental Figure 2).

Comprehensive analysis of DNA methylation revealed that methylation pattern of CML-iPSCs was different from that of original CML sample but was very similar to that of normal iPSCs although there were slight differences (Figure 3A). Previously, stem cell–specific differentially methylated regions (SS DMRs)
were identified during reprogramming process of iPSCs. Hypomethylated SS DMRs (hypo SS DMRs) in the variety of iPSCs were also hypomethylated in the CML-iPSCs including the promoters of OCT4 (Figure 3B). In the same way, hypermethylated SS DMRs (hyper SS DMRs) in the variety of iPSCs were also hypermethylated in the CML-iPSCs (Figure 3C). The promoters of hematopoietic lineage-specific marker genes, such as CD45 and CD11b, were hypermethylated in the CML-iPSCs. Thus, the methylation pattern of CML-iPSCs was confirmed to be not hematopoietic cell-like, but iPSC-like. Next, we compared the gene expression pattern among CML-iPSCs and normal iPSCs (Figure 3D). In a result, CML-iPSCs and normal iPSCs were very similar in regard to global gene expression profile. Furthermore, comparing our results with publicly available expression data of human ES cells and CML CD34+ cells, we found that CML-iPSCs were very similar to human ES cells, whereas they were different.
from CML CD34+ cells in terms of gene expression patterns (Figure 3D).

Hematopoietic differentiation of CML-iPSCs

Then we differentiated them into hematopoietic progenitors within the “unique sac-like structures” (iPS-sacs; Figure 4A-B). This method was reported to be able to produce the hematopoietic progenitors with higher efficiency than the usual embryoid body formation method using human ESCs and iPSCs.22,24 On day 15 of culture, iPSCs sacs contained round hematopoietic-like cells (Figure 4B). Then we picked up iPS-sacs with a pipette tip and dissociated them mechanically and obtained the inner round cells. Round cells, positive for a hematopoietic lineage marker CD45, and an immature marker CD34, proved to be hematopoietic progenitors (Figure 4C).

Then we characterized the CML-iPSCs derived hematopoietic cells, comparing with those derived from normal iPSCs. CFC activities were measured using the same number of CD34+ cells (Figure 4D). Hematopoietic progenitors derived from CML-iPSCs and normal iPSCs produced colonies of mature erythroid, granulocyte-macrophage, or mixed of these hematopoietic cells in growth factor-supplemented methyl cellulose medium with a similar distribution of colony size, morphologies, and kinetics of growth and maturation. The colony forming cells expressed BCR-ABL (supplemental Figure 1B and supplemental Table 2).

Next, we tested the engraftment potential of these cells. Nonobese diabetic/severe combined immunodeficiency IL2Rg deficient (NOD) mice serve as a superior host for engraftment of human normal and malignant hematopoietic cells.32 One million CD34+ cells were intravenously transplanted into NOG mice with minimal irradiation (2 Gy; supplemental Figure 3A). Only transient engraftment was observed and the recipient mice never showed CML phenotype in vivo (supplemental Figure 3B).

BCR-ABL dependence is lost in the CML-iPSCs

The restricted dependence of BCR-ABL signaling on survival of CML cells enables the disease suppression by imatinib and dramatically changed the CML treatment after the development of imatinib.33 CML patients whose cells were used for the generation of iPSCs effectively responded to imatinib therapy. However, although CML-iPSCs expressed BCR-ABL, they were resistant to imatinib (Figure 2E). Interestingly, CML-iPSC-derived hematopoietic cells recovered the sensitivity to imatinib except CD34+CD38−90+CD45+ immature cell population, which recapitulated the feature of initial CML disease (Figure 5A). Various concentrations of imatinib were added to the culture of iPSC derived hematopoietic cells. Similar kinetics of imatinib response between CML-iPSC–derived hematopoietic cells and imatinib sensitive CML cell line K562 was observed (Figure 5B). Furthermore, we generated CD34+CD38−CD90+CD45+ cells from CML-iPSCs. Surprisingly, this fraction of phenotypically immature cells showed the imatinib resistance like CML-iPSCs although more differentiated cells (CD34−CD45+) showed the sensitivity to imatinib (Figure 5C).

Then, we investigated why CML-iPSCs showed the imatinib-resistance. It was reported that imatinib resistant patients sometimes express higher BCR-ABL transcript than imatinib sensitive patients.34 In addition, CML leukemia stem cells showed higher BCR-ABL expression than differentiated CML cells.35 Therefore, we examined the BCR-ABL mRNA expression levels in the CML-iPSCs, and compared them with the primary CML sample, and CML-iPSC–derived hematopoietic cells. As a result, BCR-ABL expression was not increased in CML-iPSCs compared with the primary CML sample and CML-iPSCs-derived hematopoietic cells. (Figure 6A)

BCR-ABL activates Ras-MAPK, PI3K-AKT, JAK-STAT pathways. Among them, it was reported that STAT5, ERK1/2, JNK, and AKT are essential for the survival of BCR-ABL–dependent leukemic cells.36,37 In addition, CRKL is another direct target of BCR-ABL.38 The phosphorylation status of ERK1/2, AKT, JNK, and STAT5 in CML-iPSCs, which are essential for the survival of BCR-ABL (+) hematopoietic progenitors, were evaluated after imatinib treatment. The phosphorylation of ERK1/2, AKT, and JNK, which are also essential for the maintenance of iPSCs and ES cells,39,40 were unchanged after treatment in the CML-iPSCs although they were decreased in the CML-iPSCs–derived hematopoietic cells (Figure 6B). The phosphorylation of CRKL and STAT5, which were not activated in the normal iPSCs, was decreased in both CML-iPSCs and CML-iPSCs–derived hematopoietic cells (Figure 6B). These results showed that the signaling for iPSCs maintenance
might compensate for the inhibition of BCR-ABL in CML-iPSCs and that BCR-ABL dependence was lost in CML-iPSCs. In addition, the specific inhibitor of ERK or AKT signaling worked as expected, respectively (Figure 6C), resulting in the reduction of attached cells regardless of the addition of imatinib (Figure 6D).

**Discussion**

**Generation of CML-derived iPSCs**

We generated iPSCs from primary CML patient samples. Methylation pattern and gene expression of CML-iPSCs were very similar to those of normal iPSCs. Previously, SS DMRs were identified during reprogramming process of iPSCs.31 Hypo SS DMRs were also hypomethylated in the CML-iPSCs (Figure 3B). Among them, some genomic regions, such as the promoter of N-MYC, had already been hypomethylated in the primary CML sample. In the same way, some genes associated with hyper SS DMRs had already been hypermethylated in the primary CML sample (Figure 3C). However, we could not detect the CML-iPSC-specific DMRs in this study. Then, we redifferentiated them into hematopoietic lineage and showed the recapitulation of the features of the initial disease. In addition, although CML-iPSCs expressed BCR-ABL, it was surprising that there were no obvious differences of gene expression profile between normal iPSCs and CML-iPSCs (Figure 3D). The results that inhibition of BCR-ABL by imatinib did not affect CML-iPSC survival indicate that signaling of BCR-ABL might not be important in iPSCs. These results are consistent with the gene expression profile data in which the effect of BCR-ABL signaling was hardly observed. One possibility is that global tyrosine kinase activities and downstream signaling pathways would be so activated in iPSCs irrespective of BCR-ABL that BCR-ABL no longer adds significant effects.

CML is known to be a clonal disorder originated from hematopoietic stem cells caused by BCR-ABL fusion gene. Although BCR-ABL TKI (TAK-287) can reduce CML cells below the detection of molecular level, its discontinuation often results in the rapid relapse of leukemia.41 These results indicate the existence of CML stem cells, which are resistant to the TKI.

CML stem cells are thought to be included in the primitive population (CD34+CD38−). According to some published data, they have lost the addiction to BCR-ABL42,43. In addition, CML-iPSCs also have shown resistance to the imatinib.44 Furthermore, in our experiments, immature CD34+38−90+45+ cells differentiated from CML-iPSCs also showed imatinib resistance similar to CML-iPSCs, although more differentiated cells (CD34−CD45+) showed sensitivity to imatinib (Figure 5C). So, these immature cells showed a phenotype of CML stem cells. Imatinib treatment of CML stem cells decreased the phosphorylation of CRKL and STAT5 but not of AKT,42 as shown in the CML-iPSCs described here. There may be some shared mechanism between CML stem cells and CML-iPSCs. For example, Wnt-β-catenin signaling is essential for the maintenance of both CML stem cells and iPSCs.45,46 Using immature cells obtained in our study, the mechanism of imatinib resistance of CML stem cells can be further investigated.

Previously, it was reported that primary CML samples and the CML BC cell line KBM7 were reprogrammed and that CML-iPSCs were established.17 As shown here, KBM7-iPSCs lost the BCR-ABL dependence and became resistant to imatinib, although primary CML-derived iPSCs were not checked for the imatinib sensitivity. Cret et al argued that a specific differentiated epigenetic cell state is needed to maintain BCR-ABL dependence.47 However, they only showed the BCR-ABL expression but did not confirm BCR-ABL activation in the KBM7-iPSCs. We showed BCR-ABL specific phosphorylation of STAT5 and CRKL although they were not necessary for the survival of iPSCs and that imatinib treatment inhibits these signaling. On the other hand, Ras-MAPK and PI3K-AKT signaling were unchanged after imatinib treatment. It was reported that inhibition of caspase-mediated anoikis by bFGF is dependent on activation of ERK and AKT in human ES cells.49 We also showed that the inhibition of ERK or AKT irrespective of the presence of the imatinib resulted in the decrease of the attached cell numbers. Some key molecules essential for the maintenance of iPSCs may compensate for the BCR-ABL inhibition in the CML-iPSCs through downstream ERK and AKT signaling pathways. They may include contact-mediated signaling with stem cell niches, and may be shared with CML stem cells and CML-iPSCs.
The progression of CML from initial indolent CP to the aggressive stages, AP and BC is caused by additional gene mutations. If we introduce some additional mutation into the CML-iPSCs, the CML BC model may be generated.

Generation of hematologic malignancies derived iPSCs other than CML

Primary samples of hematologic malignancy are usually difficult to be expanded. However, after they are reprogrammed to iPSCs, they can expand unlimitedly. As a result, we can obtain the genetically abnormal hematopoietic cells continuously by redifferentiating them into hematopoietic cells and use them for the studies which require the large number of living cells, such as the analysis for proteome, epigenome, transcriptome, leukemia stem cells, or drug screening. Thus, iPSCs technology would be useful for the study of hematologic malignancy based on the patient samples.

However, reprogramming of leukemia cells may be harder than generation of normal iPSCs because of the genetic and epigenetic status of leukemia cells. To overcome the difficulty, application of other factors in addition to the Yamanaka factors may be effective, such as exogenous expression of miRNA-302, chemical compounds, such as azacitidine (DNA methyltransferase inhibitor), BIX01294 (G9a histone methyltransferase inhibitor), VPA (histone deacetylase inhibitor), and knockdown of p53, p21, and Ink4/Arf.

In addition, there may be more desirable gene delivery system for iPSC generation for the study of disease pathogenesis. The integration site of retrovirus in the iPSCs may affect the gene expression and change the disease phenotype after redifferentiating them into the original lineages. Recently, efficient induction of transgene free iPSCs, such as using Sendai virus system, was reported and will be applicable for the disease derived iPSCs. We could establish the CML-iPSCs by this system. Using the newly established CML-iPSCs with sendai virus and feeder free culture system, we confirmed the same resistance to imatinib (supplemental Figure 4). Furthermore, without feeder cells, the phosphorylation of ERK and AKT were maintained, although the phosphorylation of STAT5 and CRKL were decreased by imatinib treatment.
In addition, the sendai virus system can be applied to the establishment of other disease derived iPSCs.

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