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Development of Sendai Virus Vectors and their Potential Applications in Gene Therapy and Regenerative Medicine

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Abstract: Gene delivery/expression vectors have been used as fundamental technologies in gene therapy since the 1980s. These technologies are also being applied in regenerative medicine as tools to reprogram cell genomes to a pluripotent state and to other cell lineages. Rapid progress in these new research areas and expectations for their translation into clinical applications have facilitated the development of more sophisticated gene delivery/expression technologies. Since its isolation in 1953 in Japan, Sendai virus (SeV) has been widely used as a research tool in cell biology and in industry, but the application of SeV as a recombinant viral vector has been investigated only recently. Recombinant SeV vectors have various unique characteristics, such as low pathogenicity, powerful capacity for gene expression and a wide host range. In addition, the cytoplasmic gene expression mediated by this vector is advantageous for applications, in that chromosomal integration of exogenous genes can be undesirable. In this review, we introduce a brief historical background on the development of recombinant SeV vectors and describe their current applications in gene therapy. We also describe the application of SeV vectors in advanced nuclear reprogramming and introduce a defective and persistent SeV vector (SeVdp) optimized for such reprogramming.

Keywords: Sendai virus, gene therapy, nuclear reprogramming, induced pluripotent stem cells (iPSCs).

INTRODUCTION

Since the finding in the 1970s that cultured cells can take up nucleic acids with the aid of cationic molecules, techniques of gene delivery and expression in mammalian cells have been used widely in modern biology. Development of sophisticated gene delivery tools in the 1980s such as retroviral vectors [1, 2], adenoviral vectors [3] and cationic lipid-based reagents [4] facilitated the translation of these technologies to human gene therapy. Prototypes of the current gene delivery tools used in research and clinics were mostly established in those early periods, and then followed by significant progress in each technology [5-8].

Although various formulations of DNA–carrier complexes have been developed, recombinant viral vectors are still used as the primary choice for delivering therapeutic genes because of their efficacy. Nonetheless, the refinement of current viral vectors and development of novel viral vectors are still desired, as none of the current viral vectors satisfies all of the requirements for various applications [9, 10]. In principle, any animal virus could be tailored to form gene delivery vectors provided it can accept exogenous genes as a part of their viral genome. However, their utility is limited by various factors, including pathogenicity to humans and the availability of procedures for large-scale production. Even if candidate viruses satisfy these minimum requirements, they should have clear advantages over "classical" viral vectors in being established as practical tools. The Sendai virus (SeV) vector is a newcomer in the field with unique characteristics, making it distinct from other viral vectors.

In this review, we introduce characteristics of various SeV vectors, a unique RNA virus-based gene delivery/expression system and describe recent progress in the application of SeV vectors to molecular therapy and to advanced nuclear reprogramming.

DEVELOPMENT OF RECOMBINANT SENDAI VIRUS VECTORS

SeV (mouse parainfluenza virus type 1, hemagglutinating virus of Japan (HVJ)) is a nonsegmented negative-strand RNA virus belonging to the *Paramyxovirus* family [11] with a large spherical shape and an average diameter of 260 nm [12]. A SeV virion consists of the nucleocapsid (genomic RNA complexed with NP, P and L proteins), an envelope (a lipid bilayer with F and HN proteins) and a matrix (M protein) connecting the nucleocapsid and envelope (Fig. (**1**)).

Since its first isolation in the 1950s in Japan [13], SeV has occupied a unique position as a research tool for basic and applied biology. Long before being characterized at a molecular level, SeV particles inactivated by brief exposure to ultraviolet light or by treatment with alkylation reagents have been widely used as research tools as a fusogenic agent to make hybrid cells [14] and as a tool for delivering macromolecules (protein and nucleic acid) into mammalian cells

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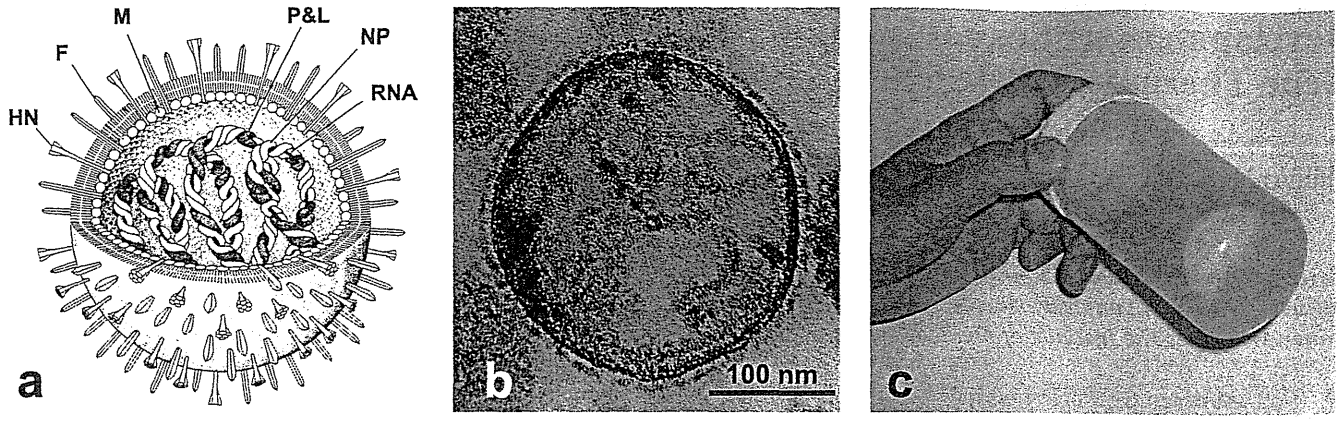


Fig (1). Characteristics of Sendai virus. (**a**) Schematic structure of Sendai virus. Reprinted with the permission of Nikkei Science, Inc. (**b**) Cross-section view of Sendai virus examined with transmission electron microscopy (courtesy of Dr. Takao Senda, Fujita Health University School of Medicine). (**c**) Purified Sendai virus in a centrifuge tube. Sendai virus was propagated in 500 fertilized chicken eggs and was purified extensively by sucrose step centrifugation. The large off-white pellet contains about 500 mg of purified Sendai virus as protein.

through membrane fusion [15, 16]. Intact live SeV was also used in the large-scale production of interferon (IFN) as an inducer of IFN expression [17, 18]. This is partly because SeV is neither tumorigenic nor pathogenic to humans and because SeV-mediated membrane fusion occurs efficiently with exceptionally low species and cell specificity. In addition, chicken egg-adapted SeV is readily propagated on the large scale: as much as one milligram (as protein) of purified SeV can be recovered from a single fertilized egg (Fig. (**1**)). All of these characteristics have made SeV a preferred research tool over other envelope viruses.

The complete genome sequence of the SeV Z strain was determined in 1986: first among the paramyxoviruses [19]. SeV has a 15,384-nucleotide single-strand RNA genome, consisting of six cistrons (Fig. (**2**)). Each cistron has concise transcription initiation and termination signals and is transcribed to mRNA encoding a single polypeptide (except for the P cistron encoding P, C and V proteins; Fig. (**2**)). This simple genome structure encouraged us to develop a recombinant SeV vector by replacing the genes dispensable for gene expression (F, HN and M genes) with therapeutic genes. However, this idea was hampered by the lack of methodology to modify the viral genome, as SeV has no DNA intermediate in its replication cycle.

Reconstitution of recombinant SeV from full-length genomic cDNA was accomplished in 1995 [20] following the establishment of a breakthrough strategy allowing the reconstitution of Rabies virus from cDNA in 1994 [21]. Since then, various SeV vectors installed with exogenous genes have been generated based on the wild-type SeV strain. In the first generation of SeV vectors, exogenous cDNA was installed between the 3′ terminus and the NP gene of a full-length SeV genome [22]. These SeV vectors were replication competent and could produce large amounts of exogenous gene products when cultured in fertilized chicken eggs [23]. For medical and other practical applications, replication-defective SeV vectors with deletion in the F gene were developed subsequently [24]. These vectors were shown to induce transient but very strong gene expression so their application as tools for gene therapy and vaccine has been explored, as described below.

APPLICATION OF SEV VECTORS IN MOLECULAR THERAPY

At the early stage of SeV vector development, the feasibility of applying SeV vectors to various cell types was examined extensively *in vitro* and *in vivo*. SeV naturally replicates in respiratory epithelial cells and is a major pathogen causing respiratory symptoms in mice. In addition to the lung/airway epithelium [25], a recombinant SeV vector can induce strong *ex*-gene expression in the cardiovascular system [26], in retinal epithelium [27], in hepatocytes [12], in colonic epithelium [28], in neurons [24], in dendritic cells [29] and in human hematopoietic stem cells [30]. This remarkably wide host range partly depends on the fact that the primary SeV receptor, sialic acid, is distributed universally among animal cells; the presence of a ubiquitous secondary receptor indispensable for SeV-mediated membrane fusion has also been suggested [31]. In addition, SeV vectors rely for their gene expression only on virus-encoded RNA polymerase and tubulin, a ubiquitous conserved cytoskeletal protein [32].

The application of SeV vectors in molecular medicine is dependent on, and is on some occasions restricted by, powerful but transient gene expression, wide host cell specificity, low pathogenicity and strong immunogenicity. To date, the feasibility for using SeV vectors clinically has been examined in the following areas: 1) as a live attenuated vaccine; 2) in gene therapy for critical limb ischemia; and 3) in cancer gene therapy.

Recombinant SeV vectors have been most intensively investigated as a vaccine platform for inducing mucosal immunity [33]. SeV was originally investigated as a xenotropic live-attenuated vaccine as it was known to have antigenicity shared with human parainfluenza virus type 1 (hPIV-1), an important human pathogen causing pneumonia and laryngotracheobronchitis. Results of a phase 1 trial where live wild-type SeV was administered intranasally showed that SeV induced anti-hPIV-1 immunity effectively without any severe adverse events [34]. This result further emphasized the nonpathogenic nature of SeV to humans, partly because of the sensitivity of SeV replication to IFN [35]. Subse-

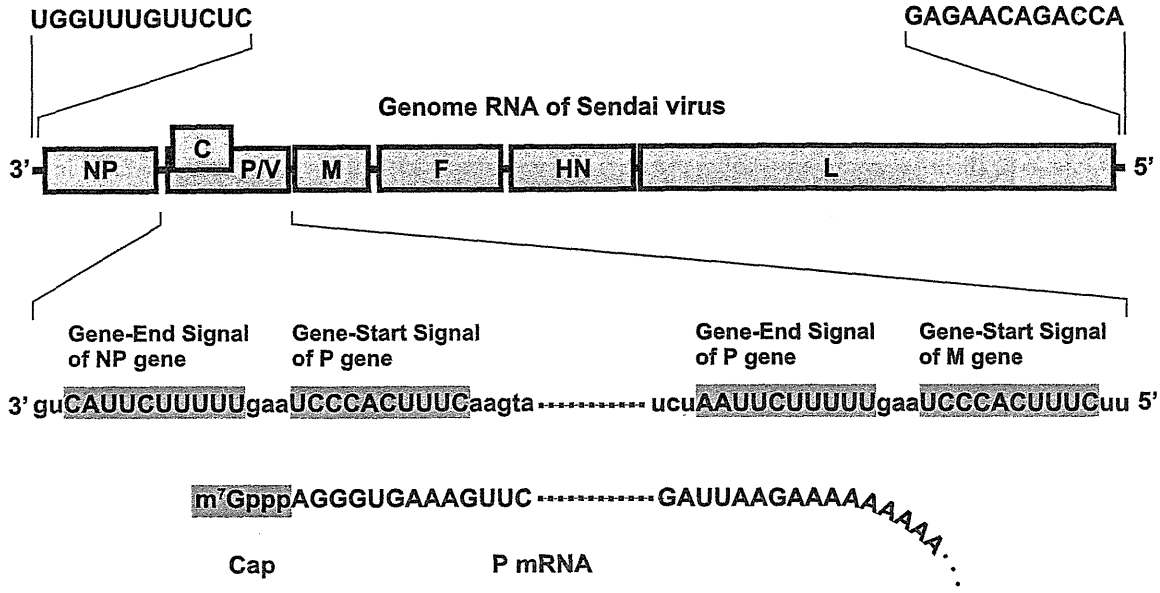


Fig. (2). Genome structure of the Sendai virus. Transcription of capped mRNAs starts from the transcription initiation signal with RNA-dependent RNA polymerase (L protein). Transcription ends at the transcription termination signal, followed by a poly-A signal.

quently, replication-competent recombinant SeV vectors expressing envelope proteins of hPIV-1, hPIV-2, hPIV-3 and respiratory syncytial virus (RSV) were developed and their effectiveness was proven in model animals [36]. SeV vectors have also been investigated as platforms for vaccines against the human immunodeficiency virus [37] and influenza viruses [38].

Replication-defective (F-defective) SeV vectors expressing the angiogenic cytokine fibroblast growth factor-2 (FGF2) have been developed for treatment of critical limb ischemia [39, 40]. A phase 1/2a clinical trial using the SeV-FGF2 vector was performed in 2006 at Kyushu University (Fukuoka, Japan) and up to 5×10^9 plaque-forming units (pfu)/60 kg (body weight) of rSeV-FGF2 were administrated intramuscularly. Although the outcomes for patients remain unpublished, no severe adverse events were reported. This trial was the first to administer a recombinant SeV vector to humans directly by injection.

Applications of SeV vectors to cancer gene therapy have been investigated at the preclinical stage. In addition to the stimulation of dendritic cells with SeV vector expressing IFNβ (rSeV-IFNβ) [29], virotherapy with the unique host-restricted SeV vector rSeV/dMFct14(uPA2) ("BioKnife") has been developed [41]. Infectivity of wild-type SeV absolutely requires the cleavage of the precursor F_0 protein to F_1 and F_2 subunits [42], resulting in exposure of the hydrophobic N-terminus of the F_1 subunit [43]. Serine protease is responsible for this cleavage in the lungs of natural hosts [44]. BioKnife is created by altering the structure of this cleavage site to that optimal to urokinase-type plasminogen activator (uPA) and by deletion of the *M* gene to interfere with virion production [41]. These modifications restrict the spread of this uPA-dependent recombinant SeV by membrane fusion between cells. As uPA is often activated on cancer cell surfaces and is responsible for metastasis, this virus spreads preferentially to metastatic tumors and destroys them by its intrinsic cytotoxic activity. BioKnife has been reported to be effective in treating intractable cancers such as malignant glioblastomas [45] and malignant pleural mesotheliomas [46] in animal models.

NUCLEAR REPROGRAMMING WITH SEV VECTORS

Gene delivery/expression technologies are also indispensable for reprogramming somatic cell genomes by the ectopic expression of transcription factors. The concept of nuclear reprogramming was first proposed in the 1970s, based on experiments with somatic cell fusion and was proven by the discovery of *MyoD*, a gene encoding a master transcription factor inducing the dynamic transition of fibroblasts to myoblasts [47]. However, no other genes encoding a master transcription factor capable of reprogramming by itself have been identified.

Reprogramming skin fibroblasts into induced pluripotent stem cells (iPSCs) by the ectopic expression of four reprogramming factors (Oct4, Sox2, Klf4 and c-Myc) opened a new era in this field [48-51]. Human iPSCs have a capacity to differentiate to all three germ layers, as do embryonic stem cells (ESCs), while tissue cells generated from iPSCs can escape from immunological rejection when transplanted to the host. In addition, using an iPSC line can avoid the ethical concern that generation of ESCs requires the destruction of normal human embryos. All of these features likely make human iPSC lines ideal sources for gene and cell therapy.

In addition to these practical aspects, the discovery of iPSCs also introduced the novel concept that cell fate conversion needs the cooperation of multiple gene products in a single cell. As genomic reprogramming is a relatively slow process, expression of the exogenous transcription factors has to be sustained for 10–20 days [52]. On the other hand, these exogenous genes should be irreversibly suppressed or—ideally—should be removed from the reprogrammed cells. This latter point is important both for avoiding unde-

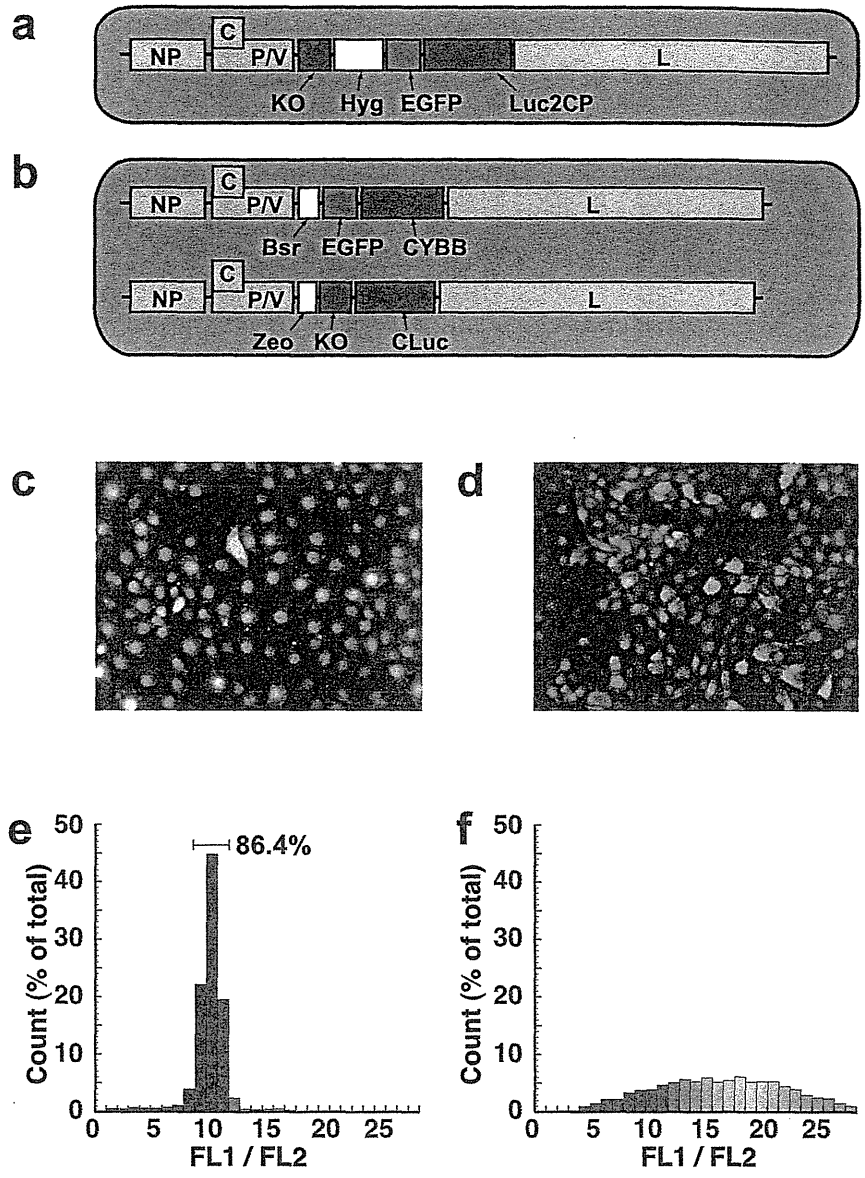


Fig. (3). Compatibility of two independent SeVdp vectors in a single cell. (**Top**) Structure of SeV vectors. cDNA sequences encoding for enhanced green fluorescent protein (EGFP) cDNA and Kusabira orange (KO) were installed on a single SeV vector SeVdp(KO/EGFP) (**a**) or on two SeV vectors SeVdp(KO) and SeVdp(EGFP) separately (**b**). (**Middle**) Fluorescence images of cells expressing KO and EGFP from these SeV vectors. Fluorescence microscopy images of KO and of EGFP were obtained separately with specific filter sets and merged after being converted to an artificial color output (green for EGFP and red for KO). Cells carry a single SeV vector SeVdp(KO/EGFP) (**c**) or mixture of two SeV vectors SeVdp(KO) and SeVdp(EGFP) (**d**). (**Bottom**) Expression levels of KO and EGFP were analyzed quantitatively using flow cytometry. The ratio of the signal intensities of EGFP and KO in each cell is shown as a histogram. Cells carry a single SeV vector SeVdp(KO/EGFP) (**e**) or mixture of two SeV vectors SeVdp(KO) and SeVdp(EGFP) (**f**). Reprinted from reference 26 with permission.

sired side effects (cell transformation and insertional mutagenesis) and for maintaining full pluripotency in these cells [53].

Following the initial successes in nuclear reprogramming using gamma-retrovirus or lentivirus vectors, both of which cause provirus insertion into host genomes, researchers have made substantial efforts to establish methods for generating *ex*-gene-free iPSCs [53]. However, it is difficult to satisfy controversial requirements described above thoroughly with conventional gene delivery/expression tools. These and other necessities have directed the attention of researchers to the gene delivery/expression technologies once again and should facilitate the development of novel technology best suited for genomic reprogramming.

The SeV vector stands unique among other vector systems in genomic reprogramming because it can express the reprogramming genes without chromosomal integration. Wild-type SeV vectors installed with *Oct4*, *Sox2*, *Klf4* and *c-Myc* cDNA were reported to generate *ex*-gene-free iPSCs, dependent on passive elimination of the genome through cell passage [54]. This prototype was replaced with a less cytotoxic backbone [55] and is now available commercially. The

SeV vector with a temperature-sensitive (*ts*) mutation was also reported to facilitate the erasure of the vector genome [56]. However, as each of the reprogramming genes is installed on separate vectors, the balance of their expression levels is likely to vary among infected cells (Fig. (3)). Therefore, the biological characteristics of each iPSC line generated by these separate SeV vectors should be examined carefully, as the balance of expression of reprogramming genes might affect the quality of the iPSC line [57].

Defective and persistent Sendai virus (SeVdp) vectors are now also recognized as a superior tool for iPSC generation thanks to their remarkably high potential and simplicity [30]. The SeVdp vector was developed by one of us (MN) based on noncytotoxic *ts* mutant SeV strain clone 151 (SeV cl.151), originally isolated in 1979 [58]. Distinct from wild-type cytotoxic SeV and from other *ts* mutant SeV strains with defects in gene expression, SeV cl.151 is unique because it readily establishes stable persistent infection in cultured cells at 37 °C and strong expression of viral genes can be sustained indefinitely [31]. We identified the mutations responsible for this phenotype [30, 59, 60] and showed that this virus escapes from the intrinsic cytotoxicity of SeV by a defect in the induction of IFNβ production in target cells [60]. This phenotype is unique among cytoplasmic RNA viruses and is ideal for stable and reproducible gene expression.

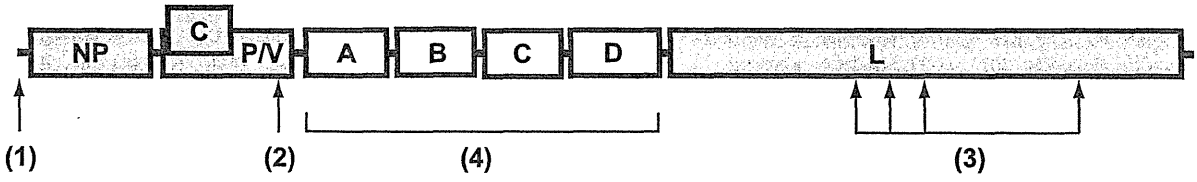
An SeVdp vector suitable for generating *ex*-gene-free iPSCs (SeVdp-iPSCs) was created by deleting the *M, F* and *HN* genes dispensable for persistent gene expression and by installing four reprogramming genes instead (Fig. (4)). This "all-in-one" genome structure is essential both for certifying simultaneous expression of all the reprogramming genes at a constant ratio in a single cell (Fig. (3)), and for preventing secondary virion production from infected cells [30]. After completion of reprogramming, the SeVdp-iPS genome can be erased through suppression of viral L protein expression with short interfering RNA (siRNA) [30]. The human iPSC lines generated are *ex*-gene free and are quite uniform in their characteristics: more than 80% of the colonies are positive for TRA-1-60, a reliable marker for pluripotency [61] (Nishimura and Nakanishi, unpublished).

A broad target cell range is another important characteristic of SeV vector-mediated nuclear reprogramming. In addition to skin fibroblasts, SeV vectors can reprogram the nuclei of various human cells, including $CD34^+$ cord blood cells [62], activated T lymphocytes [55] and monocytes (Nishimura and Nakanishi, unpublished). Generation of iPSC lines from peripheral blood cells, especially from nondividing monocytes, will be quite important for the practical use of tailor-made iPSCs in regenerative and molecular medicine.

FUTURE PERSPECTIVES

As we show in this review, recombinant SeV vectors are powerful tools in basic research, in molecular therapy and in regenerative medicine. Among various applications of SeV vectors, generation of human iPSCs by nuclear reprogramming is attracting broad interest and is highly valued. Although SeV vectors can generate *ex*-gene-free iPSCs quite efficiently compared with other gene delivery/expression systems, claims of total superiority should be treated cautiously, as "*ex*-gene free" is highly desirable but might not be sufficient for clinical-grade iPSC lines [53]. The long-term stability of genome structure and epigenetic conditions should be examined before the clinical application of iPSC lines generated using SeV vectors.

Since the discovery that the genomes of somatic cells could be reprogrammed to generate pluripotent iPSCs, the notion of direct genomic reprogramming of somatic cells to other cell lineages (either to terminally differentiated cells or to tissue stem cells) has also attracted significant interest [63]. Direct reprogramming (*trans*-differentiation) relies on the same strategy for iPSC generation, that is, ectopic expression of multiple lineage-specific protein factors in a single cell followed with culture under defined conditions optimized for each target cell type. This procedure has been investigated not only in cells cultured *in vitro* but also in disease target tissues *in situ* [64, 65]. Direct genomic reprogramming can eliminate the potential risk of tumorigenicity in iPSC-mediated tissue cells, unless the cellular life span is reset to be infinite. This approach might also accelerate the generation of target tissue cells through bypassing the time-consuming process of iPSC generation.



Key Structure of SeVdp Vector

(1) Insertion of Gene-End Signal

(2) Mutation in P gene (P517H)

(3) Mutation in L gene (V981I, S1088A, C1207S, V1618L)

(4) Deletion of M,F and HN genes, and Installation of exogenous genes

Fig. (4). Genome structure of defective and persistent Sendai virus (SeVdp) vector. SeVdp has mutations in the *L* and *P* genes, which are responsible for low cytotoxicity and for defective induction of IFNβ. The *M, F* and *HN* genes are deleted and replaced with genes of interest (A–D). SeVdp-iPS was installed with *Oct4*, *Sox2*, *Klf4* and *c-Myc* cDNAs on a single vector.

Currently, direct genomic reprogramming is investigated mostly by using classical retro/lentivirus vectors. However, low reprogramming efficiency and chromosomal integration of exogenous reprogramming genes limit their translation into clinical applications. These obstacles could be overcome with the use of SeV vectors. As described above, these have the potential to deliver exogenous genes into target tissues *in situ*. The development of less antigenic, "smart" SeV vectors equipped with the machinery for controlling gene expression and genome stability *in vivo* should contribute to translation of the current direct genomic reprogramming technologies into clinical applications. Further refinement of SeV vectors through basic research is highly desired and will bring a promising future to the fields of gene therapy and regenerative medicine.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflicts of interest.

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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. (*Blood*. 2012;119(26):6234-6242)

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 fibroblasts[22] 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 (FGF: Peprotech) on mitomycin C (MMC)–treated mouse embryo fibroblast (MEF) feeder cells.[23] Imatinib (LC Laboratories) was added to the culture medium at the various concentrations (1-10μM). U0126 and LY294004 (LC Laboratories) were used to inhibit ERK and AKT, respectively.

The mouse C3H10T1/2 cells were cultured as previously described.[24]

Production of VSV-G pseudotyped retroviral particles

Construction of pMXs vectors encoding Oct3/4, Sox2, Klf4, and c-myc were performed as previously described.[22] Highly concentrated VSV-G–pseudotyped retroviral supernatant was prepared using reported procedures. The 293GPG cells were kind gifts from Dr R. C. Mulligan (Children's Hospital Boston, Harvard Medical School, Boston, MA).[25] Stable 293GPG cell lines, each capable of producing VSV-G–pseudotyped retroviral particles on induction were established as previously described.[22,25] Retroviral supernatants were concentrated by centrifugation for 16 hours at 6000*g*.

Generation of iPSCs from CML samples

Two days before infection, cells were stimulated with cytokines as mentioned in "Cell and cell culture." For infection, each well of a 24-well dish coated with a fibronectin fragment CH296:RetroNectin (Takara-Bio) was covered with virus-containing supernatants. After the adhesion of viruses according to the manufacture's recommendation, 1×10^5 cells of $CD34^+$ CML cells or CB cells were inoculated into each well and filled with the culture medium supplemented with cytokines. The next day, concentrated viral supernatant was added to the culture. On day 3 after infection, cells were harvested with vigorous pipetting, washed by phosphate-buffered saline (PBS), and cultured with the same fresh medium for next 3 days. On day 6, cells were seeded on MMC treated MEF cells. Two to 4 days after, the medium was replaced with human ES medium as previously described with 0.5mM valproic acid (VPA; Sigma-Aldrich).[26] Subsequently, medium was changed every other day. After 20 days, ES-like colonies appeared. Using live cell imaging technology with Tra-1-60 antibody as previously described,[27] each fully reprogrammed colony was distinguished from deficiently reprogrammed colonies, and was picked up to be reseeded on new MEF feeder cells. Cloned ES-like colonies were subjected to further analysis.

Antibodies, FACS analysis, and immunocytochemistry

The following fluorescent conjugated antibodies were used for fluorescence-activated cell sorter (FACS) analysis and immunocytochemistry: anti–human stage specific embryonic antigen (SSEA)–4 conjugated with Alexa Fluor 488 (BD Bioscience), anti–human tumor related antigen (TRA)–1-60 conjugated with Alexa Fluor 555 (BD Bioscience), anti-CD34 phycoerythrin (PE) conjugated (Beckman Coulter), and anti-CD45 fluorescein isothiocyanate (FITC) conjugated (Beckman Coulter).

Cells were sorted with a FACSAria, and analysis was performed on FACS LSRII (BD Bioscience).

For immunocytochemistry, cells were fixed with 4% paraformaldehyde in PBS, after which they were labeled with an antibody against human SSEA-4 and antibody against human TRA-1-60 antibody and observed using a confocal microscope (Carl Zeiss).

Methylation profiling

Genomic DNA was extracted using the QIAamp DNA Mini Kit (QIAGEN) according to the manufacture's instruction. Methylation status was evaluated as previously reported.[28] Methylation status was analyzed using HumanMethylation27 BeadChip (Illumina). Genomic DNA for methylation profiling was quantified using the Quant-iT dsDNA BR assay kit (Invitrogen). Five-hundred nanograms of genomic DNA was bisulfite-converted using an EZ DNA methylation kit (Zymo Research). The converted DNA was amplified, fragmented and hybridized to a beadchip according to the manufacturer's instructions. The raw signal intensity for both methylated (M) and unmethylated (U) DNA was measured using a BeadArray Scanner (Illumina). The methylation level of the each individual CpG is obtained using the formula (M)/(M) + (U) + 100 by the GenomeStudio (Illumina).

Microarray analysis

Gene expression analysis was carried out as previously described[29] 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.

Hematopoietic differentiation of iPSCs

To differentiate iPSCs into hematopoietic cells, we used the same protocol previously used with ES cells and iPSCs.[22,24] In brief, small clusters of iPSCs (< 100 cells treated with PBS containing 0.25% trypsin, 1mM $CaCl_2$, and 20% KSR) were transferred onto irradiated 10T1/2 cells and cocultured in hematopoietic cell differentiation medium, which was refreshed every third day. Differentiation medium consists of Iscove modified Dulbecco medium supplemented with a cocktail of 10 μg/mL human insulin, 5.5 μg/mL human transferrin, 5 ng/mL sodium selenite, 2mM L-glutamine, 0.45mM α-monothioglycerol, 50 μg/mL ascorbic acid, and 15% highly filtered FBS in the presence of 20 ng/mL human vascular endothelial growth factor (VEGF).[24] On days 14 to 15 of culture, the iPS-sacs were collected into a 50-mL tube, gently crushed with a pipette tip and passed through a 40-μm cell strainer to obtain hematopoietic progenitors. Hematopoietic progenitors were collected by sorting with CD34 and CD45 antibodies, Giemsa stained, and then examied under a microscope. Hematopoietic progenitors were cultured in the α-medium plus 20% FCS supplemented with 100 ng/mL SCF, 10 ng/mL TPO, 100 ng/mL FL3L, 10 ng/mL IL3, and 100 ng/mL IL6.

Hematopoietic colony-forming cell (CFC) assay

CFC assays were performed in MethoCult H4434 semisolid medium (StemCell Technologies). Ten thousand hematopoietic progenitors harvested from an iPS-Sacs were plated in 1.5 mL of medium and cultivated for 14 days.

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

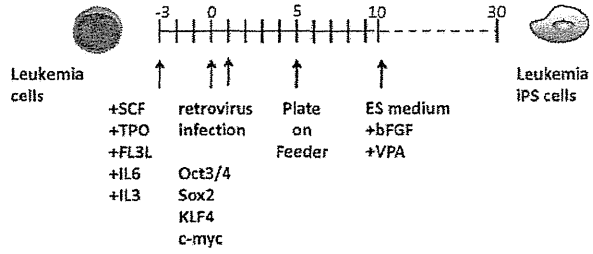

Figure 1. Experimental scheme for generating of iPSCs from the CML patient sample. After cytokine stimulation, $CD34^+$ CML cells were reprogrammed by transduction with Yamanaka factors. To improve the reprogramming, valproic acid was added to the culture.

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 pluripotency 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)

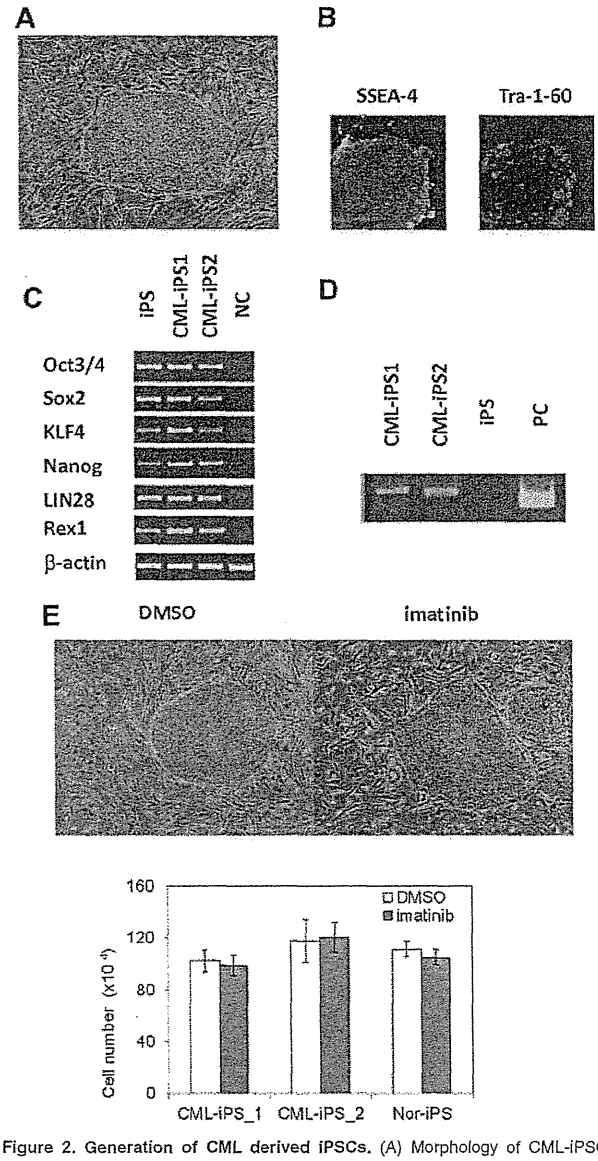

Figure 2. Generation of CML derived iPSCs. (A) Morphology of CML-iPSCs. (B) Immunofluirescence staining shows expression of pluripotent marker (left: SSEA-4 and right: Tra-1-60) in CML-iPSCs. (C) RT-PCR analysis of ES cell marker genes. Endogenous expression of these stem cell–specific genes in CML-iPSCs was verified. (D) CML-iPSCs expressed the BCR-ABL fusion transcript. (E) Imatinib (10μM) were added to the culture of iPSCs. DMSO (top left panel) and imatinib (top right panel) treated CML-iPSCs were shown. The number of alive CML-iPSCs (CML-iPS_1 and CML-iPS_2) and normal iPSCs (Nor-iPS) after 5 days treatment was calculated (bottom panel). These were the representative data from 3 independent experiments.

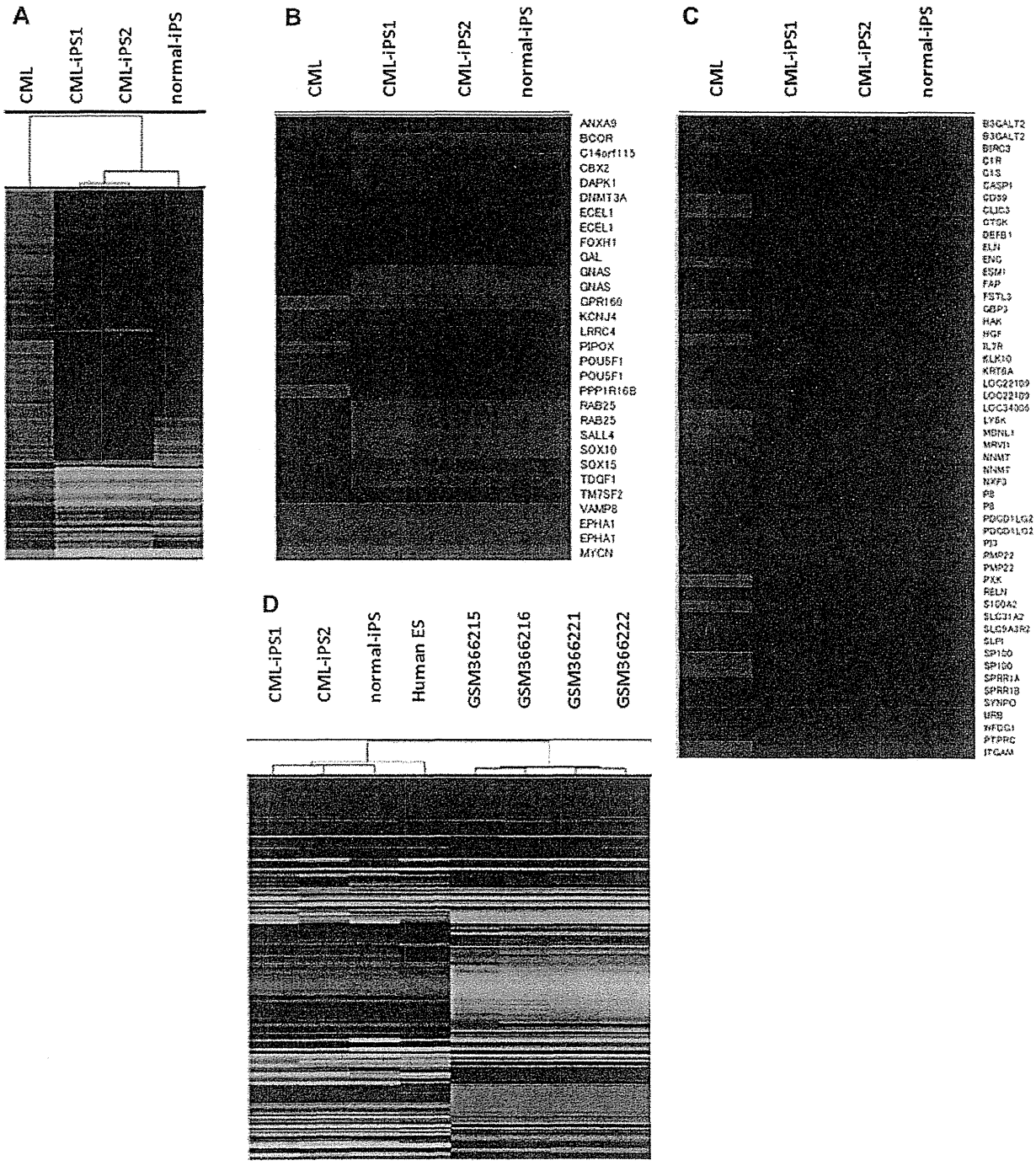

Figure 3. Comprehensive analysis of DNA methylation and gene expression. (A) Unsupervised hierarchical clustering based on differentially methylated CpGs is shown on the dendrogram. The accompanying heatmap shows the methylation status across 5001 differentially methylated CpGs. In the heatmap, red indicates a CpG methylation more than 50%, and green less than 50%. The methylation status in hypo SS DMRs (B) or hyper SS DMRs (C) was shown in the heatmap. (D) Unsupervised hierarchical clustering based on global gene expression data are shown on the dendrogram. The accompanying heatmap shows the normalized log2 transformed expression values (Z-scores) for each probe. In the heatmap, red indicates expression more than mean, and green less than mean.

were identified during reprogramming process of iPSCs.[31] 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

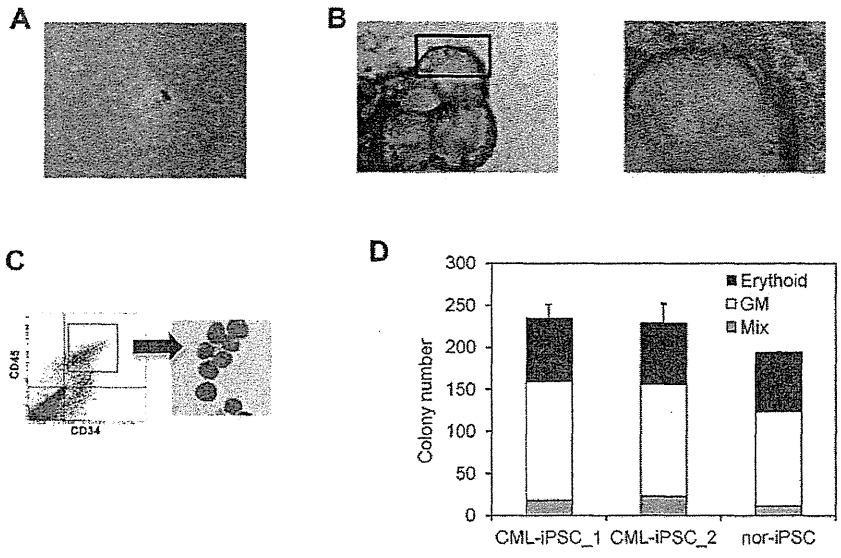

Figure 4. Hematopoietic differentiation of CML-iPSCs. CML-iPSCs were differentiated on the 10T1/2 cells. On day 7 (A), iPSCs began to mount. On day 14 of culture (B: left panel), inflated sac-like structures appeared. These sac-like structures contained the round hematopoietic cells (B: right panel: higher magnification). (C) These hematopoietic cells expressed immature marker CD34 and CD45. (D) CFC activity was estimated using 1×10^4 3CD34$^+$ CD45$^+$cells. Erythroid colonies (black bars), granulocyte-monocyte (GM) colonies (white bars), and mixed GM colonies with erythtoid cells (mix; gray bars) were plotted.

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 (NOG) 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$^+$38$^-$90$^+$45$^+$ 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

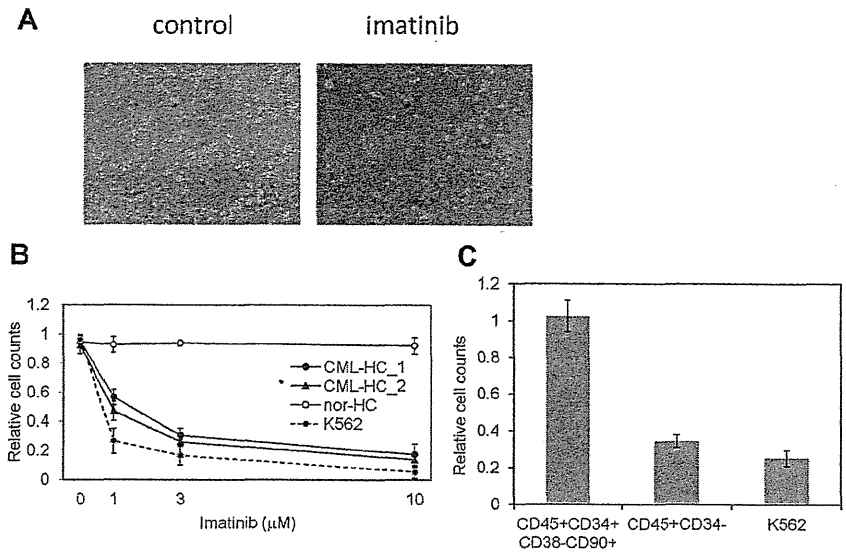

Figure 5. CML-iPSC derived hematopoietic cells recovered the sensitivity to imatinib. (A) Imatinib but not the vehicle (DMSO) decreased the growth of hematopoietic cells derived from CML-iPSCs in suspension culture. (B) Various concentrations of imatinib were added to the culture of iPSC derived hematopoietic cells for 4 days. CML-iPSC–derived CD34+ hematopoietic cells (CML-HC_1 and CML-HC_2), normal iPSC-derived hematopoietic cells (nor-HC), and K562 cells were used for analyses. Relative cell counts compared with the vehicle control were plotted. Shown is the mean of a single experiment conducted in triplicate as a representative of 3 independent experiments. (C) Imatinib (10μM) was added to the suspension culture of CML-iPSC–derived hematopoietic cells for 4 days. The immature cell fraction ($CD34^+CD38^-CD90^+CD45^+$) showed resistance similar to CML-iPSCs, although more differentiated cells ($CD34^-CD45^+$) showed the sensitivity to imatinib. Relative cell counts compared with the vehicle control was plotted.

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 imatinib 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-ABL[42,43] 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 primary CML-iPSCs[47] and KBM7-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. Carette et al argued that a specific differentiated epigenetic cell state is needed to maintain BCR-ABL dependence.[17] 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.[39] 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.

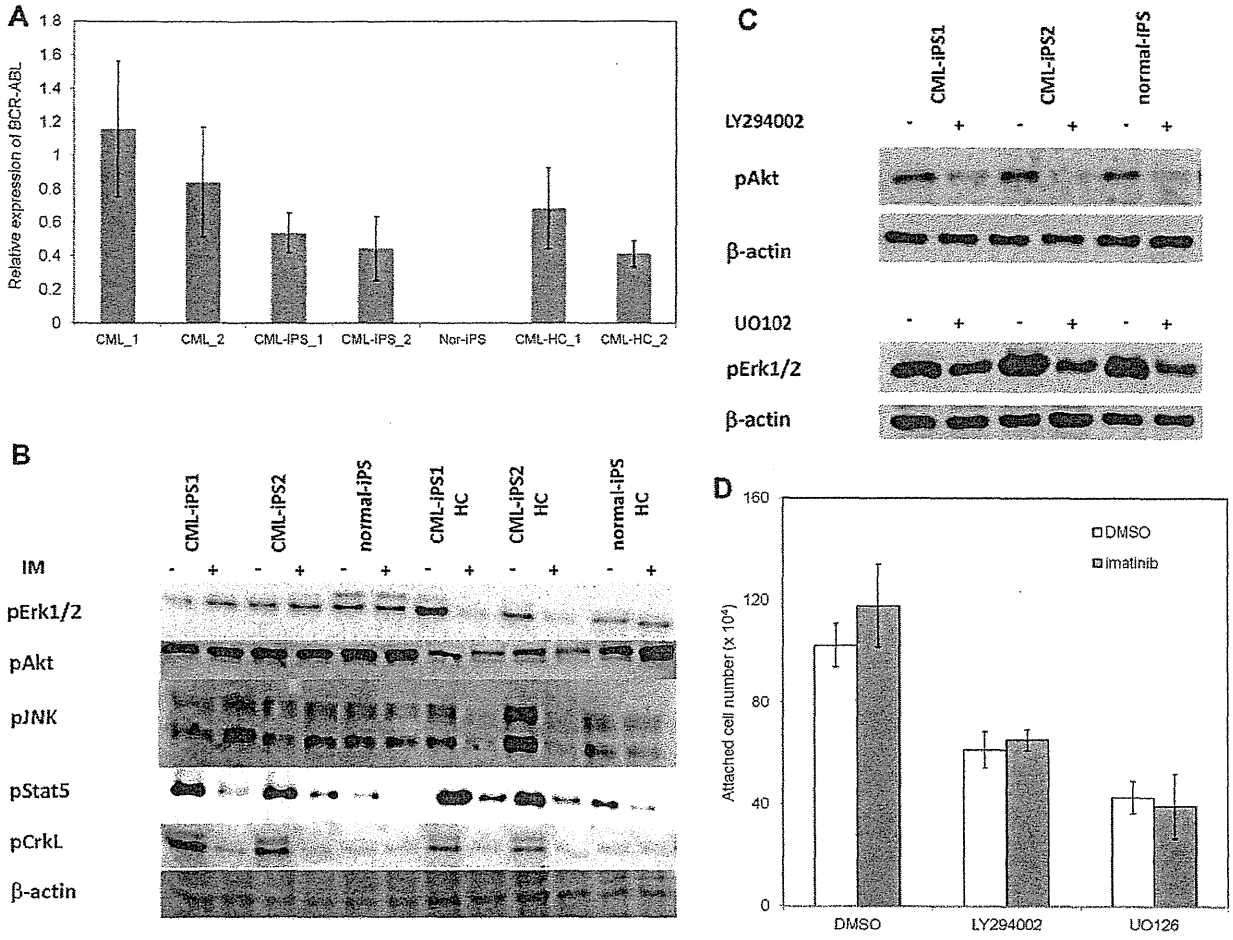

Figure 6. The mechanism of imatinib resistance in the CML-iPSCs. (A) The expression profile of BCR-ABL transcript during hematopoietic differentiation. The expression levels of BCR-ABL in the CML-iPSCs were compared with those of primary CML samples (CML_1 and CML_2), CML-iPSC–derived CD34⁺ hematopoietic cells (CML-HC_1 and CML-HC_2), and normal iPSC (nor-iPS). The expression level of the mean in the primary CML sample was set at 1. (B) BCR-ABL signaling was estimated in the CML-iPSCs after imatinib (IM) treatment. The phosphorylation state of ERK1/2, AKT, JNK, and STAT5, which are the essential for the survival of BCR-ABL (+) hematopoietic progenitors (CD34⁺CD45⁺), were evaluated after imatinib treatment in CML-iPSCs. These were the representative data from 3 independent experiments. (C-D) LY294002 and U0126 (10μM) were added to the culture of CML iPSCs to inhibit AKT and ERK, respectively with or without imatinib. (C) After 4 hours of culture, each inhibitor decreased the phosphorylation of ERK or AKT as expected. (D) The attached cell numbers after treatment with specific AKT or ERK inhibitor were shown. These were the representative data from 3 independent experiments.

The progression of CML from initial indolent CP to the aggressive stages, the 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, transciptome, 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,[48] chemical compounds, such as azacitidine (DNA metyltranferase inhibitor),[49] BIX01294 (G9a histone metyltransferase inhibitor),[50] VPA (histone deacetylase inhibitor), or TSA (histone deacetylase inhibitor),[26] and knockdown of p53, p21, and Ink4/Arf.[51,52]

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[53] 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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Authorship

Contribution: K.K. designed the research, performed experiments, and wrote the paper; S.A., M.H., K.T., N.T., M.O., G.N., K.U., K.N., and Y.K. performed experiments, K.E., H.A., and H.N. discussed the paper; and M.K. conceived and designed the research, supervised the whole project, and wrote the paper.

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Review

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Stem cell therapy: an exercise in patience and prudence

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In recent times, the epigenetic study of pluripotency based on cellular reprogramming techniques led to the creation of induced pluripotent stem cells. It has come to represent the forefront of a new wave of alternative therapeutic approaches in the field of stem cell therapy. Progress in drug development has saved countless lives, but there are numerous intractable diseases where curative treatment cannot be achieved through pharmacological intervention alone. Consequently, there has been an unfortunate rise in incidences of organ failures, degenerative disorders and cancers, hence novel therapeutic interventions are required. Stem cells have unique self-renewal and multilineage differentiation capabilities that could be harnessed for therapeutic purposes. Although a number of mature differentiated cells have been characterized *in vitro*, few have been demonstrated to function in a physiologically relevant context. Despite fervent levels of enthusiasm in the field, the reality is that other than the employment of haematopoietic stem cells, many other therapies have yet to be thoroughly proven for their therapeutic benefit and safety in application. This review shall focus on a discussion regarding the current status of stem cell therapy, the issues surrounding it and its future prospects with a general background on the regulatory networks underlying pluripotency.

1. Introduction: cellular reprogramming and half a century of stem cell therapy

In the past century, pharmacological advancements have dramatically improved the clinical outlook for patients suffering from all sorts of diseases. However, there are limitations to this approach; beyond a certain point, it becomes palliative only and no longer curative. Attempts at identifying the regulatory networks underlying pluripotency began in 1952, with cellular reprogramming experiments studying the nuclear transfer (NT) of amphibian nuclei [1]. Together with cell-fusion experiments involving embryonal carcinoma cells (ECCs) [2] and embryonic stem cells (ESCs) [3], it was demonstrated that the nuclear epigenetic modifications of somatic cells acquired during differentiation are reversible and can be rearranged to a configuration that supports a pluripotent state. At that time, 'pluripotency' was defined along the lines of 'an unlimited capacity to give rise to all cells of the embryo'. Researchers had not yet begun to consider the true therapeutic impact of this statement. A decade later in 1963, Till & McCulloch [4] demonstrated the presence of repopulating cells in mouse bone marrow (BM). This was to be the first demonstration of stem cells and the birth of regenerative medicine as it is known today. In later years, the cells were identified and characterized as haematopoietic stem cells (HSCs) and since that first demonstration, numerous other somatic stem cell (SSC) populations have been identified [5].

The connection between cellular reprogramming and the clinical application of stem cells was not immediately obvious. In many ways, they are two sides of the same coin so to speak. It was not until difficulties were encountered in handling SSCs *in vitro*, and the realization that some tissues had no resident pool of regenerating stem cells, did researchers began to consider the necessity of searching for a cell higher up in the hierarchical order of cell potency. The two were

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thus inextricably linked. Some thought the answer came in 1998, with the first *in vitro* establishment of human embryonic stem cells (hESCs) [6]. Not only were hESCs found to be pluripotent, they were to become a vital tool in further dissecting the intricacies of the regulatory networks that underlies pluripotency. However, ESCs also became something of an ethical landmine, as the derivation of these cells required the destruction of human embryos [7]. In 2006, the first generation of induced pluripotent stem cells (iPSCs) took place, which appeared to be 'ES cell-like' but without the need for instigating an ethical debate [8]. The focus in medicine, in theory at least, began shifting towards how to harness the regenerative powers of stem cells with the hope of perhaps curing intractable diseases in transplantation settings. Not only that, scientists and clinicians dared to dream and began to explore the possibilities of replacing damaged cells either at a singular level or even as whole organs. It is natural therefore to ask what are stem cells? What makes stem cells unique and how can they be used to treat patients?

2. Stem cells overview

In reviewing stem cells, one of their key defining characteristics are their ability to self-renew. 'Self-renewal' is the ability to undergo cycles of mitotic division while maintaining the same undifferentiated state as the parent cell [9]. This is particularly important in tissues where there is a resident pool of stem cells that are responsible for maintaining the lifelong homeostasis of that tissue, including HSCs [10] and neural stem cells (NSCs) [11] and epithelial stem cells (EpSCs) [12]. In the clinical situation, a potential therapeutic strategy could be to aim for the replacement or the induction of these stem cells so that when injury occurs, they can reconstitute the tissue system in question or facilitate the natural mechanisms of repair. The other defining characteristic of stem cells, and perhaps the one that has most captured the imagination of so many is the characteristic of cell potency. 'Potency', from the Latin *potens*, meaning *having power*, is a rather appropriate way to characterize stem cells because it is this power that science is trying to harness. This refers to the capacity of stem cells in being able to differentiate into different cell types (figure 1). As shown in the figure, stem cells can be assigned to a hierarchical order based on their degree of potency. At the top, cells of the morula are deemed to be totipotent in that they can differentiate into all tissue types. ESCs and iPSCs are pluripotent cells that are one magnitude short of totipotency because they cannot differentiate into placental tissue. Multipotent cells can include resident tissue-specific stem cells that are more lineage-restricted. Oligopotent cells are usually committed progenitors such as the common myeloid and common lymphoid progenitors (CMP and CLP) that are restricted towards differentiating into an even smaller subcategory of cells (myeloid and lymphoid, respectively).

In broad terms, stem cells are categorized into 'somatic' or 'embryonic' stem cells. SSCs, also know as adult stem cells, are generally multipotent and able to differentiate into any cell of a specified lineage. Notable examples include HSCs, NSCs, EpSCs in the skin [13], cornea [14], gut [15] and mesenchymal stem/stromal cells (MSCs). Their anatomical location is a reflection of the critical roles that they possess in the development, maintenance and repair of specified tissues and organs

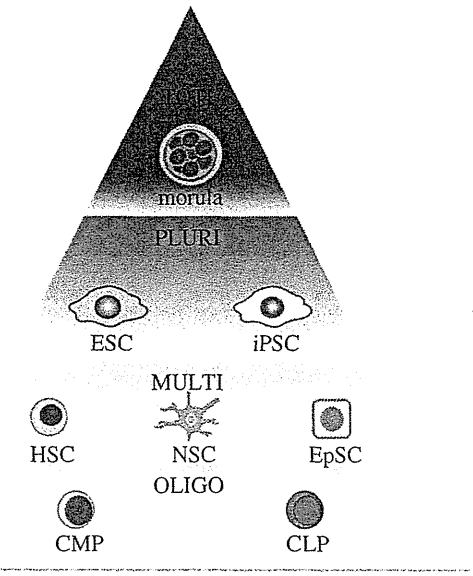

Figure 1. A pyramidal hierarchy of cell potency. Sitting atop the pyramid (red), cells of the morula are the most potent cells that can differentiate into all tissue types. ESCs and iPSCs occupy the next level, as they cannot differentiate into the placenta (orange). Tissue resident stem cells (HSCs, NSCs, EpSCs) can differentiate into multiple cell types restricted to that lineage (yellow). At the base are cells with more limited differentiation potential (CMP, CLP) usually committed progenitors (light green). TOTI, totipotent; PLURI, pluripotent; MULTI, multipotent; OLIGO, oligopotent; ESC, embryonic stem cell; iPSC, induced pluripotent stem cell; HSC, haematopoietic stem cell; NSC, neural stem cell; EpSC, epithelial stem cell; CMP, common myeloid progenitor; CLP, common lymphoid progenitor.

[16]. These locations are often loosely referred to as the 'niche', but strictly speaking, the name has a much stronger emphasis on the surrounding micro-environment and its constituent supporting and regulatory cells derived from which are extrinsic signals that can strongly influence the functions of the residing stem cell [17]. This is an important concept to keep in mind regarding SSCs because clinical strategies can be targeted at replenishing the resident cell population either through transplantation or through endogenous manipulation to positively enhance cell functions [18]. The BM, host niche for HSCs, remains the best defined and, although incompletely so, it is one of the most readily accessible niches of all. The BM–HSC relationship was the basis for the first stem cell experiments. NSCs are found in the dentate gyrus of the hippocampus and the lateral ventricle wall of the olfactory bulb [19]. These represent very sensitive parts of the brain, thereby making the prospective isolation of NSCs rather difficult and treacherous. Of the EpSCs, these are a located within the epithelia overlying external tissue surfaces such as the skin, gut and cornea. The epithelia in these tissues are subject to high rates of cell turnover; thus the role of resident EpSCs is crucial in maintaining homeostasis. For MSCs, their *in vivo* physiological functions remain unclear much less having a defined niche. Recent studies indicate that when used in a transplant setting, it is the MSCs themselves that act as supporting cells through the paracrine and anti-inflammatory effects that these cells have [20]. Use of the term niche is probably inappropriate, given the lack of understanding of the surrounding micro-environment hosting MSCs. Generically, they are referred to purely by their source of derivation for example adipose stem cells (ASCs), as in MSCs derived from

adipose tissue. However, not all tissues in the body contain a resident pool of stem cells, a notable example being the endocrine pancreas [21]. Damage to, or the defective function of these tissues can only be treated by a regimen of pharmaceutical drugs where applicable or whole organ transplantation from a HLA matched donor.

Occupying a higher order of cell potency than SSCs, the first derivation of mouse embryonic stem cells (mESCs) took place in 1981 [22,23] and then of hESCs in 1998 [6]. These are derived either from cells of the morula or from the inner cell mass (ICM) of the blastocyst stage embryo (figure 2). ESCs are pluripotent cells capable of differentiating into all cell types except for the placenta. This differentiation capacity can be assessed by testing the ability of the cells to partake in tissue development. Upon injection into a blastocyst, ESCs can contribute efficiently to the formation of all adult tissues including the germline [23]. For this reason, ESCs have become the gold standard with which all *in vitro* cultivated pluripotent cells are judged against. In the case of mESCs, it is possible to assess pluripotency by carrying out the teraploid complementation assay, which is currently the most stringent assay of testing developmental potential of its kind [24]. For ethical reasons, the same assay cannot be carried out using hESCs where the evaluation of the developmental potency of human pluripotent cells is limited to the teratoma formation assay. This assays for the spontaneous potential of the cells in differentiating into the three germ layers and hence it is not as stringent. Still, under defined conditions, hESCs can differentiate into transplantable neural precursors [25], functional hepatocytes [26], haematopoietic progenitors [27] and other tissue lineages. As such, their therapeutic potentials are clear to see, and efforts are ongoing to exploit this.

However, there are some major obstacles that have precluded the successful clinical application of ESCs. The pluripotent potential of these cells makes them therapeutically attractive but by the same token, when this potential becomes dysregulated, undifferented ESCs can form teratomas *in vivo* [28]. The ramifications could be severe particularly in cell-replacement therapy should even one undifferentiated cell enter the patient. Another point for consideration is that there is the risk of eliciting an immune response in the recipient, given the mismatch in major histocompatibility complex (MHC) class I antigens [29]. Unfortunately, the most negatively publicized of these challenges is that the *in vitro* establishment of ESCs involves destruction of the embryo. Some have cried 'the destruction of a life'. Criticism has ranged from public condemnation by the Vatican [30] to calls for restraint by the then US president George W. Bush [31]. These may not have any direct scientific or clinical relevance but some mention is warranted, given that this is an obstacle that has continued to remain insurmountable. Therefore, the application of ESCs for research or therapeutic purposes is likely to face continued restraint in developing further progress, particularly in translating into clinical trials.

In any case, none of this public negativity should serve to diminish the enormous impact that ESCs have had on studying the transcriptional regulation of pluripotency and of stem cell therapy. From a cellular reprogramming perspective, ESCs were a critical tool in achieving the seminal breakthrough of induced pluripotency [8] along with the technique of somatic cell nuclear transfer (SCNT) [32] and the study of pluripotency-associated transcription factors. In a cell-fusion experiment with ESCs and adult

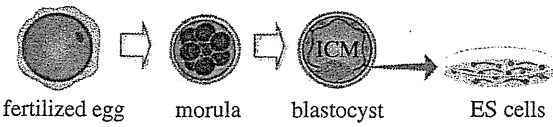

Figure 2. Derivation and establishment of embryonic stem cells (ESCs). Zygotes are maintained until reaching the morula or blastocyst stages of development. Cells from the intra cellular mass (ICM) are extracted and maintained *in vitro* on a feeder layer of cells.

thymocytes, it was demonstrated that the epigenotype of the somatic cells could be reset to a configuration that supported a pluripotent state [33]. This dominant reprogramming activity exhibited by ESCs can in part be attributable to the raised expression of master regulatory transcription factors of pluripotency such as Oct3/4, Nanog and Sox2 [34]. The work of Takahashi and Yamanaka took this idea further and identified the combination of Oct3, Sox2, Klf4 and c-Myc from a pool of 24 predominantly ES-cell specific genes. In 2007, they generated the first human iPSCs by retrovirally expressing this combination in dermal fibroblasts [35,36]. These are considered to be ESC-like pluripotent cells. When subjected to the teratoma assay, human iPSCs can also contribute to the formation of tissue from all three germ layers.

The landmark discovery of iPSCs had fuelled enthusiasm to fervent levels and since then, research in the stem cell therapy field has grown almost exponentially. There were, and still are, many reasons to feel excitement. Initially at least, iPSCs seemed identical to ESCs but its establishment did not necessitate the destruction of an embryo (figure 3). This would permit ongoing research to continue without any lingering ethical constraints. From an epigenetic standpoint, it seemed incredible that the overexpression of a simple set of genes can induce a whole genome-wide wave of epigenetic modifications that one can assume must take place to restore a differentiated somatic cell to a pluripotent state. The initial two reports of iPSC generation used integrating vector systems to reprogramme fibroblasts. Since then, induced pluripotency has also been achieved using non-integrational adenoviruses [37], and even DNA-free Sendai viruses [38]. Different somatic targets were also selected for reprogramming, including haematopoietic cells, hepatic progenitor cells [39] and adipocytes, which all led to the generation of viable iPSCs displaying the same set of characteristics as do ESCs. So the framework for a new therapeutic strategy was set. Stem cell therapy can become more personalized [40]. This is because patient autologous iPSCs can be derived from somatic cells isolated directly from the patient. In theory at least, differentiated cells could be transplanted without encountering issues relating to immune rejection. Those with an inherited genetic disorder could have their autologous iPSCs genetically modified and differentiated into functional cells to then repair damaged tissues, for example, in the treatment of primary immunodeficiencies (PIDs) [41]. Such cell-replacement-type therapies are not the only applications of iPSCs. They can also be used to recapitulate a disease phenotype for the purposes of disease modelling [42]. This has profound promise in applications such as *in vitro* drug screening and the elucidation of disease pathophysiological development. Unfortunately, the therapeutic promise of iPSCs is not reflected in terms of actual numbers of ongoing clinical trials. There are some reservations, given that similar to ESCs, iPSCs retain the ability for teratoma formation *in vivo*. To