本研究室では、希少疾患の有病率をより正確に把握するための方法論、データ管理方法論について担当する。

B. 研究方法

1. 国内外における希少疾患対策などの現状を把握する。

2. 調査を行うにあたり、個人情報及び診療情報の保護に関するポリシーを作成・徹底を図る。

3. 希少疾患であるという当該疾患領域の特殊性を考慮し、前向きコホート研究などの疫学的データの蓄積や臨床研究を実施する際の方法論に関する研究を行い、システムの整備を含めたデータ管理の標準化、効率化を目指す。

(倫理面への配慮)

本研究は世代間に伝わる遺伝子変異を解析する研究であり、ヒトゲノム・遺伝子解析研究に関する倫理指針、疫学研究に関する倫理指針、臨床研究に関する倫理指針を遵守するとともに、事前に研究代表者施設の倫理審査委員会の承認を得て行う。尚、本研究で用いる臨床検体は、患者の臨床的診断のために採取された検体の残検体や非侵襲的に得られる体細胞(口腔粘膜スメアなど)を用いるため、健康上の危険性はないが、本研究の為の解析に先立ち、書面にてインフォームド・コンセントを得て行う。尚、試料(臨床情報及び検体)の取扱いについては、参加施設の規定に従い、匿名化を図り行う。以上の手続きをとることにより、研究の対象となる患者の倫理性が確保できる。

C. 研究結果

1. 国内外における希少疾患対策の現状把握

国内外の希少疾患への対策に関する現状を把握する為に文献的レビューを実施した。米国 Natiohal Institute of Health では、有病数が 20 万人未満の有効な治療法が無い疾患が希少疾患と定義しているのに対し、日本では薬事法第 77 条の 2 での希少疾病用医薬品等として厚生労働大臣の指定を受ける要件の 1 つに記載されている「国内対象患者数 5 万人未満」が希少疾患の定義となっている。米国においては、Rare Diseases Act of 2002(希少疾患対策法)に応答する形で、Rare Diseases Clinical Research Network (RDCRN 希少疾患臨床研究ネットワーク)が組織され、米国内の臨床研究関連施設によるコンソーシアム研究として、希少疾患に特化した研究の推進と患者への情報提供などを行っている。現在 10 の研究コンソーシアと central Data and Technology Coordinating Center (DTCC, 中央データ管理部門)が存在し、20 件以上の研究を米国 50 箇所以上で行っている。いずれも希少かつ難治性疾患の把握と情報の相互リンク、情報共有を図り、研究の推進に役立てることを目的としていることを確認した。

2. 作成した個人情報保護ポリシーの実務運用と遵守徹底

患者の個人情報及び診療情報などの漏洩、混交、紛失、盗用などを防ぐ為のセキュリティ確保の方針、患者情報の不正流出の防止策、不要となった情報の取り扱い、開示の方法、研究に関わる側の情報の取り扱いなどについて具体的に方針を定めた「個人情報保護方針」に基づく活動を行った。

3. 疫学的情報の蓄積、及び継続的観察を行う為の方法論の研究とシステムの構築

FPD は、診断基準が定まっていないことも有り、疾患の有病数の正確な把握が難しい。このような稀少疾患に関する詳細な情報を収集するには、効率的な手法が必要不可欠である。当研究室では小児希少疾患のデータ管理の実務・研究の経験をもとに、希少疾患に対する疫学研究と臨床試験を融合させた仕組みを構築し、臨床試験不参加例の分析や対象患者の長期的観察を実現するための枠組みを電子的データ収集システム(EDC)開発と共に構築してきた。このような活動を通して、今期はシステム導入・利用率向上に伴う、データ管理業務効率化に関する検討を行った。

データセンターに勤務するデータマネージャー(DM)9 名に対し、1 週間の業務内容と各業務の所要時間を、EDC 導入前後で 2 回ずつ測定し、それぞれの平均値を比較した(EDC 導入前の調査時期:2010/8, 2011/10、EDC 導入後の調査時期:2012/12, 2013/1)。

データ管理を担当する研究数、登録実績とデータ管理業務時間・内訳を EDC 導入前後で比較検討した。

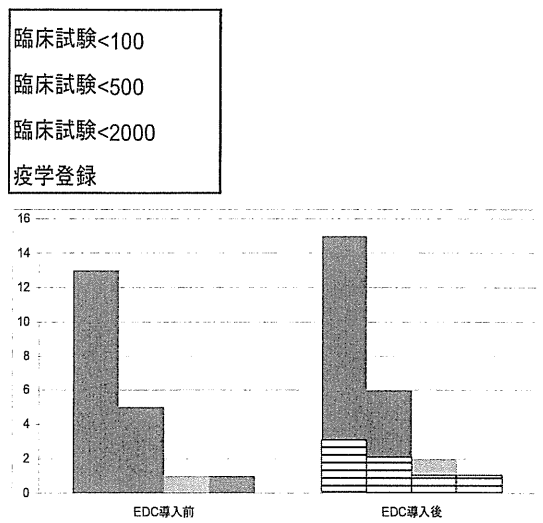
表 1:EDC 導入前後のデータ管理実務

EDC 導入 (2011.11) 以前
ローカル DB を、担当データマネージャー(DM)がシステムエンジニアと協議し、ロジックを含めて作成
紙の症例報告書(CRF)を用いてデータ収集
目視チェック/問合せ
ローカルのデータベース(DB)に入力
ロジックエラーデータの再問合せ, CRF 修正, DB 修正
紙 CRF との整合性確認
EDC 導入 (2011.11) 以後
DM による eCRF 作成、eCRF 完成と DB スキーマ完成は同時
電子的データ収集(論理チェック項目・内容は DM が設定可能)
データ内容の確認, 問合せと修正(変更履歴は全て EDC に保存)

(1) 本調査対象業務時間は EDC 導入前 362 時間、EDC 導入後 327 時間と EDC 導入後に若干減少しているものの統計学的有意差は認めなかった。

(2) 調査対象期間中の管理研究数, 登録実績・小規模(<100)臨床試験数、及び大規模臨床試験数(<2000 例)共に EDC 導入後に増加している(図 1)。

図 1. EDC 導入前後の管理研究数



(3) EDC 導入前後のデータ管理業務の比較結果を図 2 に、臨床研究開始前業務・後業務別の検討結果を図 3,4 に示す。

・臨床研究開始前業務は、 EDC 導入前に比し EDC 導入後で 25 時間の短縮を認めた(前:100 時間(全業務の 25%) vs. 後:75 時間(22%))。EDC 導入後の調査時期(2 ポイント)での「試験開始キックオフ準備」が業務短縮の約 6 割を占めていた。反対に、「プロトコール・同意説明文書等の作成支援」や、「CRF 作成支援」などに要する時間は EDC 導入前後で変わらなかった。「DB 作成業務」は、従来のローカル DB 作成時間は減少していた一方で、EDC 機能追加に伴う業務時間

の増大を認めた。

臨床試験開始後業務は、EDC 導入前に比し EDC 導入後で 75 時間の短縮を認めた(前: 253 時間(64%) vs. 後:178 時間(54%))。このうち約 8 割は「CRF 収集・問合せ・修正・入力関連業務」であり、EDC 導入による効率化が確認できた。また僅かながら、質管理業務(7.5 時間増)や、教育研究関連業務(14 時間増)の為の時間について、EDC 導入後に増加している傾向が確認できた。

図 2. EDC 導入前後の業務内訳

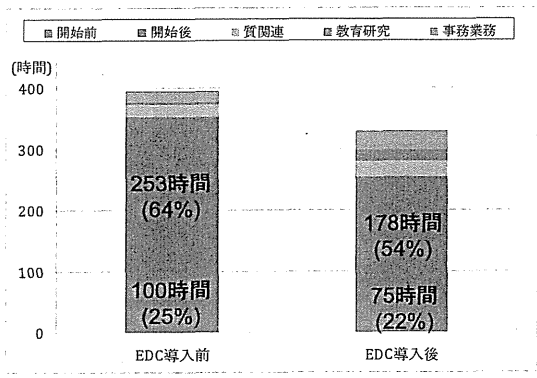


図 3. 試験開始前業務内訳

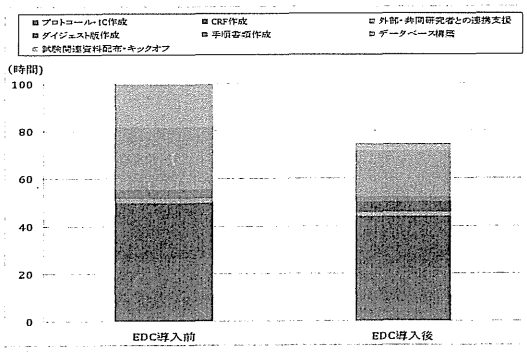
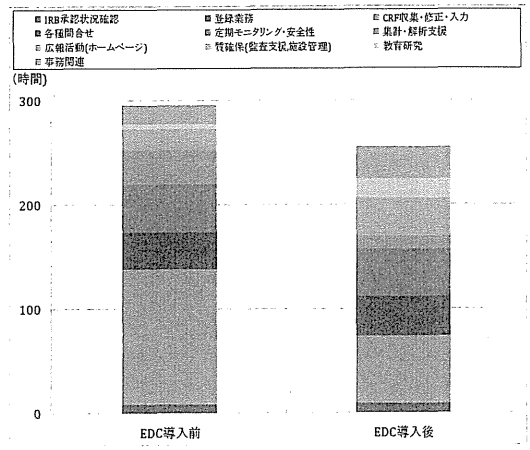


図 4. 試験開始後業務内訳



D. 考察

FPD は、血小板減少と出血傾向を伴い、患者の生活の質を著しく低下させるだけでなく、高率に白血病発症をきたすという生命予後にも重大な影響を与える難治性疾患の 1 つである。その希少性と疾病の特性に関する認識が不十分であったことから、その有病数はこれまで過小評価されてきた可能性がある。診断率の向上は言うまでもなく、将来的には治療法の開発も目指した病態研究や治療開発研究は、患者の福祉向上に必要不可欠であるものの、希少性という市場の小ささが故に、開発が遅れて来た領域であり、医師が主導となって行う研究として成立させる必要がある疾患群である。国内には、日本臨床腫瘍研究グループ(Japan Clinical Oncology Group; JCOG)のような歴史ある臨床研究グループを支えるデータセンターが存在するが JCOG データセンターが統括する臨床研究データと本研究が取り扱うデータとの決定的な違いは、“希少疾患領域であること”、“固形がんのような局所治療が主となることはなく、常に全身性疾患としての長期的な全身治療が必要になる”ことなどである。治療内容とその副作用の追跡は、必然的に複雑化・煩雑化する。一方、参加施設側の臨床研究支援体制は未だ十分とはいえず、医師が多忙な臨床業務の

傍ら、複雑な研究を行っている実情がある。従って、患者情報や予後情報などの追跡調査結果の収集は、なるべく簡便で、標準化されていることが望ましい。当研究室が開発に関わったオンライン登録システム導入前後の、データ管理業務の効率化に関する検討結果から、開発が進み難い、希少・難治疾患領域における臨床研究を効率的に進めるための策として、疫学研究と臨床研究を融合させた枠組みでのEDC導入が有効である可能性が示された。質を確保しつつ、大幅なコスト削減に繋がる可能性がある。

E．結論

希少難治性疾患としてのFPDの実態把握、及び疫学的知見を収集/追跡する為の方法論の研究により、効率的な情報収集と、質の高いデータの蓄積・情報発信を行えるよう、今後も引き続き基盤整備に努める。

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G. 知的財産権の出願・登録状況（予定を含む。）

1. 特許取得
該当なし

2. 実用新案登録
該当なし

3.その他
該当なし

III. 研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
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IV. 研究成果の刊行物・別刷

(主なもの)

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

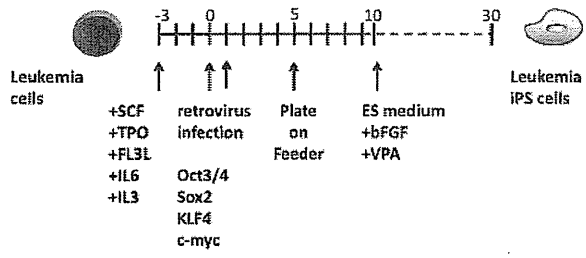

Figure 1. Experimental scheme for generating of iPSCs from the CML patient sample. After cytokine stimulation, $CD34^+$ CML cells were reprogrammed by transduction with Yamanaka factors. To improve the reprogramming, valproic acid was added to the culture.

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)

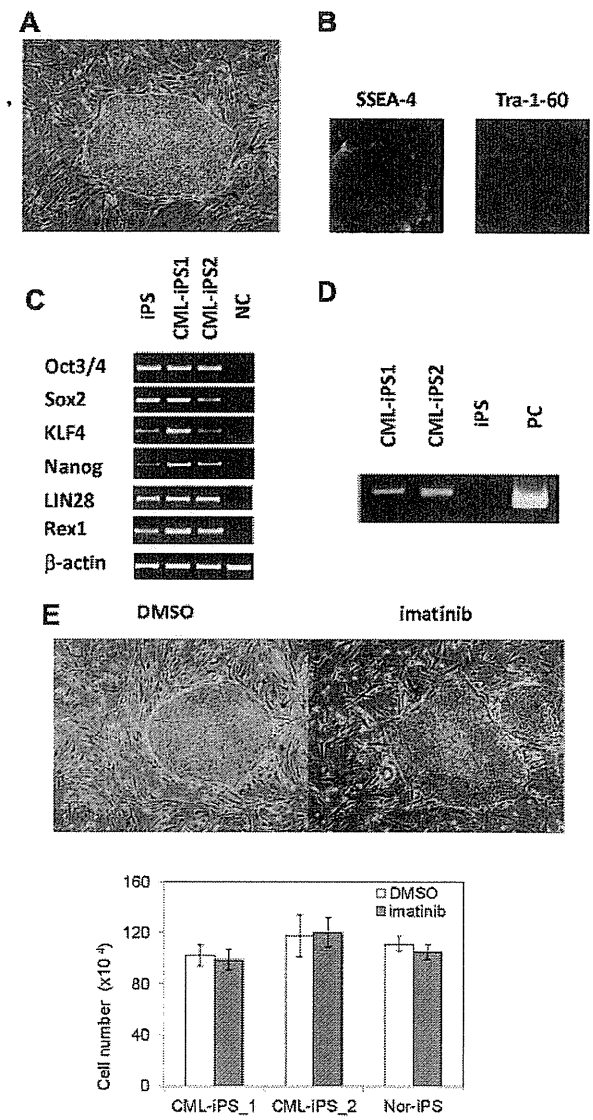

Figure 2. Generation of CML derived iPSCs. (A) Morphology of CML-iPSCs. (B) Immunofluirescence staining shows expression of pluripotent marker (left: SSEA-4 and right: Tra-1-60) in CML-iPSCs. (C) RT-PCR analysis of ES cell marker genes. Endogenous expression of these stem cell–specific genes in CML-iPSCs 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.

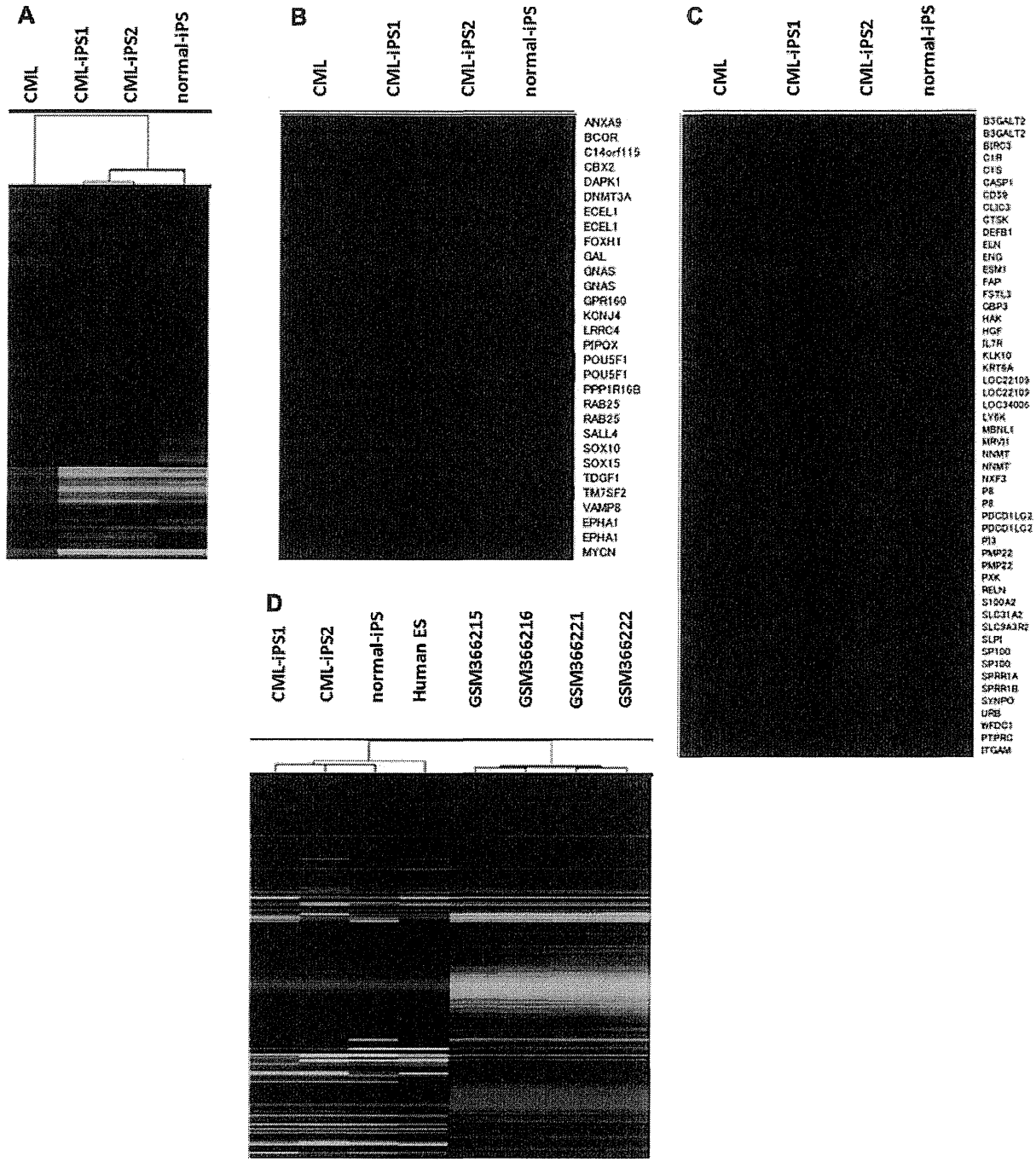

Figure 3. Comprehensive analysis of DNA methylation and gene expression. (A) Unsupervised hierarchical clustering based on differentially methylated CpGs is shown on the dendrogram. The accompanying heatmap shows the methylation status across 5001 differentially methylated CpGs. In the heatmap, red indicates 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

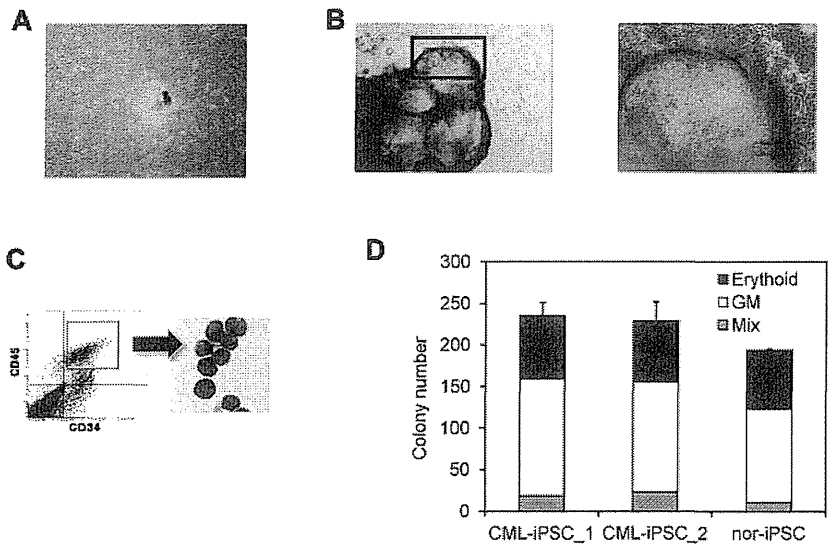

Figure 4. Hematopoietic differentiation of CML-iPSCs. CML-iPSCs were differentiated on the 10T1/2 cells. On day 7 (A), iPSCs began to mount. On day 14 of culture (B: left panel), inflated sac-like structures appeared. These sac-like structures contained the round hematopoietic cells (B: right panel: 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