Discontinuation of imatinib in Japanese patients with chronic myeloid leukemia

Naoto Takahashi,[1] Taiichi Kyo,[2] Yasuhiro Maeda,[3] Takashi Sugihara,[4] Kensuke Usuki,[5] Tatsuya Kawaguchi,[6] Noriko Usui,[7] Shinichiro Okamoto,[8] Yokiko Ohe,[9] Shigeki Ohtake,[10] Kunio Kitamura,[11] Masahide Yamamoto,[12] Hirofumi Teshima,[13] Toshiko Motoji,[14] Toshiharu Tamaki,[15] Kenichi Sawada,[1] and Kazuma Ohyashiki[16]

[1]Department of Hematology, Nephrology, and Rheumatology, Akita University; [2]IV Department of Internal Medicine, Hiroshima Red Cross Hospital and Atomic Bomb Survivors Hospital, Hiroshima City; [3]Division of Hematology, National Hospital Organization Osaka Minami Medical Center, Kawachinagano; [4]Division of Hematology, Department of Medicine, Kawasaki Medical School, Kurashiki City; [5]Division of Hematology, NTT Medical Center Tokyo, Tokyo; [6]Departments of Hematology and Infectious Diseases, Kumamoto University Hospital, Kumamoto City; [7]Division of Clinical Oncology and Hematology, Department of Internal Medicine, Jikei University School of Medicine; [8]Division of Hematology, Department of Internal Medicine, Keio University School of Medicine, Tokyo; [9]Division of Internal Medicine, Uegahara Hospital, Nishinomiya City; [10]Department of Hematology, Kanazawa University Graduate School of Medical Science, Kanazawa City; [11]Division of Hematology, Ichinomiya Municipal Hospital, Ichinomiya City; [12]Department of Hematology, Tokyo Medical and Dental University, Tokyo; [13]Division of Hematology, Osaka City General Hospital, Osaka; [14]Department of Hematology, Tokyo Women's Medical University, Tokyo; [15]Department of Hematology, Rinku General Medical Center, Osaka; and [16]I Department of Internal Medicine, Tokyo Medical University, Japan

ABSTRACT

It was recently recognized that some chronic myeloid leukemia patients with a complete molecular response could sustain that response after discontinuation of imatinib. To characterize the clinical outcomes and profiles of chronic phase chronic myeloid leukemia patients who could discontinue imatinib, we conducted a nationwide survey in Japan. Among 3,242 imatinib-treated chronic myeloid leukemia patients, we identified 50 who had discontinued imatinib for at least six months; of these we analyzed 43. Molecular recurrence was detected in 19 patients, and a complete molecular response rate was estimated to be 47% following imatinib discontinuation. Based on multivariate regression analysis, imatinib dose intensity and prior interferon-α administration were independently predictive of molecular recurrence within 12 months. The depth of the molecular response should be a factor influencing long-term sustained complete molecular response after discontinuation of imatinib. Additionally, an immunological mechanism modified by interferon-α might control chronic myeloid leukemia stem cells.

Key words: chronic myeloid leukemia, molecular recurrence, imatinib, discontinuation.

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Introduction

Imatinib treatment dramatically improves survival in chronic myeloid leukemia (CML) patients and has made imatinib the standard-of-care for chronic phase CML. But whether the effects of imatinib can be considered a cure remains controversial. This may be because primitive, quiescent, Philadelphia-positive stem cells from patients with CML are insensitive to imatinib *in vitro*,[1] and residual BCR/ABL$^+$ hematopoietic progenitors are present in patients who achieve a complete cytogenetic response (CCyR) with imatinib.[2] Recently, it was recognized that some patients with a complete molecular response (CMR) are able to sustain this response after discontinuation of imatinib.[3] In a non-randomized prospective study, Mahon *et al.* reported that among patients with a CMR lasting at least two years, the CMR was sustained in 41% after discontinuation of imatinib. However, this strategy requires further validation and much longer follow up. At present, there appear to be no patients or disease characteristics that identify in advance those who can safely discontinue imatinib. Consequently, a cure has not yet been proven and life-long therapy with imatinib is still the consensus recommendation.[4] Discontinuing imatinib in CML should only be considered in a clinical trial with strict molecular monitoring.[3,5]

Nevertheless, the literature contains several case reports of CML patients in whom imatinib had to be discontinued for various reasons.[6-13] Some of these patients had molecular relapsed but others did not. This raises the question as to how deep does the molecular response to imatinib treatment need to be and how long must imatinib treatment be continued after achieving CMR before the drug can be safely discontinued. There has been no regional retrospective survey of the clinical outcomes of CML patients after discontinuation of imatinib.

To characterize the clinical outcomes and profiles of CML patients who have been off imatinib therapy for at least six months, we conducted a nationwide survey of CML patients in Japan.

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Correspondence: Naoto Takahashi, Department of Hematology, Nephrology, and Rheumatology, Akita University, 1-1-1 Hondo, Akita City, Akita, 010-8543, Japan. E-mail: naotot@doc.med.akita-u.ac.jp

Design and Methods

Data collection

We first sent questionnaires to 780 hematologists in the Japanese Society of Hematology requesting information on the number of CML patients treated with imatinib between 2001 and 2010, and the number of patients who had been off therapy for at least six months due to any cause, except disease progression, transplantation or death. As dictated by the inclusion criteria, patients who relapsed and restarted imatinib within six months of its discontinuation were not included in the number of patients who had been off therapy. From among those contacted, 181 hematologists (23%) responded to the first questionnaire yielding a total of 3,242 CML patients who had been treated with imatinib. This is about one-third of Japanese CML patients. Among them, 50 patients (1.5%) were identified who discontinued imatinib therapy for at least six months. We then sent second questionnaires to the hematologists who treated those 50 patients, asking for information on the clinical features, treatments and clinical outcome in each patient. Forty-three CML patients were analyzed in this retrospective study. The other 7 patients among the 50 identified in the first questionnaire were not included because the hematologist either did not respond to the second questionnaire, the response was incomplete, or CMR was not confirmed at the time of imatinib cessation. All patients gave written informed consent in accordance with the Declaration of Helsinki, and this study was approved by the Akita University Research Ethics Board and Tokyo Medical University Research Ethics Board.

Molecular response

In general practice, the molecular response was assessed at least every three months. A major molecular response (MMR) was defined as a 3-log reduction in the BCR-ABL transcript (international scale <0.1%), and a complete molecular response (CMR) was defined as detection of no BCR-ABL transcript in a real-time quantitative-polymerase chain reaction (RQ-PCR) assay (n=24), nested reverse transcriptase-polymerase chain reaction (RT-PCR) assay (n=14), or a highly sensitive transcription-mediated amplification (TMA) method[14] (n=5). These PCR methods can detect at least a 4-log reduction in the BCR-ABL transcript (international scale <0.01%).

Statistical analysis

Statistical analyses were carried out using SPSS statistical software (SPSS Japan Inc., Tokyo, Japan, version 17.0). Non-parametric values or numbers were compared between two groups using the Mann-Whitney test, the χ^2 test or Fisher's exact test. Time to molecular relapse was measured from the date of imatinib discontinuation to the date of molecular recurrence, or the date of last molecular examination for patients who did not relapse. Relapse-free survival was estimated using the Kaplan-Meier method. Survival rate was compared between two groups using the log rank test. Stepwise forward selection multiple logistic analysis for molecular recurrence by 12 months was performed to determine the effect of the variables examined in a univariate analysis. $P<0.05$ was considered significant.

Results and Discussion

This is the first survey of outcomes after imatinib discontinuation in Japan. The survey is estimated to cover about one-third of Japanese CML patients. One question we asked was how many patients are able to sustain CMR after imatinib discontinuation. Only 50 patients (1.5%) who had discontinued imatinib therapy for at least six months were identified, and 43 patients were analyzed in this retrospective study. The median age at diagnosis of CML was 57 years (range 18-80). The male:female ratio was 19:24. All patients were in chronic phase, with no history of progression to the accelerated phase or blast crisis (AP/BC), and all had achieved CMR before imatinib discontinuation. Based on their Sokal scores, 25 patients were classified as low risk, 15 patients as intermediate risk, and 3 patients as high risk. The reasons for which imatinib was discontinued were adverse events (n=18), patient's request due to cost (n=14), patient's desire to become pregnant (n=3), and long undetectable residual disease (n=8). In this study, the male:female ratio was the inversion of that commonly observed. This might indicate that women are more likely than men to want to stop imatinib because of some adverse event (e.g. facial edema, skin rash), and could also reflect their desire to become pregnant.

Seventy-two percent of patients were treated with the standard or a higher dose of imatinib (<400 mg, n=12; 400 mg, n=22; >400 mg, n=9; median daily dose 400 mg; range 100-700 mg). The median duration of imatinib treatment was 45.2 months (range 4.5-92.7 months) and the estimated median total dose of imatinib was calculated to be 541 g (range 53.6-1,112.8 g). Twenty-five patients (58%) had prior IFN-α treatment, and 12 (28%) received IFN-α in combination with imatinib. Among the patients who received combination therapy, 8 had also received prior IFN-α treatment. However, no patients received maintenance therapy with IFN-α after cessation of imatinib. The median duration of imatinib treatment needed to achieve CMR was 12.6 months (range 2.0-83.3 months). The median duration of CMR before cessation was 27.4 months (range 0.9-79.6 months). The median period of cessation was 22.4 months (range 6.2-97.9 months).

Molecular recurrence was detected in 19 patients (44%); it was not associated with cytogenetic relapse in any patient. Among them, TKI treatment was restarted in 17 patients who all then recovered to CMR (13 patients) or MMR (4 patients). The remaining 2 patients had shown sustained MMR (<0.1%) for 98 months or near MMR (0.175%) for 24 months, respectively, with no therapy. No progression to AP/BC was seen after restarting TKI treatment, and all patients are still alive. The relapse-free survival (RFS) rate at five years was estimated to be 47% while median RFS was determined to be 41 months using the Kaplan-Meier method. Twenty-four patients among the 43 (56%) analyzed also showed sustained CMR without a molecular recurrence after discontinuation. Although the sample size was small, it seems clear that imatinib treatment could be stopped in some patients.

We also asked, what is the profile of patients who can sustain CMR without a molecular recurrence after discontinuation of imatinib, and how long after achieving CMR should imatinib treatment be continued before attempting cessation? Comparison of patients who did and did not show molecular recurrence within 12 months after stopping imatinib therapy revealed several significant differences between them (Table 1). First, the median duration of imatinib therapy before cessation was 51.7 months in patients without molecular recurrence, which is significantly (P=0.0228) longer than the 26.3 months of

therapy received by patients with molecular recurrence. Second, the estimated dose of imatinib before cessation was 576.8 g in patients without molecular recurrence, which is significantly (P=0.0042) greater than the 275.4 g in patients with molecular recurrence. Third, IFN-α was administered prior to cessation significantly (P=0.0102) more frequently in patients without molecular recurrence within 12 months. On the other hand, no significant differences were found with respect to age, sex, Sokal risk, imatinib daily dose, combination with IFN-α, or time to CMR. In the STIM study, the probability of a sustained CMR at 12 months differed between Sokal risk groups (P=0.008).[3] In our study, a molecular recurrence by 12 months occurred in only one of 3 patients in the high risk group, 7 of 13 patients in the intermediate risk group, and 6 of 24 patients in the low risk group. Because of the sample size in this study, the effect of the Sokal score was not significant (P=0.2135).

Importantly, the median duration of CMR before cessation was 32.5 months in patients without molecular recurrence, which was significantly (P=0.0025) longer than the 6.0 months in patients with molecular recurrence. Furthermore, there was a significant difference in the estimated RFS rates following discontinuation between patients in whom CMR was sustained for more than 24 months prior to imatinib discontinuation and those sustaining a CMR for less than 24 months (78% *vs.* 15%, P=0.0002 by log rank test, Figure 1). The duration of CMR was one of the eligibility criteria for the STIM trial.[3] Because patients who relapsed and restarted imatinib within six months after its discontinuation were not included in this study, as dictated by the inclusion criteria, we are able to show that the estimated RFS rates at five years were much higher (78%) among patients who sustained a CMR for more than 24 months before cessation of therapy in this series than in the STIM study. However, in a multivariate regression analysis, only imatinib dose intensity and prior IFN-α administration were independently predictive of a molecular recurrence within 12 months (Table 2). The identified prediction formula was: $Y = -0.0061 \times$ dose intensity of imatinib (g) $-3.17171 \times$ prior IFN-α (Yes=1/No=0) + 4.0124. If $1/(1+\exp(-1 \times Y))$ was more than 0.5, a molecular recurrence was predicted; the total accuracy rate of this formula was 82.5%.

In our series, although 56% of patients showed a sustained CMR after discontinuation of imatinib, some molecular recurrences occurred much later after cessation. The longest period between cessation and recurrence was 42 months, which suggests that there was a residual CML stem cell that was induced to begin cycling.

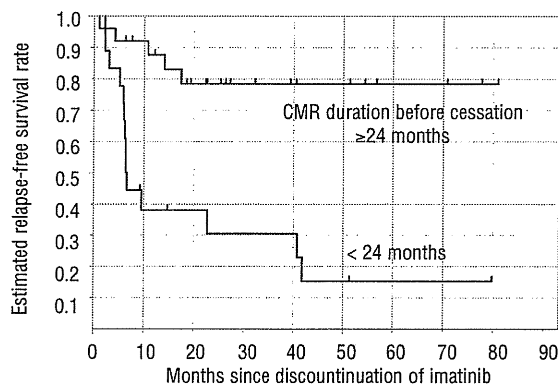

Figure 1. Relapse-free survival (RFS) plotted against CMR duration. The estimated RFS rates at five years were 78% and 15% among patients who did and did not sustain a CMR for more than 24 months before cessation of therapy (P=0.0002, log rank test).

Table 2. Multivariate regression analysis: factors predictive of a molecular recurrence within 12 months.

	Estimate	SE	P	OR (95% CI)
Imatinib dose intensity (g)	-0.0061	0.0021	0.0035	0.9940 (0.9899-0.9980)
Prior IFN-α (Yes=1/No=0)	-3.1717	1.1551	0.0060	0.0419 (0.0044-0.4035)
Intercept	4.0124	1.4810	0.0067	

SE: standard error; OR: odds ratio; CI: confidence interval.

Table 1. Patients' characteristics as related to molecular recurrence within 12 months after discontinuation of imatinib therapy.

Molecular recurrence by 12 months	Yes (14)	No (26)*	P value
Age (y.o)	61.0 (56.3-65.4)	46.6 (34.5-67.6)	0.1164
Sex (male/female)	7/7	11/15	0.6409
Sokal risk (low/intermediate/high)	6/7/1	18/6/2	0.2135
Imatinib daily dose (mg)	400 (300-400)	400 (300-500)	0.2543
≥400/<400 (yes/no)	9/5	19/7	0.4089
Duration of imatinib therapy (months)	26.3 (13.3-63.9)	51.7 (31.0-70.6)	0.0228
Imatinib dose intensity (g)	275.4 (159.2-541.5)	576.8 (364.0-846.8)	0.0042
Prior IFN-α (yes/no)	5/9	20/6	0.0102
Combination IFN-α (Yes/no)	2/12	10/16	0.1076
Time to achieve CMR (months)	11.5 (6.4-17.3)	13.1 (7.4-33.8)	0.4852
CMR duration before cessation (months)	6.0 (2.0-19.3)	32.5 (24.6-39.7)	0.0025
PCR method (RQ-PCR/nested PCR/TMA)	6/7/1	17/5/4	0.1234

** Three patients without a molecular recurrence were excluded from this group because the duration of cessation was less than 12 months. Data are medians (quartile 1- quartile 3) or number. Non-parametric values were compared between two groups using the Mann-Whitney test. CMR: complete molecular response.*

The depth of the molecular response should be one of the factors influencing long-term sustained CMR, but other factors, for example an immunological mechanism that could be modified by IFN-α, might eradicate CML stem cells in a quiescent state. Interestingly, 2 patients in the present study experienced molecular recurrence after sustaining MMR or near MMR for an extended period with no therapy. This finding is consistent with the notion that other factors might control CML stem cells. As suggested by Hochhaus *et al.*, induction of a proteinase-3-specific cytotoxic T-cell response by IFN-α may contribute to sustained remissions.[15]

Although it is still a small subset of CML patients, our data also suggest that imatinib could achieve a clinical 'cure'.

Authorship and Disclosures

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RUNX1/AML1 mutant collaborates with BMI1 overexpression in the development of human and murine myelodysplastic syndromes

Running title: RUNX1 MUTANT AND BMI1 COLLABORATE TO PROMOTE MDS

Yuka Harada[1], Daichi Inoue[2], Ye Ding[3], Jun Imagawa[3], Noriko Doki[2], Hirotaka Matsui[4], Takashi Yahata[5], Hiromichi Matsushita[5], Kiyoshi Ando[5], Goro Sashida[6], Atsushi Iwama[6], Toshio Kitamura[2], and Hironori Harada[3]

[1]Division of Radiation Information Registry, Research Institute for Radiation Biology and Medicine, Hiroshima University, Hiroshima, Japan.

[2]Division of Cellular Therapy, Advanced Clinical Research Center, Institute of Medical Science, University of Tokyo, Tokyo, Japan.

[3]Department of Hematology and Oncology, Research Institute for Radiation Biology and Medicine, Hiroshima University, Hiroshima, Japan.

[4]Department of Molecular Oncology, Research Institute for Radiation Biology and Medicine, Hiroshima University, Hiroshima, Japan.

[5]Division of Hematopoiesis, Research Center for Regenerative Medicine, Tokai University School of Medicine, Isehara, Japan.

[6]Department of Cellular and Molecular Medicine, Graduate School of Medicine, Chiba University, Chiba, Japan.

Corresponding author: Hironori Harada, Department of Hematology and Oncology, Research Institute for Radiation Biology and Medicine, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8553, JAPAN. Phone: +81-82-257-5861; Fax: +81-82-256-7108; E-mail: herf1@hiroshima-u.ac.jp.

Y.H., D.I. and Y.D. contributed equally to this work.

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Key Point

- BMI1 overexpression is one of the second hit partner genes of RUNX1 mutations that contribute to the development of MDS.

Abstract

RUNX1/AML1 mutations have been identified in myelodysplastic syndromes (MDS). In a mouse bone marrow transplantation model, a RUNX1 mutant, D171N, was shown to collaborate with Evi1 in the development of MDS, however, this is rare in humans. Using enforced expression in human CD34$^+$ cells, we showed that the D171N mutant, the most frequent target of mutation in *RUNX1* gene, had an increased self-renewal capacity, blocked differentiation, dysplasia in all 3 lineages, and tendency for immaturity, but no proliferation ability. *BMI1* overexpression was observed in CD34$^+$ cells from the majority of MDS patients with RUNX1 mutations, but not in D171N-transduced human CD34$^+$ cells. Co-transduction of D171N and BMI1 demonstrated that BMI1 overexpression conferred proliferation ability to D171N-transduced cells in both human CD34$^+$ cells and mouse bone marrow transplantation model. Stepwise transduction of D171N followed by BMI1 in human CD34$^+$ cells resulted in long-term proliferation with a retained CD34$^+$ cell fraction, which is quite similar to the phenotype in patients with higher-risk MDS. Our results indicate that *BMI1* overexpression is one of the second hit partner genes of *RUNX1* mutations that contribute to the development of MDS.

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Introduction

RUNX1/AML1 gene has been investigated in the pathogenesis of hematopoietic diseases, and point mutations of *RUNX1* have been frequently detected in patients with various types of myeloid neoplasms. A heterozygous germline mutation of the *RUNX1* gene is known to cause familial platelet disorder with a predisposition to acute myeloid leukemia (FPD/AML),[1,2] which is regarded as familial myelodysplastic syndromes (MDS).[3] *RUNX1* mutations have been detected with high frequency in MDS, MDS following AML,[4] minimally differentiated AML M0 subtypes,[2,5-7] de novo AML without recurrent or complex karyotype,[8,9] and myelodysplastic/myeloproliferative neoplasms (MDS/MPN).[10,11] Furthermore, *RUNX1* mutations are detected with high frequency in therapy-related or radiation-associated MDS and AML,[4,12-15] and leukemic transformation from MPN.[16-19]

It is intriguing how RUNX1 mutations contribute to the development of divergent hematological neoplasms. Functionally, most of the RUNX1 mutants equally show a loss of normal RUNX1 *trans*-activation potential.[4,12 20,21]The amino acid residues in the runt homology domain (RHD) of the RUNX1 protein that directly interact with DNA have been found to be frequent targets of amino acid replacement.[20,21] Mutations on other amino acids close to these DNA-contact residues are also suspected to inhibit DNA binding by an obstructive side-chain or a structural change. Therefore, amino-replacement and in-frame insertion/deletion types of mutations confer loss of DNA-binding ability and *trans*-activation potential. *RUNX1* mutations have been shown to play a pivotal role in the pathogenesis of MDS/AML in mouse bone marrow transplantation (BMT) systems.[22] Mice transduced with the D171N mutant, which harbors a mutation in the RHD of the *RUNX1* gene, exhibited hyper-proliferative AML with multilineage dysplasia in collaboration with *Evi1* overexpression. This impressive result indicates that *RUNX1* mutations may be a cause of MDS with a leukemogenic potential. However, mouse phenotypes do not always correspond to the clinical features of patients with the mutations. This may be partly due to differential gene circumstances, such as retrovirus integration sites or collaborating gene alterations, between mouse and human.[23,24] As opposed to *EVI1*, overexpression of the polycomb group gene *BMI1* is more common in MDS patients and is associated with MDS progression.[25,26] Biological analysis using human hematopoietic cells is considered to be necessary to clarify the molecular mechanisms of the *RUNX1* mutations in the pathogenesis of MDS. Enforced gene expression in human CD34$^+$ cells has been utilized to investigate the role of leukemogenic oncogenes in leukemogenesis.[27-35]

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In this study, using enforced gene expression in human CD34$^+$ cells, we demonstrated that the D171N mutant, the most frequent target of mutation in *RUNX1* gene, has an increased self-renewal capacity, blocked differentiation, dysplasia in all 3 lineages, and tendency for immaturity, but no proliferation ability. Moreover, we revealed that *BMI1* overexpression collaborates with *RUNX1* mutations and confers proliferation ability to D171N-mutated cells, which was confirmed in both human and mouse hematopoietic stem/progenitor cells. Our results indicate that *BMI1* overexpression is one of the second hit partner genes of *RUNX1* mutations in the development of MDS.

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Materials and Methods

Patients

Patients with MDS were divided into 2 groups using the International Prognostic Scoring System (IPSS): lower-risk MDS includes low- or intermediate-1-risk MDS, and higher-risk MDS includes intermediate-2- or high-risk MDS. Mutation analysis of *RUNX1* was performed as described previously.[4] The study was approved by the institutional review board at Hiroshima University. Patients gave written informed consent for the study, according to the Declaration of Helsinki.

qRT-PCR

$CD34^+$ cells were purified from patients using the CD34 MicroBead Kit and autoMACS system (Milteny Biotec, Bergisch Gladbach, Germany). Total RNA was harvested from the $CD34^+$ cells using the RNeasy Micro Kit (Qiagen, Hilden, Germany). The expression levels of *EVI1*, *BMI1*, *INK4A, ARF* and *GAPDH*, were quantified by the Applied Biosystems 7500 Real-Time PCR system using TaqMan Gene Expression Assays (Hs00602795_m1 for *EVI1*, Hs00180411_m1 for *BMI1*, Hs00923894_m1 for *INK4A*, Hs99999189_m1 for *ARF* and Hs99999905_m1 for *GAPDH*) and TaqMan Universal PCR Master Mix (Applied Biosystems). qRT-PCR in mouse cells was performed as described previously[22] with the following forward/reverse primer pairs: p16 (aatctccgcgaggaaagc/gtctgcagcggactccat), p19 (gggttttcttggtgaagttcg/ttgcccatcatcatcacct), Evi1 (atcggaagatcttagatgagttttg/cttcctacatctggttgactgg) and Gapdh (gcattgtggaagggctcatg/ttgctgttgaagtcgcaggag).

Retroviral vectors and infection

RUNX1 (wild-type (WT) and D171N mutant) cDNA with FLAG tag was subcloned into the pMXs-IRES-EGFP (pMXs.IG). *BMI1* was subcloned into the pMXs-IRES-DsRed-Express (pMXs.IR), in which the IRES-DsRed-Express fragment from the pIRES2-DsRed-Express (Clontech, Mountain View, CA) was inserted into the pMXs. FLAG-tagged D171N was also subcloned into pMYs-IRES-puro (pMYs.IP) and pMYs-IRES-EGFP (pMYs.IG), and *BMI1* into pMYs-IRES-blasticidin (pMYs.IB) and pMYs-IRES-nerve growth factor receptor (NGFR) (pMYs.IN). Plat-GP and Plat-E packaging cells were transfected with retroviral constructs using FuGENE6 (Roche, Mannheim, Germany) as described previously.[16,22]

Retrovirus transduction of human $CD34^+$ primary cells

Cord blood cells (CBs) were collected with written informed consent. $CD34^+$ cells were purified from CBs by the CD34 MicroBead Kit using autoMACS. They were precultured for 3-4 days in Stemline II

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Hematopoietic Stem Cell Expansion medium (Sigma-Aldrich, St Louis, MO) supplemented with 100 ng/ml each of FLT3 ligand, SCF and TPO (PeproTech, London, UK) (expansion medium). The cells were resuspended in new expansion medium and placed in plates coated with RetroNectin (Takara, Otsu, Japan) preloaded with virus. At 3-4 days after transduction, GFP^+ and/or $DsRed^+$ cells were sorted by FACS Aria (Becton Dickinson (BD), Franklin Lakes, NJ).

Cells were cultured continuously in IMDM containing 20% FBS and 100 ng/mL each of FLT-3 ligand, SCF and TPO. For long-term growth, IMDM was supplemented with 20% FBS, 100 ng/mL each of FLT-3 ligand, SCF, and TPO, and 20 ng/mL each of IL-6 and IL-3 (Peprotech).

Colony forming cell (CFC) replating assay

Ten thousand sorted cells were resuspended in MethoCult $H4034^+$ medium (StemCell Technologies, Vancouver, Canada) containing SCF, G-CSF, hGM-CSF, IL-3 and EPO. After 14 days in culture, colonies were counted. Cells were then suspended in methylcellulose medium, and 10^4 cells were plated again for CFC replating assay. Remaining cells were used for cell number counting and cytospin centrifuge for morphological and flow cytometry analyses.

Long-term culture-initiating cell (LTC-IC) assay

For bulk culture assay, 10,000 sorted cells were suspended in Myelocult H5100 medium (StemCell Technologies) with 1 μM hydrocortisone (StemCell Technologies). The cells were divided into two dishes precoated with MS5 stromal cells and cultured for 5 weeks. LTC-IC cultures were harvested, and clonogenic progenitors were assayed in Methocult GF^+ H4435 medium (StemCell Technologies) containing SCF, GM-CSF, IL-3, IL-6, G-CSF and EPO. After 20 days, LTC-ICs derived CFCs were counted. For limiting dilution assay, 100 to 800 sorted cells per well were plated on MS5 stromal cells in 96-well plates and cultured as above. Wells were scored as growth or no growth of colonies. LTC-IC frequency was calculated with L-Calc software (StemCell Technologies).

Retroviral transduction of 32Dcl3 cells and differentiation assay

The murine myeloid progenitor 32Dcl3 cells were infected with retrovirus as previously described.[36]

Mouse BMT

Mouse BMT was performed as described previously.[22] BM mononuclear cells were isolated from C57BL/6 (Ly-5.1) donor mice, and after stimulation with SCF, FLT3 ligand, IL-6 and TPO (R&D Systems), the cells were transduced with retrovirus constructs. Then, $3\text{-}5\times10^5$ of the non-sorted cells were injected into sublethally γ-irradiated Ly-5.2 recipient mice. These studies were approved by the Animal Care

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Committee of the Institute of Medical Science, The University of Tokyo.

Flow cytometry

Human cells were stained with the indicated PE or APC-conjugated antibodies (BD). For the cell cycle analysis, the cells were stained with APC BrdU Flow Kit (BD) or Hoechst 33342 (BD). Flow cytometry analysis was performed on a FACSCalibur (BD) or FACS Aria and data were analyzed using CELLQuest. Mouse cells were stained with the indicated PE-conjugated antibodies (eBioscience) and analyzed using FACSCalibur equipped with FlowJo Version 7.2.4 software (TreeStar). Annexin V staining was carried out with the PE Annexin V Apoptosis Detection Kit I (BD).

Immunoblot analysis

Immunoblot analysis was performed as reported previously.[12] The primary antibodies used in this study were anti-FLAG M2 (Sigma-Aldrich), anti-Bmi-1 (Upstate, Lake Placid, NY or #05-637, Millipore), anti-β-Actin (Santa Cruz) and anti-α-tubulin (Sigma-Aldrich) monoclonal antibodies.

Statistical analysis

For comparison of two independent samples, normally-distributed variables were compared by the Student's *t* test and nonnormally-distributed variables by the Mann-Whitney *U* test. For multiple pairwise comparisons, the data were analyzed by one-way analysis of variance followed by Dunnett's multiple comparison test, or differences between individual groups were estimated using the Steel-Dwass test. Survival curves were estimated by the Kaplan-Meier analysis and compared using the log-rank test. $P < 0.05$ was considered statistically significant.

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Results

RUNX1 D171 amino acid is the most frequent target of mutations

We analyzed *RUNX1* mutations in various myeloid neoplasms, mostly MDS and MDS-related AML including therapy-related cases. We found that 107 patients had *RUNX1* mutations, which were shown to be distributed throughout the full length of the RUNX1 protein (Table S1). Replacement of the D171 amino acid (D171N and D171G) was the most frequent target of mutation in the *RUNX1* gene, which was detected in 8 (7.5 %) patients. The D171 residue resides in the RHD and is one of the critical amino acid residues that directly contact DNA, however, small changes in the RHD do not influence the ability for binding CBFβ.[20,21] Moreover, the C-terminal region including the transactivation/repression domains is preserved in the mutations (Figure S1A), contrary to that in truncation type mutations. Replacement or small in-frame insertion/deletion at or around critical amino acid residues involved in DNA binding were also detected, and 41.6% of the *RUNX1* mutations (45 of 107) had impaired DNA-binding but intact C-terminal transactivation/repression domains. The D171N mutant was localized to the nucleus (Figure S1B) and showed a loss of normal RUNX1 *trans*-activation potential for the M-CSF receptor (Figure S1C). Furthermore, the mutant displayed a dominant-negative type of *trans*-activation suppression (Figure S1D), suggesting that the mutant may have some oncogenic potential in addition to the loss of normal RUNX1 function.

EVI1 overexpression collaborates with RUNX1 mutation in human MDS

Because collaboration between *RUNX1* mutations and *Evi1* overexpression has been shown in a mouse BMT model,[22] we first checked *EVI1* expression levels in selected CD34$^+$ cells from MDS patients. Most of the examined patients showed very low *EVI1* expression (Figure 1A). However, one patient whose initial diagnosis was MDS with D171G mutation displayed extremely high expression level of *EVI1*. The clinical course of the patient was unique in that a steep increase in blast cells was followed by a relatively short MDS period (Figure 1B), which was similar to that in the mouse BMT model.[22] Thus, expression of *EVI1* is generally not high in MDS patients. Then, we set out to elucidate the collaborating genes with *RUNX1* mutations in other patients.

Overexpression of D171N promotes inhibition of differentiation and increase in self-renewal capacity in human CD34$^+$ cells

To clarify the biological functions of the RUNX1 mutants itself and to identify their collaborating genes in hematopoietic stem/progenitor cells, we transduced a RUNX1 mutant into human CD34$^+$ cells to avoid

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the effect of *Evi1* overexpression. The D171N mutant, which was produced by a 1-bp replacement in exon 5, was transduced into CD34$^+$ cells from human CBs (Figure 2A). The efficiency of transduction was about 30-60% (Figure 2B), and RUNX1 expression was confirmed by Western blotting (Figure 2C).

To examine the effect of RUNX1 expression on cell differentiation, we performed CFC assay by plating sorted cells in methylcellulose medium. The number of burst forming unit-erythroid (BFU-E) colonies was significantly decreased in both WT- and D171N-transduced cells, while the number of granulocyte-macrophage colony-forming unit (CFU-GM) colonies was not significantly different (Figure 2D). On the other hand, the individual BFU-E colonies in the WT plates and individual CFU-GM colonies in the D171N plates were larger in size (Figure 2E), in addition to the presence of significantly more growing cells as compared with control (Figure 2F). GPA$^+$ erythroid cells were dominant in the WT group, while most of the D171N-transduced cells expressed myeloid lineage markers (Figure 2G). To determine whether D171N have self-renewal advantage, we performed replating CFC assay. The plates of the D171N mutant contained approximately half the total number of colonies as the pMXs.IG plates in the 1st assay, while total cell numbers were comparable between the D171N and pMXs.IG plates (Figure 2H). Unlike pMXs.IG and WT, D171N showed replating capacity for three replatings. To confirm the presence of progenitors with long-term self-renewal capabilities, LTC-IC assay was conducted. Cells transduced with D171N showed a drastic increase in the number of colonies (Figure 2I).

D171N-transduced cells lack long-term proliferation ability in human CD34$^+$ cells

To determine proliferative and survival advantages of RUNX1-mutated cells, transduced non-sorted human CD34$^+$ cells were cultured in liquid medium (Figure 3A). The percentage of GFP-expressing cells in the WT, D171N, and pMXs.IG-transduced control cultures gradually decreased over time in culture. For further evaluation of the proliferation ability of the transduced cells, we sorted GFP$^+$ cells and performed long-term culture. The WT-transduced cells hardly proliferated, while the D171N-transduced cells proliferated slightly, exhibiting lower proliferation ability than pMXs.IG cells (Figure 3B). These results indicate that the D171N cells have no proliferation ability. To determine whether these differences were due to increased apoptosis or cell cycle inhibition, we confirmed by cell cycle analysis. On day 53 when the D171N cells stopped proliferating, most of the cells accumulated in G1 phase (Figure 3C). The percentage of CD34$^+$ cells among the D171N cell population increased slightly, but gradually decreased with a maximum around day 35 (Figure 3D). At this point, although the percentage of CD34$^+$/CD38$^+$ cells within the D171N cell population did not increase in comparison to the pMXs.IG group, the percentage of

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$CD34^{+}/CD38^{-}$ cells increased to a maximum around 4 % (Figure 3E). On day 35, a vast majority of the pMXs.IG cells and all of the WT cells terminally differentiated into mature myeloid cells and monocytes, whereas the D171N cells contained a large number of immature cells (Figure 3F). The cells transduced with D171N mutant displayed morphological abnormalities in all 3 hematopoietic lineages (Figure 3G). These findings indicate that RUNX1 mutations probably give rise to the multi-lineage dysplasia of hematopoietic cells with increase in the number of blasts that is the main characteristic of MDS. However, the D171N-transduced cells did not expand in liquid media. Furthermore, the D171N mutant abrogated engraftment potential of human stem/progenitor cells in NOD/Shi-scid, $IL2R\gamma c^{null}$ (NOG) mice[37] (Table S2). Thus, it is suspected that the mutant requires additional gene alterations for the development of MDS.

BMI1 is overexpressed in $CD34^{+}$ cells from MDS patients with RUNX1 mutations, while it is repressed in D171N-transduced human $CD34^{+}$ cells

We focused on *BMI1* as a candidate of the additional partner gene alterations because this gene is known to be overexpressed in some MDS patients. *BMI1* expression levels were analyzed in selected $CD34^{+}$ cells from MDS patients (Figure 4A). Patients with *RUNX1* mutations displayed a significantly higher expression level of *BMI1* compared with normal control and lower-risk MDS patients, and 14 of 20 (70 %) of the RUNX1-mutated patients showed *BMI1* overexpression that exceeded the range of normal control, as opposed to only one *EVI1* overexpression. Next, we examined *BMI1* expression levels in the D171N-transduced $CD34^{+}$ cells over a time course. Unexpectedly, the cells with D171N mutation showed a lower expression level of *BMI1*, especially during a long culture period (Figure 4B). These results indicate that the high *BMI1* expression in patients with RUNX1 mutations is not induced by direct effects of the mutations, but rather, the RUNX1-mutated cells may require the acquisition of *BMI1* overexpression for the development of MDS. To confirm the effects of additional *BMI1* overexpression on D171N-transduced cells, we antecedently analyzed BMI1-transduced $CD34^{+}$ cells.

Enforced BMI1 expression shows no leukemogenic ability in human $CD34^{+}$ cells

To evaluate the biological significance of BMI1 overexpression in stem/progenitor cells, BMI1 was cloned into a retrovirus vector (Figure 4C), and transduced into $CD34^{+}$ cells (Figure 4D). BMI1 was endogenously expressed in cells transduced with pMXs.IR, while a strong upregulation of this protein was observed in the BMI1-transduced cells (Figure 4E). The number of colonies of the BMI1-transduced $CD34^{+}$ cells was almost equal to that of the pMXs.IR-transduced cells, while the replating efficiency of

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the BMI1 cells was slightly increased (Figure 4F). The BMI1-transduced cells exhibited greater proliferation than the pMXs.IR-transduced cells (Figure 4G), but the cells proliferated with myeloid terminal differentiation without dysplasia or increase in $CD34^+$ cells (Figure 4H). Thus, BMI1-overexpressed cells showed a slight increase in self-renewal capacity and an increase in proliferation ability. However, BMI1 did not block differentiation at all. Overexpression of BMI1 may have limited proliferation ability without differentiation inhibition, suggesting that BMI1 alone does not have enough potential for MDS-genesis.

These results raised the possibility that BMI1 may be overexpressed by additional molecular abnormalities and may work as a proliferative activator in the stem/progenitor cells with RUNX1 mutations. Since the D171N mutation itself was not proliferogenic, our next investigation was undertaken to determine whether BMI1 is required for leukemogenesis in the D171N-mutated cells.

BMI1 overexpression confers survival advantage but does not inhibit G-CSF–induced differentiation of 32D cells

To confirm the collaboration of D171N with BMI1, we first transduced both the D171N mutant and BMI1 into 32Dcl3 cells. The 32Dcl3 cells were transduced with pMYs.IP/IB, pMYs.IP/BMI1, D171N/pMYs.IB or D171N/BMI1, and the infected cells were subjected to drug selection by puromycin and blasticidin. G-CSF treatment induced terminal differentiation of 32Dcl3 cells transduced with pMYs.IP/IB, as indicated by the appearance of polymorphonucleated neutrophils and up-regulation of CD11b on the surface, and pMYs.IP/BMI1-transduced cells were also terminally differentiated as well (Figure 5A). However, the differentiation was weakly inhibited by D171N/pMYs.IB, and D171N/BMI1 showed no additional effect on differentiation compared with D171N/pMYs.IB (Figure 5A), indicating that BMI1 did not block differentiation at all. Growth rate was comparable among the indicated transfectants after 3 days of culture with the presence of IL-3 (Figure 5B). However, annexin V positivity in the D171N/BMI1-transduced cells was at the same level as that in pMYs.IG/IB- or pMYs.IP/BMI1-transduced cells, and it was significantly lower than that in D171N/pMYs.IB-transduced cells after 36 hours of culture without IL-3 (Figure 5C). These data raised a possibility that BMI1 may add some survival effects to D171N-transduced cells.

Double transduction of D171N and BMI1 into human $CD34^+$ cells leads to proliferation with differentiation

To investigate whether BMI1 can add growth advantage to RUNX1-mutated cells, we performed

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simultaneous double transduction of D171N mutant with BMI1 into human CD34^{+} cells. Human CB CD34^{+} cells were transduced with pMXs.IG/IR, pMXs.IG/BMI1, D171N/pMXs.IR or D171N/BMI1. BMI1 was endogenously expressed in cells transduced with pMXs.IG/IR or D171N/pMXs.IR, while a strong upregulation of this protein was observed in the BMI1-transduced cells (Figure 5D). In the CFC replating assay, all of the double-transduced cells formed fewer colonies than single transduced cells (Figure 5E, compared with Figures 2H and 4F). Both colony number and replating ability were altered by the double transduction of D171N and BMI1 compared with D171N/pMXs.IR transduced cells. The presence of stem/progenitor cells with long-term self-renewal capabilities was confirmed by the LTC-IC assay. In both limiting dilution and bulk assays, increased stem/progenitor cell frequencies were observed in D171N/BMI1-transduced cells (Figure 5F). In addition, a dramatic change was observed in the liquid culture. Double-transduced cells with pMXs.IG/IR or D171N/pMXs.IR hardly proliferated in the long-term culture medium, and their proliferation folds were less than 100 (Figure 5G). However, double-transduced cells containing BMI1 exhibited an altered effect on cell proliferation. In particular, the D171N/BMI1 double-transduced cells spontaneously started to proliferate around day 20, a phenomenon that was earlier and greater than the cells with pMXs.IG/BMI1. The D171N/BMI1 cells markedly grew until day 70, resulting in the same proliferation ability when compared with pMXs.IG/BMI1 transduced cells (Figure 5G). Thus, the D171N/BMI1-transduced cells had a strong growth advantage compared with the D171N/pMXs.IR-transduced cells. The BMI1 double-transduced cells showed an increase in S phase; however, the cells did not harbor an expansion of a population of CD34^{+} or blast cells, and most of the cells differentiated to various stages of myeloid cells with dysplasia (Figure 5H). These results indicate that BMI1 overexpression confers proliferation ability with differentiation to D171N-mutated cells. To assess the impact of BMI1 on the proliferation and survival of the progenitor cells, we analyzed the effect of *BMI1* expression on *INK4A/ARF* (*p16/p14*) locus expression in the CD34^{+} cells. *INK4A/ARF* expression was downregulated by BMI1 (Figure 5I), which may have contributed to the proliferation and survival of D171N-transduced cells. Significant enrichments of BMI1 on *Ink4a/Arf* (*p16/p19*) promoter regions were detected in both BMI1-transduced 32D cells and BMI1/D171N-transduced 32D cells, but not on the beta-actin promoter region (Figure S2A). A physical association *in vivo* between BMI1 and D171N was observed in 293T cells, which was comparable to that between BMI1 and wild type RUNX1 (Figure S2B).

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INK4A/ARF expression levels were also lower in MDS patients with RUNX1 mutations than in those without mutations in this gene (Figure S3A). To clarify the role of microRNAs associated with BMI1 and polycomb-repressive complex (PRC) 1/2 in the patients with RUNX1 mutations, we analyzed microRNA levels in $CD34^+$ cells from MDS patients; however, no remarkable difference was detected between $CD34^+$ cells from patients and normal BM (Figure S3B). Furthermore, we analyzed the effects of BMI1 knockdown by short hairpin RNA in the $CD34^+$ cells from MDS/AML patients with RUNX1 mutations and high BMI1 expression. BMI1 knockdown resulted in impaired cell proliferation on MS5 stromal cells (Figure S3C). These results indicate that expansion of RUNX1-mutated $CD34^+$ cells depends on BMI1 expression, which coincides with repression of the cell cycle regulators INK4A/ARF.

Collaboration of D171N mutant and BMI1 in a mouse BMT model

Nevertheless, even the D171N/BMI1-transduced human $CD34^+$ cells did not develop MDS/AML in NOG mice (Table S2). Therefore, to confirm the collaboration of BMI1 overexpression with D171N mutant *in vivo*, we performed mouse BMT using BM cells transduced with both D171N and BMI1. We previously reported that most of the mice that received D171N-transduced BM cells died of MDS/AML, and collaboration between D171N and Evi1 overexpression was confirmed in a BMT model where coexpression of D171N and Evi1 induced MDS/AML with much shorter latencies.[22] To investigate whether high expression of BMI1 can also collaborate with D171N, Ly-5.1 murine BM mononuclear cells were infected with retrovirus harboring pMYs.IG/IN, pMYs.IG/BMI1, D171N/pMYs.IN or D171N/BMI1. The efficiency of retrovirus infection was 35%-45% of $GFP^+/NGFR^+$ cells (Figure S4A and Table S3), and non-sorted cells were transplanted into sublethally irradiated syngeneic Ly-5.2 mice. Each cell population was successfully engrafted (Figure S4B), and in time, the proportion of $GFP^+/NGFR^+$ cells gradually increased in the mice that were transplanted with D171N/BMI1-transduced cells (Figure S4C).

Mice that received transplants of pMYs.IG/BMI1-transduced cells remained healthy over the observation period (n=12/12), as well as those that were transplanted with pMYs.IG/IN-transduced cells (n=4/4). Most of the mice that received transplants of D171N/pMYs.IN-transduced cells developed MDS/AML mainly 6-8 months after transplantation (n=6/11, $P<0.0001$, Figure 6A), as observed in the previous report.[22] Of note, mice that received transplants of BM cells expressing D171N/BMI1 developed MDS/AML with significantly shorter latencies (mainly 3-5 months) compared with the D171N/pMYs.IN group (n=12/12, $P=0.001$, Figure 6A). Expression of the transduced D171N and BMI1 was confirmed by Western blot analysis, and endogenous Bmi1 expression could be detected in the

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D171N/pMYs.IN cohort (Figure 6B). Morbid mice with D171N/pMYs.IN or D171N/BMI1 exhibited similar phenotypes, characterized by leukocytosis, anemia, and marked splenomegaly, while the mice with pMYs.IG/BMI1 or pMYs.IG/IN, sacrificed 8 months after BMT, showed none of these phenotypes (Table 1 and Figure 6C). In the leukemic mice with D171N/pMYs.IN or D171N/BMI1, BM and spleen were occupied by immature myeloid cells including myeloid blasts (Figure 6D). More myeloblasts in BM were observed in D171N/BMI1 mice than in D171N/pMYs.IN ones (Table 1). The leukemic cells displayed similar morphological abnormalities and surface markers: GFP^{+}/$NGFR^{+}$ leukemic cells were $CD11b^{low\ to\ high}$, $Gr\text{-}1^{low}$, $B220^{low}$ and $c\text{-}kit^{low\ to\ high}$ (Figures 6E, S4D and Table S2), although the expression level of c-kit tended to be higher in the D171N/BMI1 cohort than that in the D171N/pMYs.IN group. The normal structure of the spleen was completely destroyed with massive blast and immature myeloid cell infiltration, and these cells also invaded into the hepatic portal areas in the liver (Figure 6F). Meanwhile, GFP^{+}/$NGFR^{+}$ cells in pMYs.IG/BMI1-induced mice were very few, indicating that pMYs.IG/BMI1-transduced BM cells did not become dominant *in vivo* (Figure 6E). In addition, myeloid cells showed normal differentiation into segmented cells in the BM, and most of the nucleated cells in the spleen were found to be small lymphocytes as observed in the mice with pMYs.IG/IN (Figure 6D). Collectively, BMI1 overexpression has a strong potential to induce MDS/AML in concert with D171N in a mouse BMT model, although BMI1 overexpression by itself does not result in maturation block or leukemogenesis. Furthermore, *Ink4a/Arf* (*p16/p19*) expression in D171N/BMI1-transduced mice was significantly lower than that in the D171N/pMYs.IN-transduced mice (Figure 6G). However, most of the D171N/BMI1 mice still showed high expression of *Evi1* which was relatively lower than that in the D171N/pMYs.IN group (Figure 6H). Therefore, *Evi1* overexpression is suspected to play a critical role along with the RUNX1 D171N mutation in the development of MDS/AML in the mouse system.

Stepwise transduction of D171N mutant and BMI1 leads to MDS-like long-term proliferation in human $CD34^{+}$ cells

Our results showed that simultaneous transduction of D171N and BMI1 can induce MDS/AML, while FPD/AML patients who have congenital RUNX1 mutations develop MDS/AML after decades of latency period. This raises the possibility that additional gene abnormalities occur afterwards in the RUNX1-mutated cells for the development of MDS. To clarify the effect of BMI1 in RUNX1-mutated $CD34^{+}$ cells, we next performed stepwise transduction of D171N mutant and BMI1 (Figure 7A).

We first transduced D171N into $CD34^{+}$ cells. Sorted GFP^{+} cells were cultured for 28 days more,

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until the percentage of the D171N-transduced $CD34^+$ cells, especially the $CD34^+/CD38^-$ population, was maximal (Figure 3D and 3E). Then, we selected for the $CD34^+$ cells again, followed by BMI1 transduction. $GFP^+/DsRed^+$ cells were sorted and cultured in methylcellulose or long-term culture medium. In the CFC assay, pMXs.IR-transduced cells seemed to have very low colony forming ability, whereas the stepwise BMI1-transduced D171N cells displayed an increase in both colony forming ability and replating capacity (Figure 7B). Moreover, the $CD34^+$ cell population remained in the stepwise BMI1-transduced D171N cells (Figure 7C). Furthermore, long-term proliferation with a retained $CD34^+$ cell fraction was observed in the stepwise BMI1-transduced D171N cells, and morphological findings showed myeloid cell dysplasia with increased blast cells (Figure 7D and 7E). These findings are quite similar to that seen in human patients with higher-risk MDS. Thus, our results demonstrate that the MDS phenotype could be reproduced in human hematopoietic cells by stepwise transduction of D171N mutant followed by BMI1.

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Discussion

RUNX1 mutations have been detected in nearly 20% patients with higher-risk MDS. Biochemically, RUNX1 mutants show loss of normal RUNX1 function, and some mutants have dominant-negative *trans*-activation potential similar to leukemogenic chimeras such as CBFβ-MYH11. The biological functions of RUNX1 mutants, which have already been demonstrated using the mouse BMT model, include increase in leukemogenic potential.[22] In this model, however, the retrovirus frequently integrated into the chromosome near the *Evi1* locus, resulting in its high expression. We checked *EVI1* expression levels in MDS patients, however, most of the examined patients showed very low *EVI1* expression except only one patient with *RUNX1* mutation, who rapidly progressed from MDS to AML with hyperblastcytosis. Because collaboration between *RUNX1* mutations and *EVI1* overexpression does not appear to be common in MDS patients, we tried to clarify the biological significance of RUNX1 mutants in stem/progenitor cells using human CBs without the effect of *Evi1* overexpression.

The cells transduced with wild-type RUNX1 quickly differentiated into mature myeloid/monocytoid cells without proliferation in both colony forming and liquid culture assays. This result suggests that overexpression of wild-type RUNX1 in stem/progenitor cells promotes terminal differentiation without self-renewal, blocks cell proliferation, and has no oncogenic potential. These data can explain the reduction in wild-type RUNX1-transduced cells in a mouse BMT model.[22,38] On the other hand, the D171N mutant, the most common mutation in RUNX1 that is caused by only a 1-bp replacement in the RHD, has increased self-renewal capacity, mildly blocked differentiation, dysplasia in all 3 lineages, and slight tendency for immaturity, but no proliferation ability. Although a stem/progenitor cell with the D171N mutation is suspected to have MDS-genic potential of cell dysplasia and self-renewal capacity, it induces G1 arrest and cannot develop MDS due to lack of proliferation ability. Thus, additional gene alterations that induce proliferation activity seem to be necessary for development of MDS. *BMI1* overexpression was suspected as a candidate collaborator, since upregulated BMI1 level was observed in higher-risk MDS patients with *RUNX1* mutations, even though the D171N mutant itself does not induce BMI1 expression. The molecular mechanism of high BMI1 expression in RUNX1-mutated patients is not due to microRNAs either. A previous study showed that forced expression of activated N-RAS mutant induced overexpression of Bmi1 in mouse c-Kit^{+} cells, especially in Runx1$^{-/-}$ cells.[39] Thus, the gene mutations that induce activation of the RAS signaling pathway, which