In vitro expansion of CD34^{+}CD38^{-} cells under stimulation with hematopoietic growth factors on AGM-S3 cells in juvenile myelomonocytic leukemia

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Abstract

Using serum-containing culture, we examined whether AGM-S3 stromal cells, alone or in combination with hematopoietic growth factor(s), stimulated the proliferation of CD34$^+$ cells from patients with juvenile myelomonocytic leukemia (JMML). AGM-S3 cells in concert with stem cell factor plus thrombopoietin increased the numbers of peripheral blood CD34$^+$ cells to approximately 20-fold the input value after 2 weeks in 9 JMML patients with either *PTPN11* mutations or *RAS* mutations who received allogeneic hematopoietic transplantation. Granulocyte-macrophage colony-stimulating factor (GM-CSF) also augmented the proliferation of JMML CD34$^+$ cells on AGM-S3 cells. The expansion potential of CD34$^+$ cells was markedly low in 4 patients who achieved spontaneous hematological improvement. A large proportion of day 14-cultured CD34$^+$ cells were negative for CD38 and cryopreservable. Cultured JMML CD34$^+$CD38$^-$ cells expressed CD117, CD116, c-mpl, CD123, CD90, but not CXCR4, and formed GM and erythroid colonies. Day 7-cultured CD34$^+$ cells from 2 of 3 JMML patients injected intrafemorally into immunodeficient mice stimulated with human GM-CSF after transplantation displayed significant hematopoietic reconstitution. The abilities of OP9 cells and MS-5 cells were one-third and one-tenth, respectively, of the value obtained with AGM-S3 cells. Our culture system may provide a useful tool for elucidating leukemogenesis and for therapeutic approaches in JMML.

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Introduction

Juvenile myelomonocytic leukemia (JMML) is a fatal, mixed myeloproliferative and myelodysplastic disorder that occurs in infancy and early childhood. Children with JMML have mutually exclusive genetic abnormalities in granulocyte-macrophage colony-stimulating factor (GM-CSF) signaling pathways: inactivation of the *NF1* gene or mutations in *NRAS, KRAS, PTPN11*, and *CBL* genes (ref. 1, 2). These specific defects in the RAS signaling pathway make JMML cells hypersensitive to GM-CSF. More recently, Sakaguchi et al. (ref. 3) demonstrated, using whole-exome sequencing, that together with the high frequency of RAS pathway mutations, mutations of *SETBP1* and *JAK3* are common recurrent secondary events presumed to be involved in tumor progression and are associated with poor clinical outcomes.

We previously reported that the combination of stem cell factor (SCF) and thrombopoietin (TPO) stimulates the GM-CSF-dependent proliferation of primitive hematopoietic progenitors in JMML (ref. 4). Nevertheless, clonogenic JMML progenitors cannot be maintained in culture because they differentiate, and within a few weeks the leukemic clone is lost. This makes it difficult to identify the cell that initiates and maintains the disease in the patients. Additionally, no JMML cell lines have been developed so far. Using in vivo models, Lapidot et al. (ref. 5) demonstrated that only CD34$^+$CD38$^-$ cells could initiate the disease after transplantation of peripheral blood (PB), bone marrow (BM), or spleen cells from JMML patients into sublethally irradiated severe combined immunodeficient (SCID) mice.

The aorta-gonad-mesonephros (AGM) region is involved in the generation and

maintenance of the first definitive hematopoietic stem cells. It has been demonstrated that a stromal cell line, AGM-S3, derived from AGM region of a 10.5-day postcoitum C3H/HeN mouse embryo, supports the development of normal human hematopoietic stem cells as well as murine hematopoietic stem cells (ref. 6, 7). Taken together with our previous findings that the JMML clone may arise prior to birth (ref. 8), we anticipated that AGM-S3 cells could support the proliferation of JMML CD34$^+$ cells.

We here report that AGM-S3 cells in combination with hematopoietic growth factors expand CD34$^+$CD38$^-$ cells of JMML patients with *PTPN11, NRAS,* or *KRAS* mutation in fetal bovine serum (FBS)-containing culture.

Materials and Methods

This study was approved by the institutional review board of Shinshu University School of Medicine. Informed consent was obtained from the guardians of the patients following institutional guidelines.

Cell preparation

JMML CD34$^+$ cells were enriched from PB mononuclear cells (MNCs) by means of positive immunomagnetic selection with MACS beads (ref. 9). Cord blood (CB) CD34$^+$ cells were purchased from Riken BioResource Center (Tsukuba, Japan). BM CD34$^+$ cells were obtained from normal healthy volunteers. Ninety-nine percent of the isolated cells were positive for CD34 according to flow cytometric analysis.

Hematopoietic growth factors

Recombinant human SCF, TPO, and GM-CSF (Miltenyi Biotec Inc., Auburn, CA) were used at a concentration of 10 ng/mL, which induced the optimal response in culture of human hematopoietic progenitors (ref. 4).

Murine stromal cells

The establishment of AGM-S3 cells and their potential to support human hematopoiesis have been reported elsewhere (ref. 6). AGM-S3 cells (kindly provided by Kyowa Hakko Kirin Co., Ltd, Tokyo, Japan) within 10 passages were used throughout our experiments. AGM-S3 cells (1–2×10^5) were cultured in 35-mm gelatin-coated dishes (Sumitomo Bakelite Co., Ltd., Tokyo, Japan) overnight to make a confluent feeder layer. The next day, they were exposed to 25 Gy of radiation.

OP9 cells were purchased from Riken BioResource Center. MS-5 cells were kindly provided by Dr. Kazuhiro J. Mori (Niigata University, Niigata, Japan).

Suspension cultures

One $\times 10^4$ JMML CD34$^+$ cells were transferred in 35-mm gelatin-coated dishes that had been covered with irradiated confluent AGM-S3 cells, and were cultured in 10% FBS (HyClone Laboratories, Logan, UT) containing alpha-medium supplemented with 10 ng/mL SCF, 10 ng/mL TPO, and 10 ng/mL GM-CSF, alone or in combination. Half of the culture medium was replaced every 3 days with fresh medium containing the factor(s). The plates were incubated at 37ºC in a humidified atmosphere flushed with 5% CO_2 in air. On days 14 and 28, cultured cells were collected by vigorous pipetting. The number of viable cells was determined by a trypan-blue exclusion test using a hemocytometer.

Flow cytometric analysis

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For the analysis of surface markers on the cultured cells, 1 to 2×10^5 cells were collected in plastic tubes and incubated with a combination of an appropriately diluted fluorescein isothiocyanate (FITC)-conjugated anti-CD34 monoclonal antibody (mAb, clone 8G12, BD Biosciences, San Jose, CA) and phycoerythrin (PE)-conjugated anti-CD38 mAb (clone T16, Beckman Coulter, Marseille, France), as described previously (ref. 9). After the cells were washed twice, their surface markers were analyzed with FACSCalibur (BD Biosciences) using CellQuest Pro. Viable cells were gated according to their forward light-scatter characteristics (FSC) and side-scatter characteristics (SSC). The proportion of positive cells was determined by comparison with cells stained with FITC- and PE-conjugated mouse isotype-matched IgG (BD Biosciences and Beckman Coulter, respectively). The expression of CD117 (c-kit), CD116 (GM-CSF receptor alpha), CD90, and CXCR4 on day 14-cultured $CD34^+CD38^-$ cells generated with SCF+TPO on AGM-S3 cells was analyzed, using FITC-conjugated anti-CD34 mAb, allophycocyanin (APC)-conjugated anti-CD38 mAb (clone HB7), and PE-conjugated mAb against CD117 (clone 104D2), CD116 (clone hGMCSFR-M1), CD90 (clone CE10), or CXCR4 (12G5). All mAbs were purchased from BD Biosciences. The expression of CD123 (IL-3 receptor alpha) on day 14-cultured $CD34^+CD38^-$ cells was analyzed, using PE-conjugated anti-CD34 mAb (clone HPCA, BD Biosciences), APC-conjugated anti-CD38 mAb, and FITC-conjugated anti-CD123 mAb (clone 6H6, eBioscience, San Diego, CA).

Cytochemical staining

Five $\times 10^3$ cultured cells were spread on glass slides using a Cytospin II (Shandon Southern, Sewickly, PA), and stained with α-naphthyl butyrate esterase (ANB) or naphthol AS-D chloroacetate esterase (NASDCA), as described previously (ref. 10)

Clonal cell culture

Clonal cell cultures were carried out in 35-mm Lux suspension culture dishes (#153066; Nunc, Roskilde, Denmark), as described previously (ref. 4, 9, 10). Five hundred $CD34^+CD38^-$ cells, $CD34^-CD38^+$ cells, and $CD34^-CD38^-$ cells were plated in a dish containing methylcellulose medium supplemented with GM-CSF, SCF, IL-3, and erythropoietin (Methocult GF H4434, Stemcell Technlogies Inc., Vancouver, Canada). Dishes were incubated at 37°C in a humidified atmosphere flushed with 5% CO_2. On day 14, GM colonies and erythroid colonies were scored in situ on an inverted microscope. Then, GM colonies were individually lifted with an Eppendorf micropipette and prepared as single-cell suspensions. Sequence analyses were performed on each colony-constituent cells.

Transplantation into NOD/SCID/γc [null] mice

NOD/SCID/γc [null] (NOG) mice were developed at the Central Institute of Experimental Animals (Kawasaki, Japan), as described previously (ref. 11). All mice were kept under specific pathogen-free conditions in accordance with the guidelines of the facility. Xeno-transplantation and analysis of JMML cells were performed by a modified version of methods reported previously (ref.12). In brief, 1×10^5 day 14-cultured JMML $CD34^+$ cells were transplanted into 2.4 Gy-irradiated 8- to 12-week-old NOG mice through the tail vein. In addition, we injected day 7- or day 14-cultured JMML cells directly into the

femoral bone marrow cavity of irradiated NOG mice (ref. 13). After transplantation,

some mice received intraperitoneal injection of human GM-CSF (5 μg per mouse) 3

times per week. For screening of JMML cells, BM cells of NOG mice were aspirated

from the tibia every 4 weeks. To analyze leukemic cells, mice were sacrificed by cervical

dislocation. BM samples were stained with antibodies after the isolation of MNCs. Dead

cells were excluded by 4′-6-diamidino-2-phenylindole staining. Samples were analyzed

using a FACS LSR and Cell Quest software (Becton Dickinson) according to the

manufacturer's protocol. For flow cytometric analysis, anti-mouse CD45 mAb (APC),

anti-human CD45 mAb (FITC), and anti-human CD34 mAb (PE) were used.

To examine whether the patient-derived cells were present in recipient mice, we used

real-time PCR analyses for human albumin, *PTPN11* mutation and *NRAS* mutation (ref.

8).

RNA extraction and Taqman gene expression analysis

Total RNA from CD34$^+$CD38$^-$ cells was extracted using Isogen reagent according to

the manufacturer's instructions. Approximately 1 to 2 μg of total RNA from each sample

was converted to cDNA using PrimeScript II 1st strand cDNA Synthesis Kit (Takara Bio

Inc., Otsu, Japan) in a volume of 20 μL. For Taqman gene expression assay (Applied

Biosystems, Carlsbad, CA), 2.5 μL of diluted cDNA was mixed with primer and c-MPL

probe (Assay ID Hs00180489_m1) in a total reaction volume of 25 μL and assayed on a

StepOnePlus Real-Time PCR system (Applied Biosystems).

PCR and quantitative real-time PCR

DNA was extracted from cultured cells and PBMNCs of children with JMML. Exons 3, 8, and 11 of *PTPN11*, and exon 1 (codons 12 and 13) and exon 2 (codon 61) of *NRAS* and *KRAS* genes were amplified by PCR, using primer pairs described previously (ref.8, 14,15). The PCR products were subjected to direct sequencing from both directions on an automatic DNA sequencer.

In quantitative real-time PCR analysis for human albumin, *PTPN11* 1508G>C and *NRAS* 38 G>A mutation, we used primers and probes as described previously (ref. 8).

Statistical analysis

Values are expressed as means ± SD. To determine the significance of differences, we used one-way analysis of variance, followed by post hoc contrasts with Bonferroni limitation. Statistical analysis was performed with IBM SPSS 18.0 (IBM Corp., New York, NY), and statistical significance was defined as $p < 0.05$.

Results

In vitro expansion of JMML CD34^{+} cells under stimulation with SCF+TPO or GM-CSF on AGM-S3 cells

A murine stromal cell line, AGM-S3, has been shown to support the survival/self-renewal of normal human long-term repopulating hematopoietic stem cells with the potential to reconstitute hematopoiesis in NOD/SCID mice (ref. 6). Using FBS-containing culture, we examined whether AGM-S3 cells alone or in combination with hematopoietic growth factor(s) stimulated the proliferation of PB CD34^{+} cells obtained at diagnosis from JMML patients. Although AGM-S3 cells alone failed to stimulate the growth of JMML CD34^{+} cells, the addition of either SCF or TPO to culture containing AGM-S3 cells resulted in significant proliferation of CD34^{+} cells after 2 weeks of culture. The combined use of the two growth factors additively increased the numbers of JMML CD34^{+} cells. Cobblestone-like colonies appeared under the stromal layer at day 14 of culture. As shown in Figure 1, SCF+TPO-mediated increase of CD34^{+} cells on AGM-S3 cells was 23.0-fold in a JMML patient with *PTPN11* mutation (1508G>C) and 42.6-fold in a JMML patient with *NRAS* 35G>A mutation. Interestingly, GM-CSF also augmented the proliferation of JMML CD34^{+} cells on AGM-S3 cells (29.3-fold and 10.4-fold, respectively). A large proportion of cultured cells grown by SCF+TPO on AGM-S3 cells could be more easily peeled off the murine cell layer by pipetting compared with the case for cultured cells grown by GM-CSF on AGM-S3 cells. Accordingly, we used the combination of SCF+TPO and the murine stromal cells for assessment of in vitro JMML CD34^{+} cell expansion in subsequent studies. As shown in

Figure 2, the mean fold increase of CD34$^+$ cells was 22.5 ± 9.9 in 9 JMML patients who

received allogeneic hematopoietic stem cell transplantation (HSCT). There was no

significant difference in proliferative potentials of CD34$^+$ cells between JMML patients

with *PTPN11* mutations and those with *RAS* mutations: the mean fold increase of CD34$^+$

cells was 17.8 ± 7.6 in 5 patients with *PTPN11* mutations, and 28.3 ± 10.1 in 4 patients

with *RAS* mutations. To examine which subpopulation of CD34$^+$ cells yielded day

14-cultured CD34$^+$ cells, CD34$^+$CD38$^-$ cells, CD34$^+$CD38$^+$ cells, CD34$^-$CD38$^+$ cells, and

CD34$^-$CD38$^-$ cells were individually sorted from PBMNCs of a patient with *PTPN11*

mutation (226G>A), and cultured in the presence of SCF+TPO on AGM-S3 cells. As

presented in Figure 3, fresh CD34$^+$CD38$^-$ cells had the highest potential to generate

CD34$^+$ cells on day 14. Similar results were obtained from a patient with *NRAS* mutation.

When 2×10^4 day 14-cultured CD34$^+$ cells were harvested and re-incubated with

SCF+TPO on AGM-S3 cells for 2 weeks, $0.8 \pm 0.9 \times 10^4$ CD34$^+$ cells (more than 80%

were negative for CD38) were generated in 3 patients with JMML.

The SCF+TPO-mediated increase of CD34$^+$ cell number on AGM-S3 cells was 3.92 ±

2.23 of the input value in CB, and 0.11 ± 0.02 of the input value in normal BM (Figure 2).

There was a significant difference in the proliferative potential among JMML, CB, and

normal BM CD34$^+$ cells.

Expression of CD38, CD117 (c-kit), c-mpl, CD116 (GM-CSF receptor alpha), CD123 (IL-3 receptor alpha), CD90, and CXCR4 of day 14-cultured JMML CD34$^+$ cells

We examined the expression of CD38 antigen on day 14-cultured CD34$^+$ cells. As

presented in Figure 4, $95.0 \pm 3.0\%$ of day 14-cultured $CD34^+$ cells generated with

SCF+TPO on AGM-S3 cells were negative for CD38 antigen in 9 JMML patients.

Similarly, a great majority of cultured $CD34^+$ cells grown by GM-CSF on AGM-S3 cells

did not react with anti-CD38 mAb. In addition, more than 90% of cultured $CD34^+$ cells

were negative for CD11b. On the other hand, most $CD34^+$ cells generated with

SCF+TPO in the absence of AGM-S3 cells expressed CD38 antigen.

   We then evaluated the expression of CD117 (c-kit), c-mpl, CD116 (GM-CSF receptor

alpha), CD123 (IL-3 receptor alpha), CD90, and CXCR4 on day 14-cultured JMML

$CD34^+CD38^-$ cells, and compared it with the values of fresh $CD34^+CD38^-$ cells obtained

from PBMNCs at diagnosis of the patients. As shown in Figure 5, CD117 was apparently

expressed on $CD34^+CD38^-$ cells generated with SCF+TPO on AGM-S3 cells. Real-time

PCR revealed that the quantity of the c-mpl gene normalized using the level of β-actin

was 0.005 to 0.011 in sorted day 14-cultured $CD34^+CD38^-$ cells, whereas c-mpl

expression was undetectable in $CD34^-CD38^+$ cells and $CD34^-CD38^-$ cells. A large

proportion of day 14-cultured $CD34^+CD38^-$ cells generated with SCF+TPO on AGM-S3

cells were positive for GM-CSF receptor alpha.   Surface expressions of CD123 and

CD90 on the cultured $CD34^+CD38^-$ cells were modest and moderate, respectively.

Expression levels of CD116, CD117, CD123, and CD90 on day 14-cultured JMML

$CD34^+CD38^-$ cells were similar to those on fresh $CD34^+CD38^-$ cells. On the other hand,

the cultured $CD34^+CD38^-$ cells displayed no substantial expression of CXCR4, different

from the case for fresh $CD34^+CD38^-$ cells. Similar results were obtained from the other 3

JMML patients.

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Cytochemical staining revealed that $91.3 \pm 7.0\%$ of $CD34^-CD38^+$ cells and $97.0 \pm$ 4.1% of $CD34^-CD38^-$ cells sorted using a flow cytometer were stained with NASDCA and ANB, respectively, in 3 patients with JMML. On the other hand, nearly all day 14-cultured JMML $CD34^+CD38^-$ cells were negative for both NASDCA and ANB.

The karyotype of day 14-cultured cells of all 7 JMML patients whose results of G-banding could be obtained was identical to that of marrow cells at diagnosis. Additionally, the genetic mutations in day 14-cultured cells were consistent with those detected at diagnosis in all 9 JMML patients according to direct sequencing. Identical genetic mutations were observed in 20 GM colonies generated from day 14-cultured $CD34^+$ cells in each of 4 children with JMML (2 patients with *PTPN11* mutations and 2 patients with *NRAS* mutations).

Clonogenic progenitors in day 14-cultured JMML $CD34^+CD38^-$ cells

We examined the frequencies of GM progenitors and erythroid progenitors in subsets of expanded JMML $CD34^+$ cells. $CD34^+CD38^-$ cells, $CD34^-CD38^+$ cells, and $CD34^-CD38^-$ cells were individually sorted from day 14-cultured cells that had been frozen and kept at $-80°C$. Total numbers of hematopoietic progenitors generated from day 14-cultured $CD34^+CD38^-$ cells surpassed those from $CD34^-CD38^+$ cells and those from $CD34^-CD38^-$ cells in 4 JMML patients with either *PTPN11* or *RAS* mutations [101.2 ± 55.9 (24 to 156) from 500 $CD34^+CD38^-$ cells; 3.4 ± 3.3 (1 to 8) from 500 $CD34^-CD38^+$ cells; 1.4 ± 1.3 (0 to 3) from 500 $CD34^-CD38^-$ cells]. Five hundred day 14-cultured JMML $CD34^+CD38^-$ cells yielded 31.4 ± 13.5 (21 to 55) GM colonies under stimulation with GM-CSF alone. Additionally, small numbers of GM colonies were grown in the

absence of growth factors [7.0 ± 3.9 (3 to 13)].

Proliferative potential of CD34$^+$ cells in 4 patients with JMML who achieved hematological improvement despite no chemotherapy

Since we previously reported that JMML patients with *NRAS* or *KRAS* 34G>A achieved hematological improvement despite no chemotherapy during a 7- to 10-year follow up (ref. 14, 16), proliferative potentials of CD34$^+$ cells derived from these 4 patients with JMML were compared with those of CD34$^+$ cells obtained at diagnosis from JMML patients who received allogeneic HSCT. As presented in Figure 2, the fold increase of CD34$^+$ cells obtained 1 year after diagnosis in patient no. 10 was 5.1. CD34$^+$ cells obtained 5 to 8 years after the diagnosis from PBMNCs of 4 JMML patients exerted no significant proliferative potential. Accordingly, there was a marked difference in expansion potential of CD34$^+$ cells between the patients who received allogeneic HSCT and those who achieved spontaneous hematological improvement.

Different potential of 3 types of murine stromal cells to expand JMML CD34$^+$ cells in concert with SCF+TPO

We then examined whether, in the presence of SCF+TPO, JMML CD34$^+$ cells exhibited different proliferation among 3 types of murine stromal cells (AGM-S3, OP9, and MS-5 cells). The results are presented in Figure 6. At 2 weeks of culture, the fold increase of CD34$^+$ cells on OP9 cells was one-third of the value of AGM-S3 cells. Approximately 70% of day 14-cultured CD34$^+$ cells grown on OP9 cells were negative for CD38. On the other hand, the potential of MS-5 cells to expand CD34$^+$ cells was markedly lower than that of AGM-S3 cells (one-tenth of the value of AGM-S3 cells). In

addition, a majority of the cultured CD34$^+$ cells grown on MS-5 cells were positive for CD38.

Transplantation of cultured JMML CD34$^+$ cells into NOG mice

We investigated whether cultured CD34$^+$ cells grown by SCF+TPO on AGM-S3 cells contained transplantable JMML stem cells. Although 1×10^5 day 14-cultured CD34$^+$ cells from 2 patients with JMML were injected intravenously into irradiated NOG mice, there was no significant growth of human CD45$^+$ cells. Since expression of CXCR4 was not detectable on day 14-cultured JMML CD34$^+$CD38$^-$ cells as described above, these CD34$^+$ cells might decrease or lose the homing ability. Accordingly, we performed intrafemoral injection of 1.1 to 3.0×10^5 day 14-cultured JMML CD34$^+$ cells generated by SCF+TPO on AGM-S3 cells. Nevertheless, no significant proportion of cells positive for human CD45 were found in the transplanted mice. Similar results were obtained from day 14-cultured JMML CD34$^+$ cells generated by SCF+TPO on OP9 cells or MS-5 cells.

Since proliferative potential of cultured JMML CD34$^+$ cells generated by SCF+TPO on AGM-S3 cells decreased with the culture period, we next examined reconstitution ability of day 7-cultured JMML CD34$^+$ cells. After 2.3 to 6×10^5 CD34$^+$ cells from 3 patients were injected into the femoral bone marrow cavity of 8 irradiated NOG mice (3 mice for *PTPN11* 1508G>C, 3 mice for *NRAS* 38G>A and 2 mice for monosomy 7), all mice were treated with human GM-CSF after transplantation (intraperitoneal injection of 5 µg of human GM-CSF per mouse 3 times per week), based on the report of Lapidot et al (ref. 5). Up to 4 weeks, $29.1 \pm 29.5\%$ (7.3% to 84.3%) of human CD45$^+$ cells were detected in BM cells of the injected right femur of all 5 recipient mice transplanted with

the cultured CD34$^+$ cells of 2 patients (*PTPN11* 1508G>C and monosomy 7).

Approximately 10% to 25% of human CD45$^+$ cells were positive for human CD34

antigen. The representative results are shown in Figure 7. Human CD45$^+$ cells infiltrated

into BM cells of the non-injected left femur. In BM cells of 2 NOG mice transplanted

with day 7-cultured CD34$^+$ cells of a patient with *NRAS* 38G>A, human CD45$^+$ cells were

detected at approximately 10% at 2 weeks after transplantation, but decreased to the

negligible levels at 4 weeks following transplantation. Additionally, there was no

substantial post-transplant growth of human CD34$^+$ cells in these 2 mice. In the

remaining mouse, neither human CD45$^+$ cells nor CD34$^+$ cells were found up to 4 weeks

after transplantation. PCR products of the mutated *PTPN11* gene, but not those of the

mutated *NRAS* gene, were detected in murine BM cells at 4 weeks after transplantation.

Discussion

   The first definitive long-term repopulating hematopoietic stem cells emerge from and

undergo rapid expansion in the embryonic AGM region. A stromal cell line, AGM-S3,

established from the AGM region of a 10.5-day postcoitum mouse embryo, has been

shown to support survival/self-renewal of human CB long-term repopulating

hematopoietic stem cells with the potential to reconstitute hematopoiesis in NOD/SCID

mice (ref. 6). In the present study, AGM-S3 cells in concert with SCF+TPO increased the

numbers of CD34$^+$ cells from 9 JMML patients with either *PTPN11* mutations or *RAS*

mutations who underwent allogeneic HSCT to approximately 20-fold of the input value

after 2 weeks of culture. Among subpopulations of CD34$^+$ cells, CD34$^+$CD38$^-$ cells had

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the highest potential to generate CD34$^+$ cells on day 14. The ability of OP9 cells (established from a newborn B6C3F1-op/op mouse calvaria) to support JMML CD34$^+$ cell expansion in the presence of SCF+TPO was one-third of the value obtained with AGM-S3 cells. The expansion potential of MS-5 cells (established from C3H/HeN S1c adult mouse bone marrow cells) was one-tenth. It is of particular interest that a large proportion of day 14-cultured CD34$^+$ cells grown on AGM-S3 cells were negative for CD38, whereas a majority of day 14-cultured CD34$^+$ cells grown on MS-5 cells were positive for it. In OP9 cells, the proportion of the CD38-negative subpopulation in cultured CD34$^+$ cells was approximately 70%. These results imply that, in the presence of SCF+TPO, JMML CD34$^+$ cells can expand on fetal murine stromal cells to a greater extent than on adult murine stromal cells. We detected *PTPN11* or *RAS* mutations in DNA extracted from dried blood on Guthrie cards from all 7 patients with JMML whose diagnosis was made at the age of 2 to 19 months, suggesting that the JMML clone arises prior to birth (ref. 8). Taking these findings together, the fetal microenvironment may play an important role in the initiation/development of JMML.

Yoshida et al. (ref. 17) demonstrated that the overall survival of patients with *PTPN11* mutations was significantly inferior to that of patients with *RAS* mutation or without any aberrations. Furthermore, the presence of *PTPN11* mutations was the factor most significantly associated with overall survival after allogeneic HSCT. Comparative analysis revealed no significant difference in the mean fold increase of CD34$^+$ cells induced under stimulation with SCF+TPO on AGM-S3 cells between JMML patients with *PTPN11* mutations and those with *RAS* mutations. A prognostic difference may not

result from the proliferative potential of leukemic CD34$^+$ cells conferred by the transforming ability of the genetic mutations on the GM-CSF pathway.

Lapidot et al. (ref. 5) described that CD34$^+$CD38$^-$ cells can initiate the disease after transplantation of PB, BM, or spleen cells from JMML patients into sublethally irradiated SCID mice. In the present study, a large proportion of day 14-cultured CD34$^+$ cells grown with SCF+TPO on AGM-S3 cells from JMML patients were negative for CD38 antigen. There was, however, no significant hematopoietic reconstitution ability of day 14-cultured JMML CD34$^+$ cells injected intravenously into NOG mice. Since CXCR4 necessary for homing and engraftment (ref. 18) was not detectable on day 14-cultured JMML CD34$^+$CD38$^-$ cells, these CD34$^+$ cells might decrease or lose the homing ability. On the other hand, day 7-cultured CD34$^+$ cells from 2 of 3 patients with JMML injected intrafemorally into irradiated NOG mice followed by intraperitoneal administration of human GM-CSF displayed significant engraftment of JMML CD45$^+$ cells including CD34$^+$ cells. These results suggest that cultured CD34$^+$ cells grown by SCF+TPO on AGM-S3 cells contained transplantable JMML stem cells.

GM-CSF also augmented the proliferation of JMML CD34$^+$CD38$^-$ cells on AGM-S3 cells in culture. According to flow cytometric analysis, both fresh JMML CD34$^+$CD38$^-$ cells and day 14-cultured CD34$^+$CD38$^-$ cells generated with SCF+TPO on AGM-S3 cells possessed GM-CSF receptor alpha on their surface. Additionally, some day 14-cultured CD34$^+$CD38$^-$ cells formed GM colonies in the presence of GM-CSF alone. Collectively, GM-CSF may play an indispensable role at the leukemic stem cell level as well as the progenitor level in JMML.

The karyotype and genetic mutation of day 14-cultured cells and GM colonies were identical to those of marrow cells at diagnosis in all 7 JMML patients examined. It is important that cryopreserved day 14-cultured CD34$^+$ cells could retain proliferative potential. Accordingly, our culture system of JMML CD34$^+$ cells with the murine stromal cells in the presence of hematopoietic growth factors may provide a useful tool for elucidating leukemogenesis and for therapeutic approaches in this disorder.

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Conflict-of-interest disclosure

The authors declare no competing financial interests.

References

1. Koike K, Matsuda K. Recent advances in the pathogenesis and management of juvenile myelomonocytic leukaemia. Br J Haematol. 2008; 141: 567-75.

2. Loh ML. Recent advances in the pathogenesis and treatment of juvenile myelomonocytic leukaemia. Br J Haematol. 2011; 152: 677-87.

3. Sakaguchi H, Okuno Y, Muramatsu H, Yoshida K, Shiraishi Y, Takahashi M et al. Exome sequencing identifies secondary mutations of SETBP1 and JAK3 in juvenile myelomonocytic leukemia. Nat Genet. 2013; 45: 937-41.

4. Sawai N, Koike K, Higuchi T, Ogami K, Oda M. Thrombopoietin enhances the production of myeloid cells, but not megakaryocytes, in juvenile chronic myelogenous leukemia. Blood. 1998; 91: 4065-73.

5. Lapidot T, Grunberger T, Vormoor J, Estrov Z, Kollet O, Bunin N et al. Identification of human juvenile chronic myelogenous leukemia stem cells capable of initiating the disease in primary and secondary SCID mice. Blood. 1996; 88: 2655-64.

6. Xu MJ, Tsuji K, Ueda T, Mukouyama YS, Hara T, Yang FC et al. Stimulation of mouse and human primitive hematopoiesis by murine embryonic aorta-gonad-mesonephros-derived stromal cell lines. Blood. 1998; 92: 2032-40.

7. Ma F, Kambe N, Wang D, Shinoda G, Fujino H, Umeda K et al. Direct development of functionally mature tryptase/chymase double-positive connective tissue-type mast cells from primate embryonic stem cells. Stem Cells. 2008; 26: 706-14.

8. Matsuda K, Sakashita K, Taira C, Tanaka-Yanagisawa M, Yanagisawa R, Shiohara M et al. Quantitative assessment of PTPN11 or RAS mutations at the neonatal period and during the clinical course in patients with juvenile myelomonocytic leukaemia. Br J Haematol. 2010; 148: 593-9.

9. Sakashita K, Koike K, Kinoshita T, Shiohara M, Kamijo T, Taniguchi S et al. Dynamic DNA methylation change in the CpG island region of p15 during human myeloid development. J Clin Invest. 2001; 108: 1195-204..

10. Sawai N, Koike K, Ito S, Mwamtemi HH, Kurokawa Y, Kinoshita T et al. Neutrophilic cell production by combination of stem cell factor and thrombopoietin from CD34(+) cord blood cells in long-term serum-deprived liquid culture. Blood. 1999; 93: 509-18.

11. Ito M, Hiramatsu H, Kobayashi K, Suzue K, Kawahata M, Hioki K et al. NOD/SCID/gamma(c)(null) mouse: an excellent recipient mouse model for engraftment of human cells. Blood. 2002; 100: 3175-82.

12. Hiramatsu H, Nishikomori R, Heike T, Ito M, Kobayashi K, Katamura K et al. Complete reconstitution of human lymphocytes from cord blood CD34+ cells using the NOD/SCID/gammacnull mice model. Blood. 2003; 102: 873-80.

13. Mazurier F, Doedens M, Gan OI, Dick JE. Rapid myeloerythroid repopulation after intrafemoral transplantation of NOD-SCID mice reveals a new class of human stem cells. Nat Med. 2003; 9: 959-63.

14. Matsuda K, Shimada A, Yoshida N, Ogawa A, Watanabe A, Yajima S et al. Spontaneous improvement of hematologic abnormalities in patients having juvenile myelomonocytic leukemia with specific RAS mutations. Blood. 2007; 109: 5477-80.

15. Matsuda K, Matsuzaki S, Miki J, Hidaka E, Yanagisawa R, Nakazawa Y et al. Chromosomal change during 6-mercaptopurine (6-MP) therapy in juvenile myelomonocytic leukemia: the growth of a 6-MP-refractory clone that already exists at onset. Leukemia. 2006; 20: 485-90.

16. Matsuda K, Yoshida N, Miura S, Nakazawa Y, Sakashita K, Hyakuna N et al. Long-term haematological improvement after non-intensive or no chemotherapy in

juvenile myelomonocytic leukaemia and poor correlation with adult myelodysplasia spliceosome-related mutations. Br J Haematol. 2012; 157: 647-50.

17. Yoshida N, Yagasaki H, Xu Y, Matsuda K, Yoshimi A, Takahashi Y et al. Correlation of clinical features with the mutational status of GM-CSF signaling pathway-related genes in juvenile myelomonocytic leukemia. Pediatr Res. 2009; 65: 334-40.

18. Lapidot T, Kollet O. The essential roles of the chemokine SDF-1 and its receptor CXCR4 in human stem cell homing and repopulation of transplanted immune-deficient NOD/SCID and NOD/SCID/B2m(null) mice. Leukemia 2002; 16: 1992-2003.

Figure Legends

Figure 1 In vitro expansion of JMML CD34$^+$ cells under stimulation with SCF+TPO or GM-CSF on AGM-S3 cells

Numbers of CD34$^+$ cells generated from 1×10^4 PB CD34$^+$ cells of a JMML patient with *PTPN11* mutation (1508G>C) and a JMML patient with *NRAS* mutation (35G>A) in 10% FBS-containing culture supplemented with either 10 ng/mL SCF plus 10 ng/mL TPO or 10 ng/mL GM-CSF on irradiated AGM-S3 cells were evaluated on days 14 and 28. (A) Day 14-cultured cells were stained with FITC-conjugated anti-CD34 mAb. (B) Solid circles, numbers of CD34$^+$ cells; open circles, numbers of total cells.

Figure 2 Comparison of SCF+TPO-dependent proliferative potentials on AGM-S3 cells among JMML, CB, and BM CD34$^+$ cells

Fold increase of CD34$^+$ cells under stimulation with SCF+TPO on AGM-S3 cells on day 14 was compared between 9 JMML patients who received HSCT (patients no. 1 to 9) and 4 JMML patients who achieved spontaneous hematological improvement (patients no. 10 to 13). Patient no. 1, *PTPN11* 227A>G; patients no. 2, 3, and 5, *PTPN11* 226G>A; patient no. 4, *PTPN11* 1508G>C; patient no. 6, *NRAS* 182A>G; patient no. 7, *NRAS* 38G>A; patient no. 8, *NRAS* 37G>C; patient no. 9, *NRAS* 35G>A. Patient no. 10 (*KRAS* 34G>A) was examined 1 year (10[a]) and 7 years (10[b]) after the diagnosis of JMML. Patients no. 11 to 13 (NRAS 34G>A) were examined 5 to 8 years after the diagnosis. The proliferative potential of CB and BM CD34$^+$ cells was also evaluated.

Figure 3 Proliferative potential of 4 subpopulations of CD34$^+$ cells under stimulation with SCF+TPO on AGM-S3 cells

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(A) One $\times$ 10^4 CD34$^+$CD38$^-$ cells, CD34$^+$CD38$^+$ cells, CD34$^-$CD38$^+$ cells, or

CD34$^-$CD38$^-$ cells sorted from PBMNCs of a patient with *PTPN11* mutation (226G>A)

were cultured in the presence of SCF+TPO on AGM-S3 cells. (B) On day 14, fold

increases of total cells and CD34$^+$ cells for the input values were determined. (C) We also

examined CD38 expression on cultured CD34$^+$ cells.

Figure 4 Expression of CD38 antigen on day 14-cultured CD34$^+$ cells generated in

the presence of SCF+TPO with or without AGM-S3 cells

A great majority of cultured CD34$^+$ cells generated with SCF+TPO on AGM-S3 cells

were negative for CD38 antigen. The results shown were derived from one representative

experiment out of nine.

Figure 5 Expression of CD116, CD117, CD123, CD90, and CXCR4 on day

14-cultured JMML CD34$^+$CD38$^-$ cells generated with SCF+TPO on AGM-S3 cells

According to flow cytometric analysis, the expression levels of CD116, CD117, CD123,

CD90, and CXCR4 on day 14-cultured JMML CD34$^+$CD38$^-$ cells generated with

SCF+TPO on AGM-S3 cells were compared with the values of fresh CD34$^+$CD38$^-$ cells

obtained from PBMNCs at diagnosis of the patients.

Figure 6 Different potential of 3 types of murine stromal cells to expand JMML

CD34$^+$ cells in concert with SCF+TPO

We examined whether JMML CD34$^+$ cells exhibited different proliferation among three

types of murine stromal cells (AGM-S3, OP9, and MS-5 cells) under stimulation with

SCF+TPO. At 2 weeks of culture, the fold increase and CD38 expression of CD34$^+$ cells

were compared.

Figure 7 Flow cytometric analysis of BM cells of NOG mice transplanted with day 7-cultured CD34$^+$ cells of a patient with JMML

For flow cytometric analysis, anti-mouse CD45 mAb (APC), anti-human CD45 mAb (FITC), and anti-human CD34 mAb (PE) were used. Significant growth of human CD45$^+$ cells and CD34$^+$ cells was found in murine BM cells at 2 weeks after transplantation of day 7-cultured CD34$^+$ cells from a patient with JMML (*PTPN11* 1508G>C).
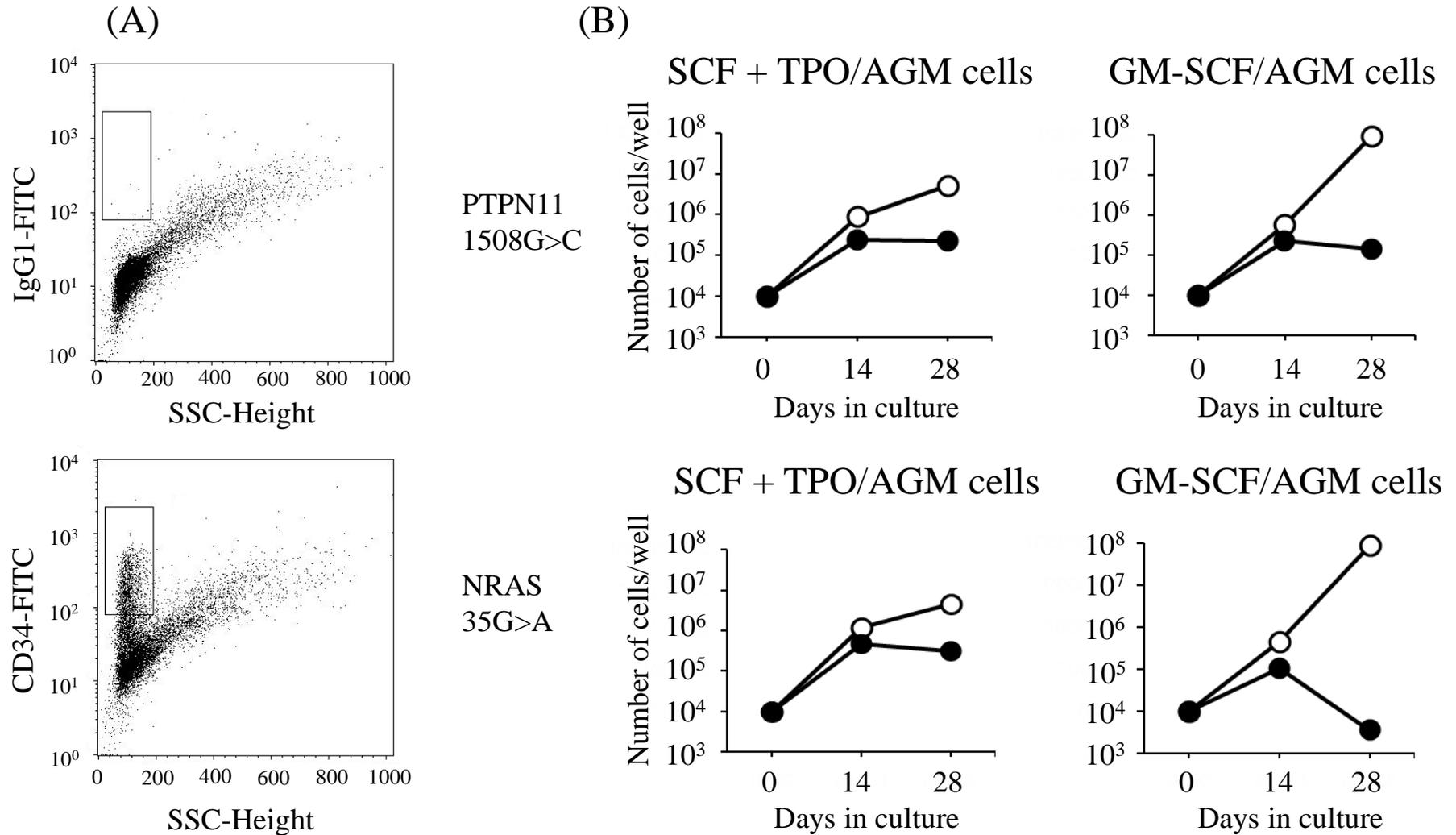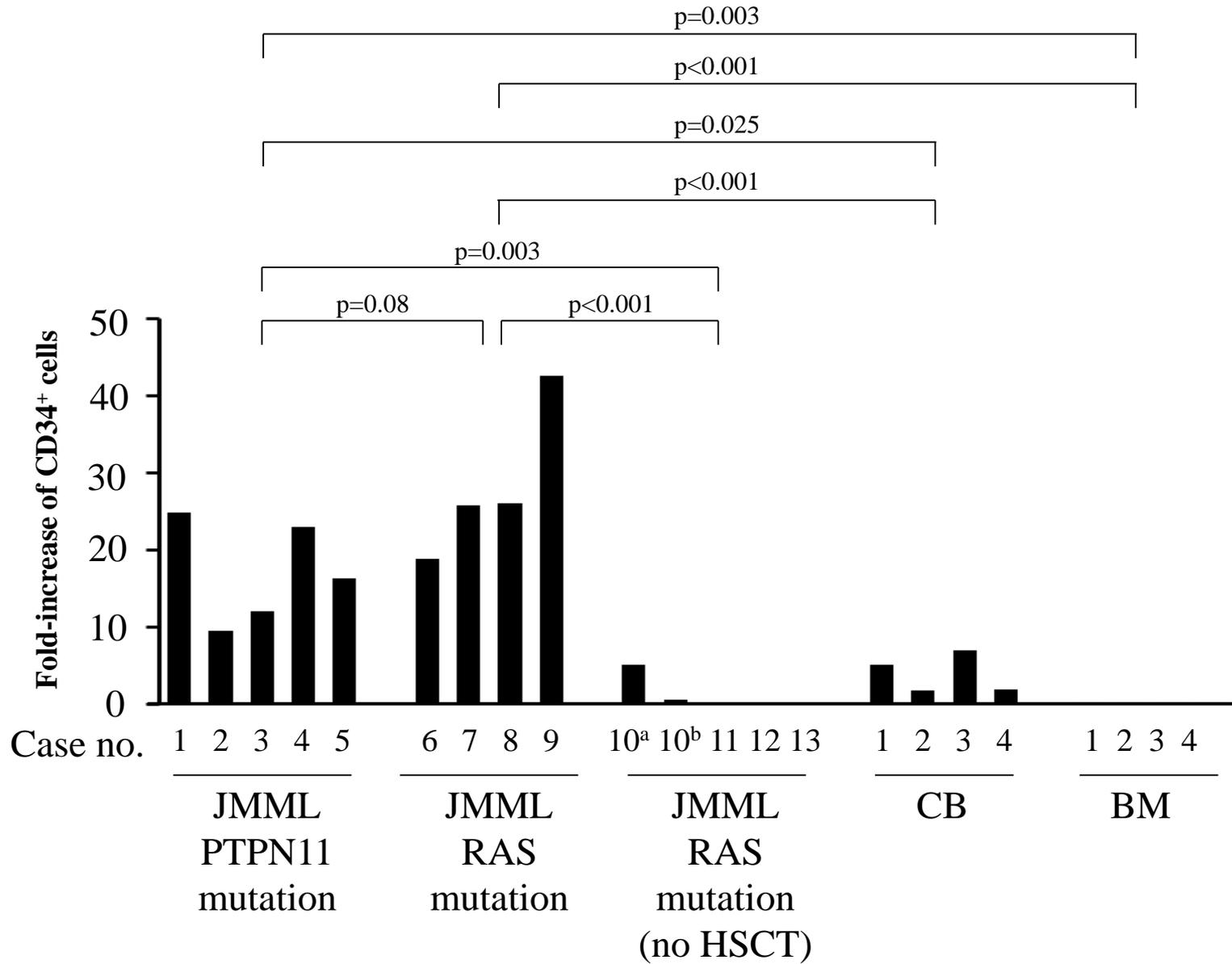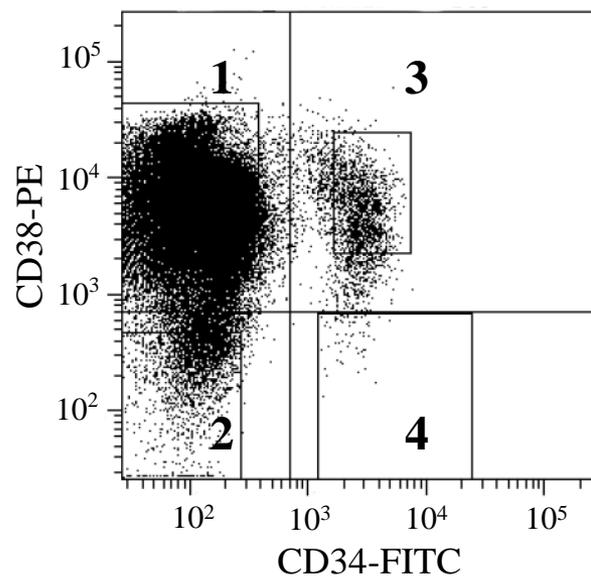
Figure 1

Figure 2

Figure 3

(A)

(B) Generation of CD34+ cells after 14 days
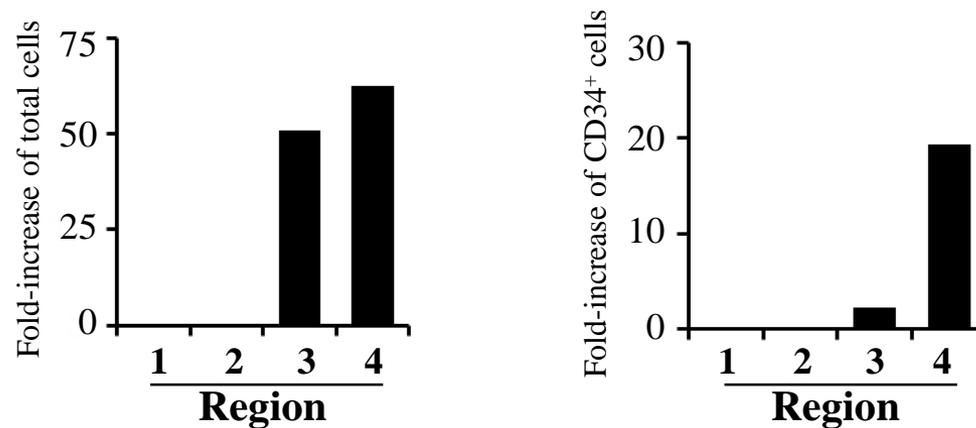
(C) CD38 expression on day 14-cultured CD34+ cells

Region 3

0.1%

3.6%

Region 4

0.2%

18.7%
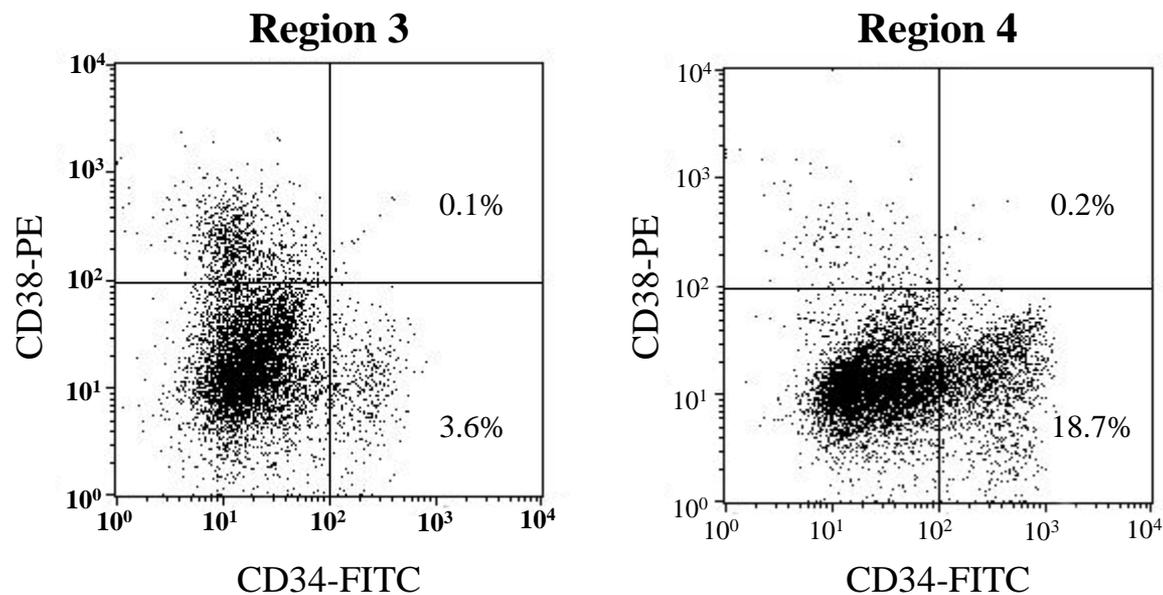
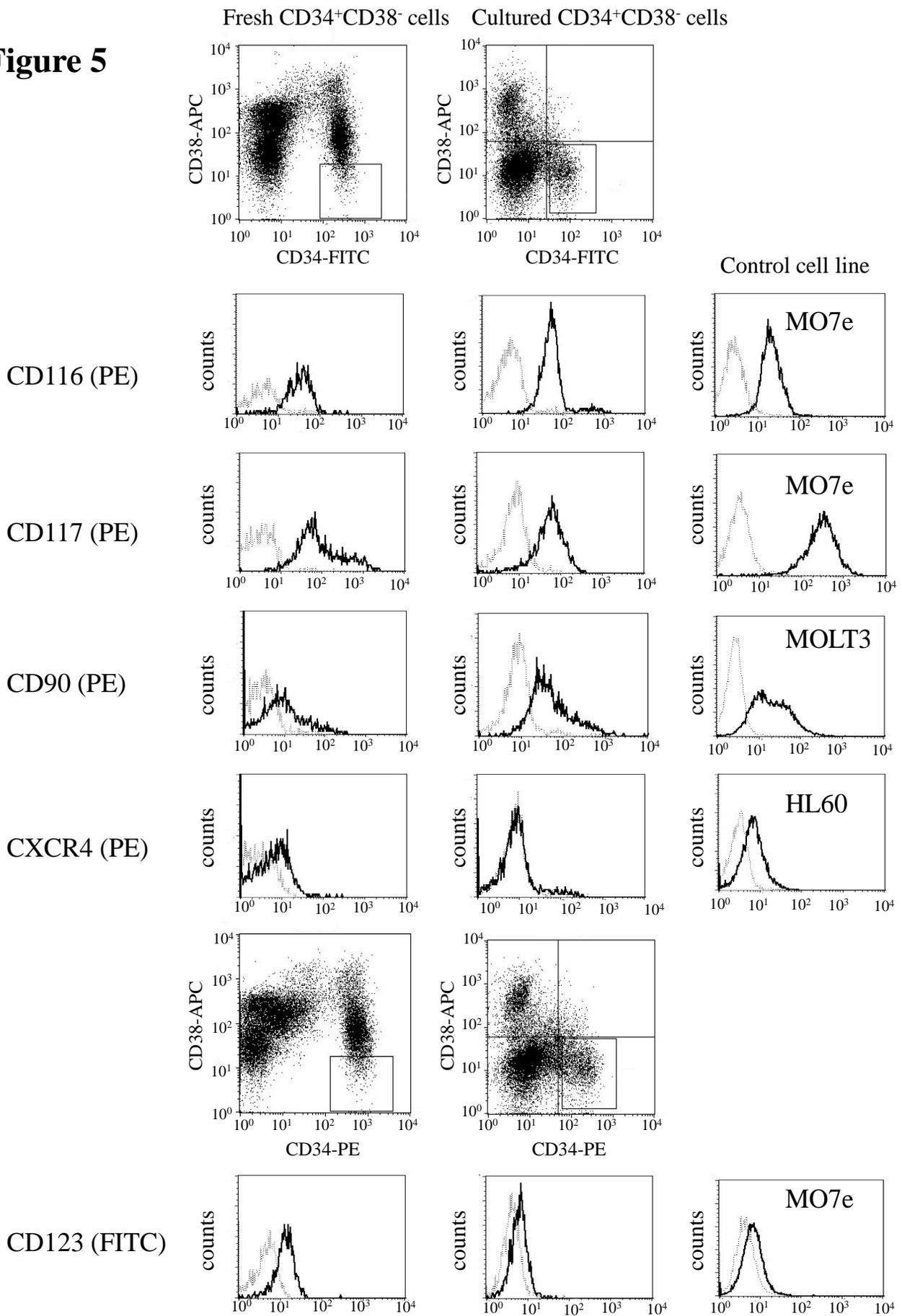# Figure 4

(A) SCF + TPO

(B) SCF + TPO / AGM cells

Figure 5

Fresh CD34⁺CD38⁻ cells   Cultured CD34⁺CD38⁻ cells

Control cell line

CD116 (PE) — MO7e

CD117 (PE) — MO7e

CD90 (PE) — MOLT3

CXCR4 (PE) — HL60

CD123 (FITC) — MO7e

Figure 6

(A)

(B)

Figure 7