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Rap1 signal modulators control the maintenance of hematopoietic progenitors in bone marrow and adult long-term hematopoiesis

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Adult long-term hematopoiesis depends on sustaining hematopoietic stem/progenitor cells (HSPC) in bone marrow (BM) niches, where their balance of quiescence, self-renewal, and hematopoietic differentiation is tightly regulated. Although various BM stroma cells that produce niche factors have been identified, regulation of the intrinsic responsiveness of HSPC to the niche factors remains elusive. We previously reported that mice deficient for *Sipa1*, a Rap1 GTPase-activating protein, develop diverse hematopoietic disorders of late onset. Here we showed that transplantation of BM cells expressing membrane-targeted *C3G (C3G-F)*, a Rap1 GTP/GDP exchanger, resulted in the progressive decline of the numbers of HSPC repopulated in BM with time and impaired long-term hematopoiesis of all cell lineages. *C3G-F*/HSPC were sustained for months in spleen retaining hematopoietic potential, but these cells inefficiently contributed to overall hematopoietic reconstitution. *C3G-F*/HSPC showed enhanced proliferation and differentiation with accelerated progenitor cell exhaustion in response to stem cell factor (SCF). Using a Ba/F3 cell line, we confirmed that the increased basal Rap1GTP levels with *C3G-F* expression caused a markedly prolonged activation of c-Kit receptor and downstream signaling through SCF ligation. A minor population of *C3G-F*/HSPC also showed enhanced proliferation in the presence of thrombopoietin (TPO) compared to *Vect*/HSPC. Current results suggest an important role of basal Rap1 activation status of HSPC in their maintenance in BM for sustaining long-term adult hematopoiesis.

KEYWORDS
bone marrow transplantation, c-Kit, hematopoietic progenitor, rap signal, stem cell factor

1 | INTRODUCTION

Long-term hematopoietic reconstitution with BMT depends on the proper homing of transplanted HSC and their repopulation at specific niche sites in the BM microenvironment.[1,2] The process involves a number of complex molecular interactions between the HSC and niche stroma cells, which produce a series of crucial hematopoietic factors called 'niche factors' represented by SCF and CXCL12.[3] Although the expression of niche factors is not confined to hematopoietic tissues, their production in the context of

Abbreviations: 5-FU, 5-fluorouracil; ATx, adult-thymectomized; BM, bone marrow; BMC, bone marrow cell; BMT, bone marrow transplantation; C3G-F, farnesylated form of C3G; CFU, colony-forming unit; CM, conditioned medium; CTV, Cell Trace Violet; CXCL12, chemokine C-X-C motif ligand 12; EGFP, enhanced green fluorescent protein; EMH, extramedullary hematopoiesis; EPO, erythropoietin; GAP, GTPase-activating protein; GEF, GTP-GDP exchange factor; HSC, hematopoietic stem cell; HSPC, hematopoietic stem/progenitor cell; IL, interleukin; IRES, internal ribosome entry site; LSK, Lin^- $Sca\text{-}1^+$ $c\text{-}Kit^+$ cell; MDS, myelodysplastic syndrome; MPN, myeloproliferative neoplasm; PB, peripheral blood; PTK, protein tyrosine kinase; SCF, stem cell factor; T-ALL, T-cell acute lymphoblastic leukemia; TPO, thrombopoietin.

hematopoietic niches is essential for constitutive hematopoiesis from HSPC in adults.[3] Recent extensive studies have identified various cellular components comprising functional hematopoietic niches and producing niche factors in an overlapping manner in BM.[3,4] Accordingly, adult long-term hematopoiesis may also depend on the expression of niche factor receptors and their signaling in HSPC.[5-8] In ontogeny, it is reported that the cycling states of HSC in fetal liver are markedly altered soon after birth in BM partly as a result of changes in the responsiveness to niche factor SCF.[9] The balance of quiescence and proliferation/differentiation of HSPC is tightly controlled at steady state hematopoiesis,[10,11] but the intrinsic regulation of cell signaling in response to niche factors in adult HSPC remains elusive.

Rap1 is a molecular switch of the Ras family that regulates cell signaling through diverse cellular receptors; it is activated to a GTP form by specific GEF linked to various receptors, whereas it is swiftly inactivated to a GDP form by GAP, represented by C3G and Sipa1, respectively.[12,13] Although C3G is expressed rather ubiquitously in many tissues, Sipa1 is predominantly expressed in hematopoietic tissues, including both hematopoietic cells[12] and stroma cells.[14,15] We previously reported that *Sipa1*-deficient mice develop quite diverse late-onset hematopoietic disorders, ranging from pancytopenia resembling MDS to overt MPN.[16] We also reported that BM HSPC in *Sipa1*$^{-/-}$ mice tended to be decreased with increased genetic stress preceding overt hematopoietic disorders.[17] As wild-type BMC also developed MDS/MPN of late onset in *Sipa1*$^{-/-}$ recipients, the overall effects in *Sipa1*-deficiency were, in part, attributed to dysregulated niche cell function.[15] Thus, involvement of Rap1 signaling in regulating HSPC per se remains to be verified.

In the current study, we directly addressed the role of Rap1 signaling in HSPC using C3G-F, which is autonomously targeted to membranes and activates endogenous Rap1 signaling bypassing extracellular stimuli.[18] We show that adult *C3G-F*/HSPC show splenic preference and decreasing sustenance in BM with time after BMT, resulting in compromised long-term hematopoiesis. Expression of *C3G-F* caused a markedly prolonged activation of SCF-mediated c-Kit receptor and the downstream signaling pathways, resulting in enhanced proliferation and differentiation with apparent exhaustion of the progenitor pool. We propose that basal Rap1 activation status in HSPC regulates their sustenance in the BM, in part, by regulation of the responsiveness to SCF.

2 | MATERIALS AND METHODS

2.1 | Mice

C57BL/6 (B6) CD45.2 and congenic B6 CD45.1 mice were purchased from Japan SLC (Shizuoka, Japan). All mice were maintained under specific pathogen-free conditions at the Institute of Laboratory Animals, Graduate School of Medicine, Kyoto University, Kyoto, Japan, according to the University's guidelines for the treatment of animals. All protocols were approved by the committee on the ethics of animal experiments of Kyoto University (Permit Number: MedKyo14049).

2.2 | Cell lines

Interleukin-3-dependent Ba/F3 cell line stably transfected with *c-Kit* (c-Kit-Ba/F3) was provided by Dr Tetsuo Sudo, Torey Research Institute, Torey Co. Ltd, Fujisawa, Japan, and was maintained in RPMI-1640 supplemented with 10% FCS, 10^{-5} mol/L 2-mercaptoethanol, antibiotics, and 10% WEHI3 cell CM. The c-Kit-Ba/F3 cell line was infected with IRES-EGFP-retroviral plasmid, either empty or containing *C3G* cDNA tagged with a CAAX motif of K-Ras at the C-terminus (*C3G-F*)[18] and was similarly maintained in the presence of WEHI3 CM, in which both cell lines proliferated comparably. For stimulation with SCF, *Vect/* and *C3G-F*/c-Kit-Ba/F3 cells were washed four times with PBS, starved for 4 hours in the absence of growth factors, and cultured in the presence of 100 ng/mL SCF (Peprotech, Rocky Hill, NJ, USA). A hematopoietic stroma cell line, OP9, was maintained in α-minimum essential medium (MEM; Invitrogen, Carlsbad, CA, USA) supplemented with 20% FBS as described previously.[19]

2.3 | Flow cytometry

Multicolor flow cytometry analysis was carried out with FACSCanto II (Becton Dickinson, San Jose, CA, USA) as previously described.[20] Antibodies included anti-CD150 (TC15-12F12.2), anti-CD48 (HM48-1), anti-c-Kit (2B8), anti-Sca1 (D7), anti-CD45 (30-F11), anti-CD45.1 (A20), and anti-CD45.2 (104). Lineage marker antibodies included PE or biotin-conjugated anti-NK1.1 (PK136), anti-Mac1 (M1/70), anti-B220 (RA3-6B2), anti-CD19 (1D3, 6D5, or MB19-1), anti-CD4 (GK1.5), anti-CD8a (53-6.7), anti-CD3e (145-2C11), anti-Gr1 (RB6-8C5) and anti-TER119 (TER119). Biotin-conjugated antibodies were visualized using APC, APC-C7, or BV510-conjugated streptavidin. All antibodies were purchased from BioLegend (San Diego, CA, USA), BD Biosciences (San Jose, CA, USA), or Thermo Fisher Scientific (Waltham, MA, USA). To detect cell divisions, the cells were labeled with CTV (Thermo Fisher Scientific) according to manufacturer's instructions before culture, and the cell divisions were evaluated by dilution of CTV with FACSCanto II. For analysis of cell cycle status, sorted Lin$^-$ BM cells were stained with APC-Cy7 conjugated streptavidin and antibodies against Sca-1 and c-Kit, fixed, and permeabilized, followed by intracellular staining with anti-Ki-67 (SolA15) and 7-AAD, with the use of APC BrdU Flow Kit (BD Biosciences).

2.4 | Retroviral infection and bone marrow transplantation

Bone marrow cells were harvested from 4- to 8-week-old mice that had been treated with 150 mg/kg 5-FU (Kyowa Hakko Kirin, Tokyo, Japan) and the lineage-negative (Lin$^-$) population was enriched by depleting Lin$^+$ cells with the use of a cocktail of antibodies containing anti-CD4,

anti-CD8, anti-Gr1, anti-Mac1, anti-B220, and anti-TER119 and anti-Rat IgG-coated magnetic beads (Dynabeads M-450; Thermo Fisher Scientific). Lin$^-$ BMC were infected with either empty, *Sipa1*- or *C3G-F*-containing IRES-EGFP-retroviral plasmid using Lipofectamine 2000 (Thermo Fisher Scientific), and were cultured for 48 hours in complete BXH-2 medium in the presence of 50 ng/mL SCF, 10 ng/mL Flt-3 ligand, 10 ng/mL IL-6 (Wako, Osaka, Japan) and 10 ng/mL IL-11 (Peprotech) as previously reported.[18] Infection efficiencies were 42.4 ± 9.6% for empty virus and 39.6 ± 8.8% for *C3G-F*-retrovirus, with essentially similar intensities of GFP expression. GFP$^+$ cells or GFP$^+$ LSK were sorted with FACSAria II and transplanted i.v. into 8.5 Gy γ-ray irradiated B6 mice at 2 × 10^4 cells/mouse. Expression levels of *C3G* and *Sipa1* in GFP$^+$ cells were assessed with quantitative PCR as reported previously.[13] Primers were as follows: *C3G*, 5′-CACATGCTGGCCTACATGC-3′ and 5′-CTTGTTCTTCTGCTGGTAG-3′; *Sipa1*, 5′-GACCCGGACCACAG CTTAC-3′ and 5′-GCCCAAAGAAGTTCTGGTGT-3′; and cyclophilin, 5′-GCCAACGATAAGAAGAAGGGACC-3′ and 5′-AGTCCAAAGACGAC TCGTCCTAC-3′. For analysis of long-term hematopoietic reconstitution (>3 months), B6 mice thymectomized at approximately 4 weeks of age (ATx) were used as recipients to circumvent the development of T-ALL in the thymus.[18] We never observed T-ALL development in ATx-recipients of BMT with C3G-F/BMC during the study periods. In a competitive setting, Lin$^-$ BMC from (CD45.1 × CD45.2) F1 and CD45.1 B6 mice were infected with empty and *C3G-F*-retrovirus, respectively, and the sorted GFP$^+$ cells were co-injected into irradiated B6 recipients at equal cell numbers (1 × 10^4/mouse).

2.5 | Cell cultures

Lin$^-$ BMC were infected with empty, or *C3GF*-containing retrovirus in the presence of mixed hematopoietic factors, and the GFP$^+$ LSK were sorted with FACSAria II. The sorted cells were labelled with CTV, starved for 1 hour and cultured in complete BXH-2 medium in the absence or presence of 100 ng/mL SCF or 25 ng/mL TPO in 96-well plates at 2.3 × 10^3 cells/well, or with OP9 cell monolayers at 4 × 10^3 cells/dish.

2.6 | Colony assay

Sorted GFP$^+$ LSK cells were cultured in methylcellulose medium (MethoCult 3434; Stem Cell Technologies, Vancouver, BC, Canada) containing SCF, IL-3, IL-6, and EPO at the indicated cell numbers. For the replating assay, primary colony cells were dispersed and recultured in MethoCult 3434 to develop secondary colonies under the same conditions. Colony numbers were counted on day 12. When indicated, the colony cells were dispersed and FACS-analyzed for expression of lineage markers.

2.7 | Cell migration assay

Cell migration assay was carried out using 24-well plates and 6.5-mm Transwell with 8.0-μm pore polycarbonate membrane inserts (Corning, Corning, NY, USA). When indicated, the inserts were coated with 10 μg/mL fibronectin (Takara Bio, Shiga, Japan) for 1 hour. Cells were added to the upper chamber (5 × 10^5 cells) in 1% BSA RPMI-1640 medium. The lower chambers were filled with 1% BSA RPMI-1640 containing SCF at 100 ng/mL. After 3 hours of incubation in 5% CO_2 at 37°C, the cells that migrated into the lower chambers were counted with FACSCanto (BD Biosciences).

2.8 | Immunoblotting and pull-down assay

Immunoblotting was carried out as previously described.[21] Antibodies included anti-ERK2 (C-14), anti-Akt (H-136), anti-C3G (C-19), anti-Actin (I-16) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-AKT1 (phospho S473, EP2109Y) (Abcam, Cambridge, MA, USA) and anti-phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) (Cell Signaling Technology, Danvers, MA, USA). To detect c-Kit phosphorylation, the cells were lysed with lysis buffer (25 mmol/L Tris-HCl, 150 mmol/L NaCl, 1% NP-40, 1% Na deoxycholate, 0.1% SDS, protease and phosphatase inhibitors), and immunoprecipitated with anti-c-Kit antibody (AF1356; R&D Systems, Minneapolis, MN, USA) and protein G-beads, followed by immunoblotting with anti-phosphotyrosine antibody (No. 05-321, clone 4G10; Merck, Darmstadt, Germany). Rap1GTP was assessed by a pull-down assay using an Active Rap1 Detection Kit (Cell Signaling Technology) according to manufacturer's instructions. Fold increases in the signals were determined using a densitometer with Multi Gage V3.2 software (Fujifilm, Tokyo, Japan).

2.9 | Statistical analysis

Statistical analysis was carried out using Welch's *t* test for the hematopoietic data of BMT recipients. For in vitro proliferation, migration, and colony formation data, two-tailed Student's *t* test was used.

3 | RESULTS

3.1 | Bone marrow transplantation of BMC expressing *C3G-F* results in the progressive decline of HSPC in BM with time and compromised long-term hematopoiesis

Bone marrow cells enriched for lineage-negative (lin$^-$) cells were infected with either empty (*Vect*) or *C3G-F*-containing IRES/EGFP-retrovirus. The GFP$^+$ fractions tended to contain more c-Kit$^+$ cells and less CD48$^-$ LSK than GFP$^-$ fractions for both *Vect*/BMC and *C3G*/BMC reflecting the higher retroviral infection efficiency in cycling cells, but overall phenotypic profiles of BMC were similar between GFP$^+$ and GFP$^-$ fractions in both groups (Figure S1A). It was confirmed that *C3G*/BMC showed approximately 35-fold more *C3G* transcripts than *Vect*/BMC (Figure S1B). The sorted GFP$^+$ cells were transplanted into 8.5 Gy-irradiated mice at 2 × 10^4 cells/mouse. Because the *C3G-F*/BMC recipients often developed T-ALL after 3 months,[18] ATx mice were used as recipients for analysis at terms longer than 3 months. The reconstitution rates of *Vect*/BMC and *C3G-F*/BMC in BM and spleen were comparable at 1 week after BMT (Figure 1A). At 8 weeks through 5 months, the recipients of *Vect*/

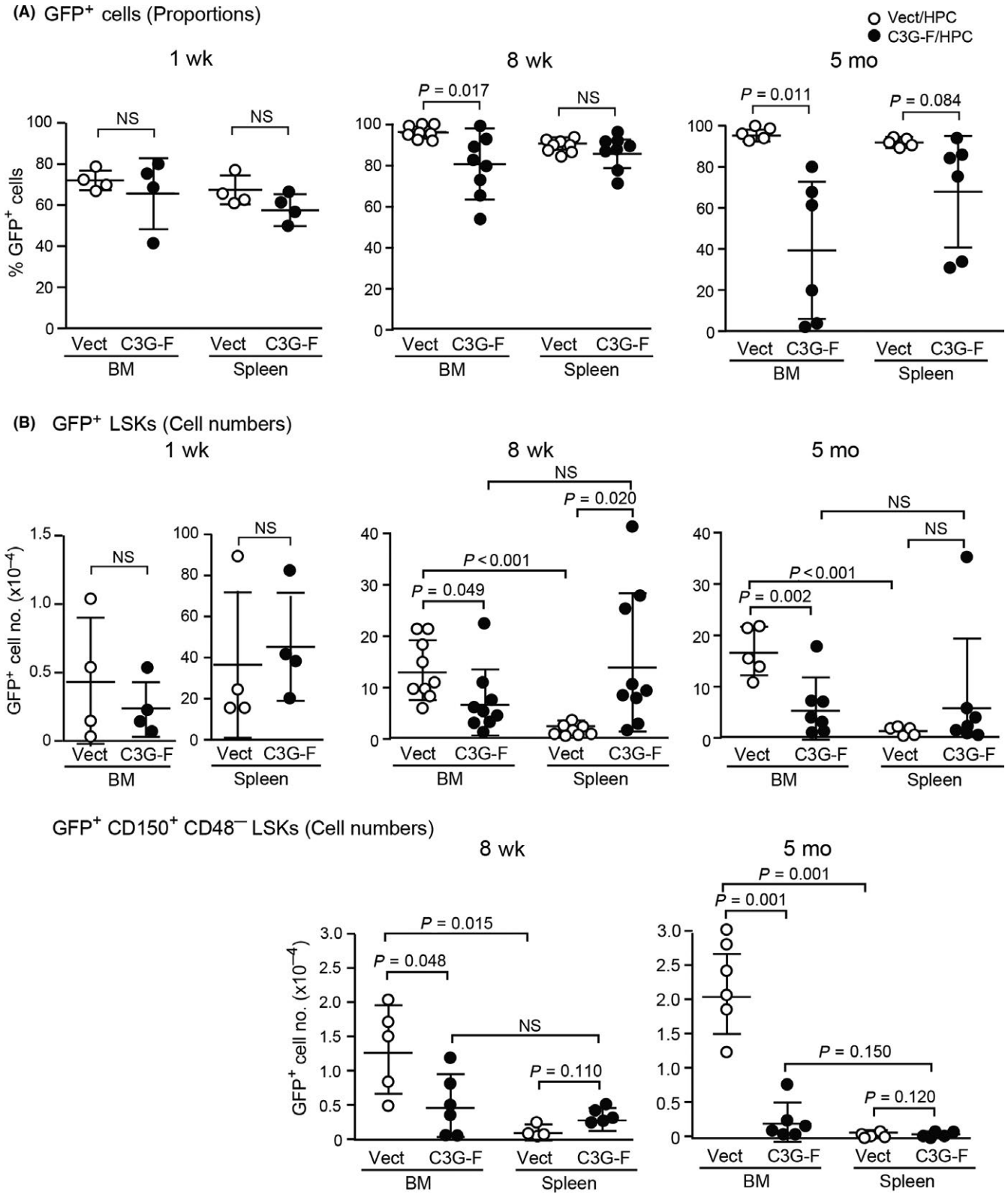
BMC showed nearly 100% reconstitution rates in the BM and spleen as expected (Figure 1A). However, the reconstitution rates in the recipients of *C3G-F*/BMC declined progressively in the BM and, to a lesser extent, in the spleen until 5 months (Figure 1A) and, concordantly, numbers of mature GFP$^+$ leukocytes in PB were significantly decreased in *C3G-F*/BM recipients compared to those in *Vect*/BM recipients (Figure S2A, left). Relative proportions of lymphoid and myeloid cells in the peripheral GFP$^+$ cell population were largely comparable between the two groups, indicating little biased effect on selective cell lineages (Figure S2A, right). At 1 week, numbers of GFP$^+$ LSK representing HSPC were comparable in the BM and spleens of *Vect*/BMC and *C3G-F*/BMC recipients, indicating no gross difference in the initial homing of HSPC to the hematopoietic organs (Figure 1B, upper). At 2 months through 5 months, *Vect*/LSK were stably repopulated in BM with negligible numbers remaining in the spleen (Figure 1B, upper, Figure S2B). However, numbers of *C3G-F*/LSK in BM were significantly less than those of *Vect*/LSK, whereas considerable *C3G-F*/LSK were retained in the spleen even at 2 months and later (Figure 1B, upper, Figure S2B). Essentially similar repopulation profiles were found for more primitive *C3G-F*/CD150$^+$ CD48$^-$ LSK that include HSC[22] (Figure 1B, lower, Figure S2B). Average overall repopulation rates of *C3G-F*/HSPC and *C3G-F*/HSC in the BM were 50% and 38% at 8 weeks, and 38% and 10% at 5 months, respectively, of the control counterparts (Figure 1B).

To confirm the findings, we carried out competitive repopulating analysis of *C3G-F*/LSK and *Vect*/LSK in the same recipients. Lin$^-$ BMC from (CD45.1 × CD45.2) B6 F1 mice and CD45.2 B6 mice were transduced with empty or *C3G-F*-retrovirus, respectively, and the sorted GFP$^+$ cells were cotransplanted at equal numbers (10^4 cells/mouse) into 8.5 Gy-irradiated B6 recipients (Figure 2A, left). We confirmed that the relative proportions of *C3G-F*$^+$ cells were consistently lower than those of *Vect*$^+$ cells in PB throughout 12 weeks after BMT (Figure 2A, right, Figure S3A). Again, the effects were evident for all hematopoietic cell lineages, except for T-lineage cells at 12 weeks (Figure S3B). Recipients without ATx were used in these experiments, and the effect on T cells probably reflected enhanced Notch-mediated T-cell development rather than the lineage commitment of HSPC, although no overt T-ALL was observed at this stage.[18] In the BM, proportions of total *C3G-F*$^+$ cells progressively decreased, and the effects were preceded by the prominent decrease of *C3G-F*/LSK and, to an even greater extent, *C3G-F*/CD48$^-$ LSK (Figure 2B, upper, Figure S3A). In the spleen, however, *C3G-F*/LSK rather predominated over *Vect*/LSK, although total GFP$^+$ cells were comparable, in agreement with their ineffective hematopoietic contribution (Figure 2B, lower). These results suggested that *C3G-F*/HSPC show splenic preference and impaired sustenance in BM, leading to compromised overall long-term hematopoiesis.

3.2 | Splenic *C3G-F*/HSPC retain hematopoietic potential comparable to BM *C3G-F*/HSPC on secondary BMT

Sustenance of *C3G-F*/LSK in the recipient spleens for more than 8 weeks after BMT was unexpected, and we addressed whether these cells, indeed, represented functional HSPC. *Vect*/Lin$^-$ BMC or *C3G-F*/Lin$^-$ BMC were transplanted into 8.5-Gy irradiated mice and, 9 weeks later, GFP$^+$ cells were recovered from the BM and spleens and retransplanted to the secondary irradiated recipients without ATx at 2×10^6 cells/mouse (Figure 3A). Because spleen cells from the *Vect*/BMC recipients contained almost negligible GFP$^+$/LSK (see Figure 1B) like normal spleen cells, we omitted this group from the study. Transplantation with splenic *C3G-F*$^+$ cells caused significant hematopoietic reconstitution in PB at 12 weeks comparable to BM *C3G-F*$^+$ cells, although the efficiency was again less than that of BM *Vect*$^+$ cells (Figure 3B, left). There was no significant lineage bias, although the T-cell proportions again tended to be higher in the non-ATx recipients of BM *C3G-F*$^+$ cells (Figure S4). Consistently, splenic *C3G-F*/LSK were significantly repopulated in the BM of the secondary recipients, to a similar extent as BM *C3G-F*/LSK (Figure 3B, center). Again, the splenic *C3G-F*/LSK were considerably retained in the spleens even at 12 weeks, whereas BM *Vect*/LSK were detected exclusively in the BM (Figure 3B, right). We also examined colony-forming activity in vitro. In the methylcellulose cultures with a mixture of hematopoietic cytokines (SCF, IL-3, IL-6, EPO), splenic *C3G-F*/LSK developed slightly fewer yet significant numbers of colonies (CFU) with Lin$^+$ myeloid cell differentiation (Figure 3C); in this culture condition, neither *Vect*/LSK nor *C3G-F*/LSK developed detectable B-cell colonies. These results indicated that the *C3G-F*/LSK that were sustained in the spleens for more than 2 months retained hematopoietic progenitor activity, being capable of initiating effective hematopoiesis upon relocation in the BM microenvironment with secondary transplantation.

FIGURE 1 Bone marrow transplantation with the farnesylated form of C3G/bone marrow cells (*C3G-F*/BMC) results in the progressive decline of BM hematopoietic stem/progenitor cells with splenic preference and compromised long-term hematopoiesis. A,B, Lin$^-$ cells enriched for hematopoietic progenitor cells (HPCs) were enriched from BMC obtained from 5-fluorouracil-treated B6 mice using magnetic beads coated with a mixture of lineage marker antibodies and were infected with empty (*Vect*) (open circles) or *C3G-F*-containing internal ribosome entry site/enhanced green fluorescent protein-retrovirus (solid circles) in BXH-2 medium supplemented with stem cell factor, interleukin (IL)-6, IL-11, and Flt3-L with spinoculation. After 48 hours, GFP$^+$ cells were sorted using FACSAria II and transplanted into 8.5 Gy-irradiated mice at 2×10^4 cells/mouse. For the analysis at 5 months after BMT, adult-thymectomized mice were used as recipients to circumvent T-cell acute lymphoblastic leukemia development from the thymus. At 1 week, 8 weeks, and 5 months after BMT, the spleen and BM cells were multi-color analyzed for the expression of GFP and hematopoietic cell markers with FACS. GFP$^+$ cell reconstitution rates in peripheral blood (A) as well as absolute GFP$^+$ cell numbers of Lin$^-$ Sca-1$^+$ c-Kit$^+$ cells (LSK) (B, upper) and CD150$^+$ CD48$^-$ LSK (B, lower) in the BM and spleens of the recipients were determined. The experiments were done independently three to five times, and pooled data are indicated. Total mouse numbers were four mice for 1 week, nine mice for 8 weeks, and six mice for 5 months for each group. Mean values and SD of individual mice are indicated. Statistical analysis was done with Welch's *t* test. NS, not significant



3.3 | *C3G-F*/LSK show accelerated proliferation/differentiation in response to SCF and TPO

We noted that surface c-Kit expression in the *C3G-F*/LSK cell population was considerably decreased compared to the control population in the presence of hematopoietic cytokines including SCF (Figure 4A). It is reported that c-Kit expression is downregulated depending on the extent of receptor ligation with SCF,[23] and this finding prompted us to examine the responsiveness of *C3G-F*/LSK cells to SCF. Lin$^-$ BMC were transduced with *Vect* or *C3G-F*, and the sorted GFP$^+$ LSK were labeled with CTV, starved for cytokines, and cultured in the presence or absence of SCF. The *C3G-F*/LSK showed significantly enhanced cellular

expansion compared to *Vect*/LSK in the presence of SCF (100 ng/mL), whereas both populations rapidly died out without SCF (Figure 4B). With CTV dye dilution analysis, we confirmed that total *C3G-F*$^+$ cells and *C3G-F*/LSK showed increased cycles of cell divisions compared to the *Vect*$^+$ counterparts at day 4, although both populations experienced more than maximally detectable cell cycles (8 times) by day 8 (Figure 4C, left). However, when the cells were gated at the most primitive CD48$^-$ LSK, a considerable proportion of *Vect*/CD48$^-$ LSK remained to experience only limited cell divisions of <4 times even at day 8, whereas the great majority of *C3G-F*/CD48$^-$ LSK underwent more than 8 times cell divisions (Figure 4C, left). In agreement with the findings, *C3G-F*/LSK showed accelerated differentiation into Lin$^+$ cells mostly consisting of Gr-1$^{+/-}$ Mac1$^+$ myeloid cells with smaller proportions of residual Lin$^-$ c-Kit$^+$ cells than those of *Vect*/LSK (Figure 4C, right). In the presence of OP9 hematopoietic stroma cells additionally expressing abundant IL-7,[24] *C3G-F*/LSK also showed markedly enhanced expansion with significant differentiation to B cells like *Vect*/LSK (Figure S5A), indicating no intrinsic bias for myeloid versus lymphoid differentiation. Further, in the methylcellulose culture containing SCF, the sorted *C3G-F*/LSK, which initially contained CFU comparable to *Vect*/LSK, developed larger but significantly fewer CFU than *Vect*/LSK, when the colony cells were replated in the secondary cultures (Figure 4D). We noted that *C3G-F*/LSK also showed enhanced cell cycling rates in culture in the presence of TPO (25 ng/mL) compared to *Vect*/LSK (Figure 4E); however, the net increase in cell numbers was observed only after a marked decrease of total GFP$^+$ cells, suggesting that the proportion responsive to TPO in the LSK was much smaller than that of SCF (Figure S5B). In any case, in agreement with the enhanced responsiveness to both SCF and TPO, GFP$^+$ LSK in the BM and spleens of BMT recipients with *C3G-F*/LSK showed higher cycling states in vivo than those in the BM of *Vect*/LSK recipients (Figure S5C). These results suggested that *C3G-F*/HSPC show significantly enhanced proliferation and differentiation in response to major niche factors such as SCF and TPO with accelerated progenitor exhaustion.

3.4 | Bone marrow transplantation with *Sipa1*/BMC shows increased HSPC repopulation in BM

To confirm that the effects of *C3G-F* expression on HSPC were directed to Rap1 signaling, we also examined the effects of *Sipa1* overexpression in BMC, which rather promotes Rap1 inactivation (Figure 5A, left). We confirmed that sorted *Sipa1*/BMC expressed approximately

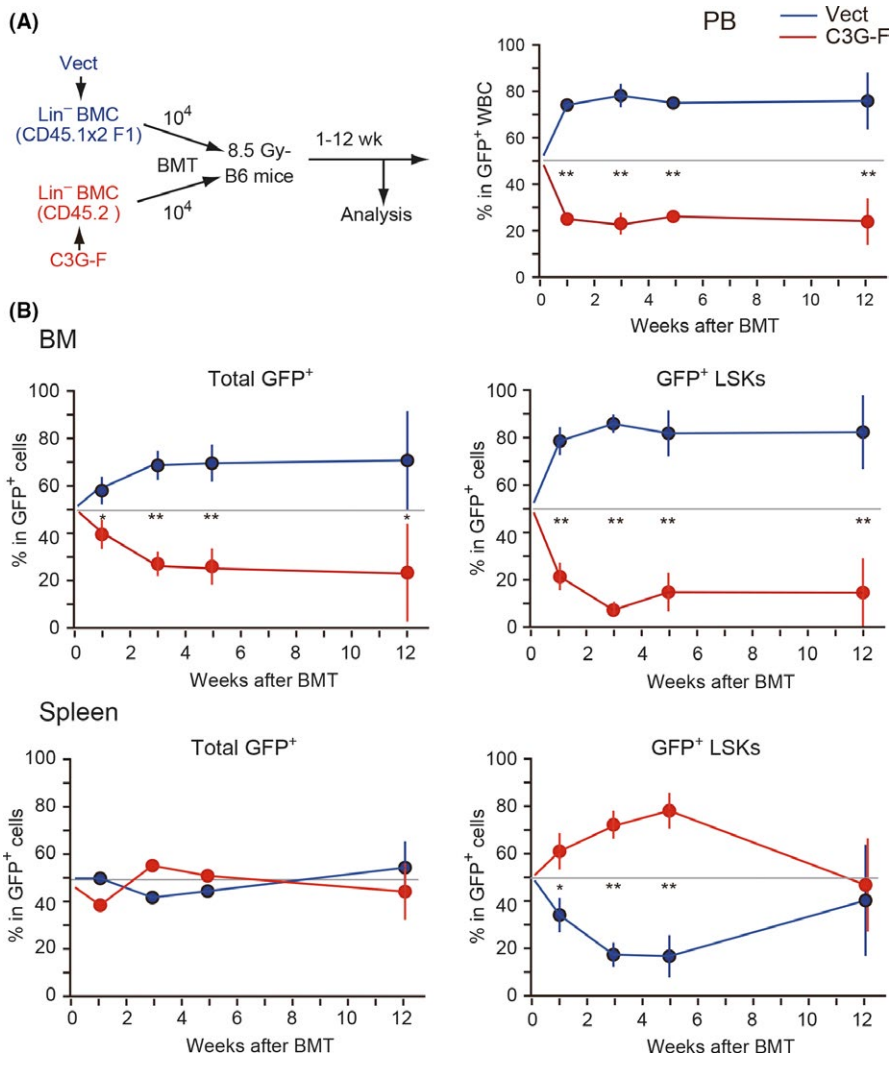


FIGURE 2 Hematopoietic reconstitution activity of the farnesylated form of C3G/bone marrow cells (*C3G-F*/BMC) is inferior to that of *Vect*/BMC in the same recipients. A, Schematic presentation of the experimental procedures (left). At varying periods after bone marrow transplantation (BMT), peripheral blood (PB) leukocytes were analyzed for the expression of GFP at the gates of CD45.1$^+$ CD45.2$^+$ (blue symbols) and CD45.1$^-$ CD45.2$^+$ (red symbols) cells, and the relative proportions of them were determined (right). B, At varying periods after BMT, the mice were sacrificed, and the BM (upper) and spleen (lower) cells were analyzed for expression of GFP in total and Lin$^-$ Sca-1$^+$ c-Kit$^+$ cells (LSK) at CD45.1$^+$ CD45.2$^+$ (blue symbols) and CD45.1$^-$ CD45.2$^+$ (red symbols) cell gates, and the relative proportions were determined. Recipient numbers were three mice for the analysis at 1, 3, and 5 weeks, and four mice for 12 weeks. Means and SD are indicated, and statistical analysis was done with Welch's *t* test. **P* < .05, ***P* < .01

55-fold more *Sipa1* transcripts than *Vect*/BMC (Figure S6A). Curiously, BMT with *Sipa1*/BMC also resulted in compromised reconstitution rates of mature peripheral leukocytes in PB, although to a lesser extent than that with *C3G-F*/BMC (Figure 5A, right). As opposed to *C3G-F*/LSK, however, numbers of *Sipa1*/LSK in the BM were significantly greater than those of *Vect*/LSK at 4 months after BMT, whereas these cells were negligible in the spleen (Figure 5B). Despite the increase in *Sipa1*/LSK, numbers of Lin^+ cells, particularly Gr-1^+ granulocytic cells, were significantly decreased in BM (Figure 5C), suggesting reduced rates of proliferation and differentiation of *Sipa1*/HSPC. Consistently,

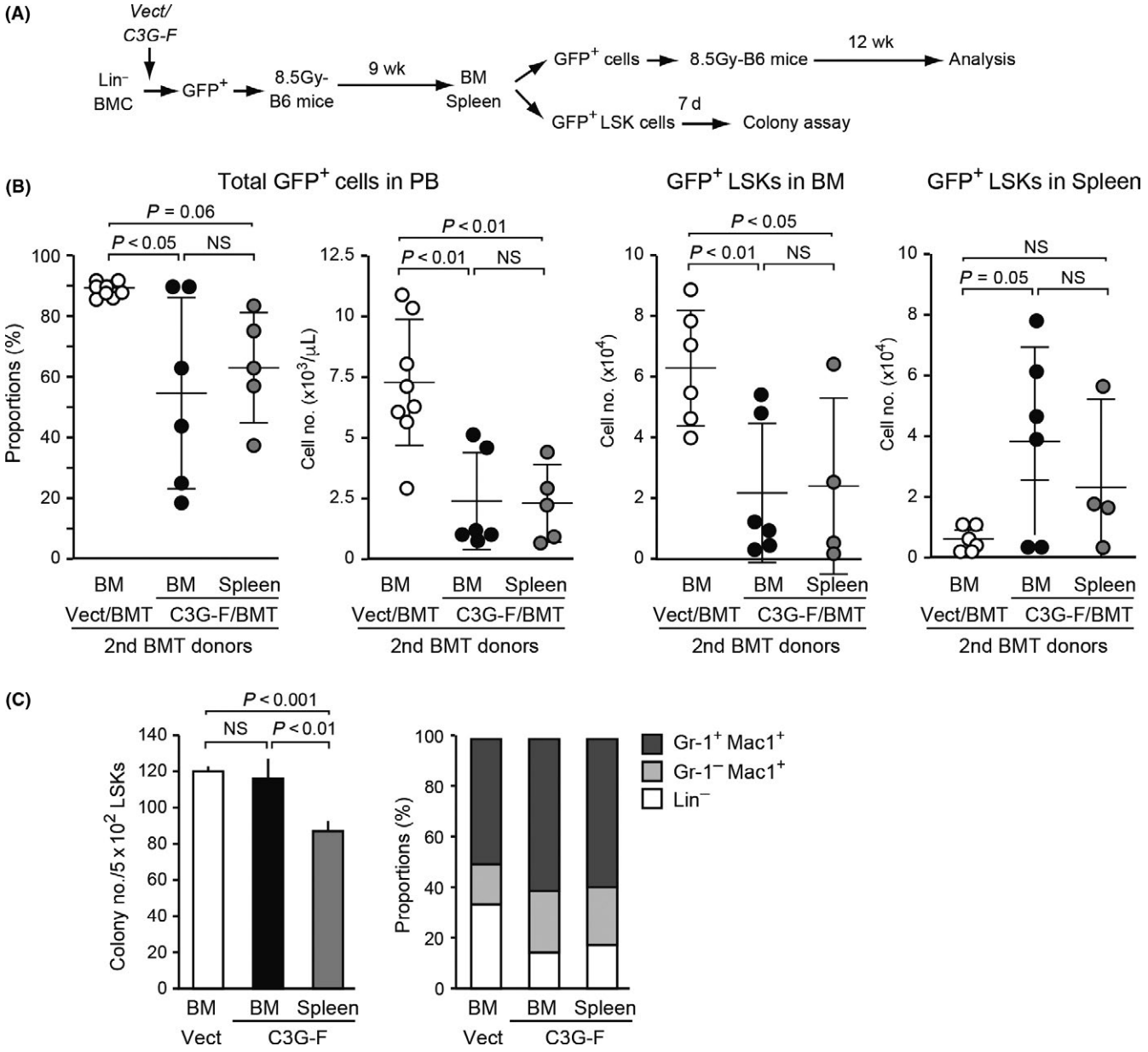


FIGURE 3 Farnesylated form of C3G/Lin^- $Sca\text{-}1^+$ c-Kit^+ cells (*C3G-F*/LSK) sustained in the spleen retain hematopoietic potential comparable to bone marrow (BM) *C3G-F*/LSK. A, Schematic presentation of the experimental procedures. B, Cells from peripheral blood (PB), BM, and spleens of the secondary recipients at 12 weeks after bone marrow transplantation (BMT) were multi-color analyzed with FACS for the expression and GFP and hematopoietic cell markers. Proportions and cell numbers of GFP^+ leukocytes in PB (left) as well as cell numbers of GFP^+ LSK in BM and spleens (right) at 12 weeks after secondary BMT were determined in the recipients of GFP^+ bone marrow cells (BMC) from the primary *Vect*/BMC hosts (open circles), GFP^+ BMC from the primary *C3G-F*/BMC hosts (solid circles), and GFP^+ splenocytes from the primary *C3G-F*/BMC hosts (grey circles). Data were pooled from two independent experiments. Means and SD are indicated, and statistical analysis was done with Welch's *t* test. NS, not significant. C, Sorted GFP^+ LSK cells from the BM of primary *Vect*/BMC hosts (open column), BM of the primary *C3G-F*/BMC hosts (solid black column), and spleens of the primary *C3G-F*/BMC hosts (grey column) were cultured in methylcellulose medium containing a mixture of hematopoietic cytokines (stem cell factors, interleukin (IL)-3, IL-6, erythropoietin) at 500 cells/dish for 7 days, and the colony numbers were counted. Means and SE of triplicate cultures are shown, and statistical analysis was done with two-tailed Student's *t* test (left). All colonies of each group were pooled and analyzed for expression of GFP and lineage markers with FACS, and the proportions of $Gr1^+$ $Mac1^+$, $Gr1^-$ $Mac1^+$, and Lin^- cells were determined (right)

the sorted *Sipa1*/LSK showed reduced proliferative response to SCF in culture compared to *Vect*/LSK as opposed to *C3G-F*/LSK (Figure S6B). These results are consistent with the primary involvement of Rap1 activation status in HSPC in controlling their steady state sustenance in hematopoietic tissues.

3.5 | Increased basal Rap1GTP leads to prolonged SCF-mediated c-Kit receptor activation

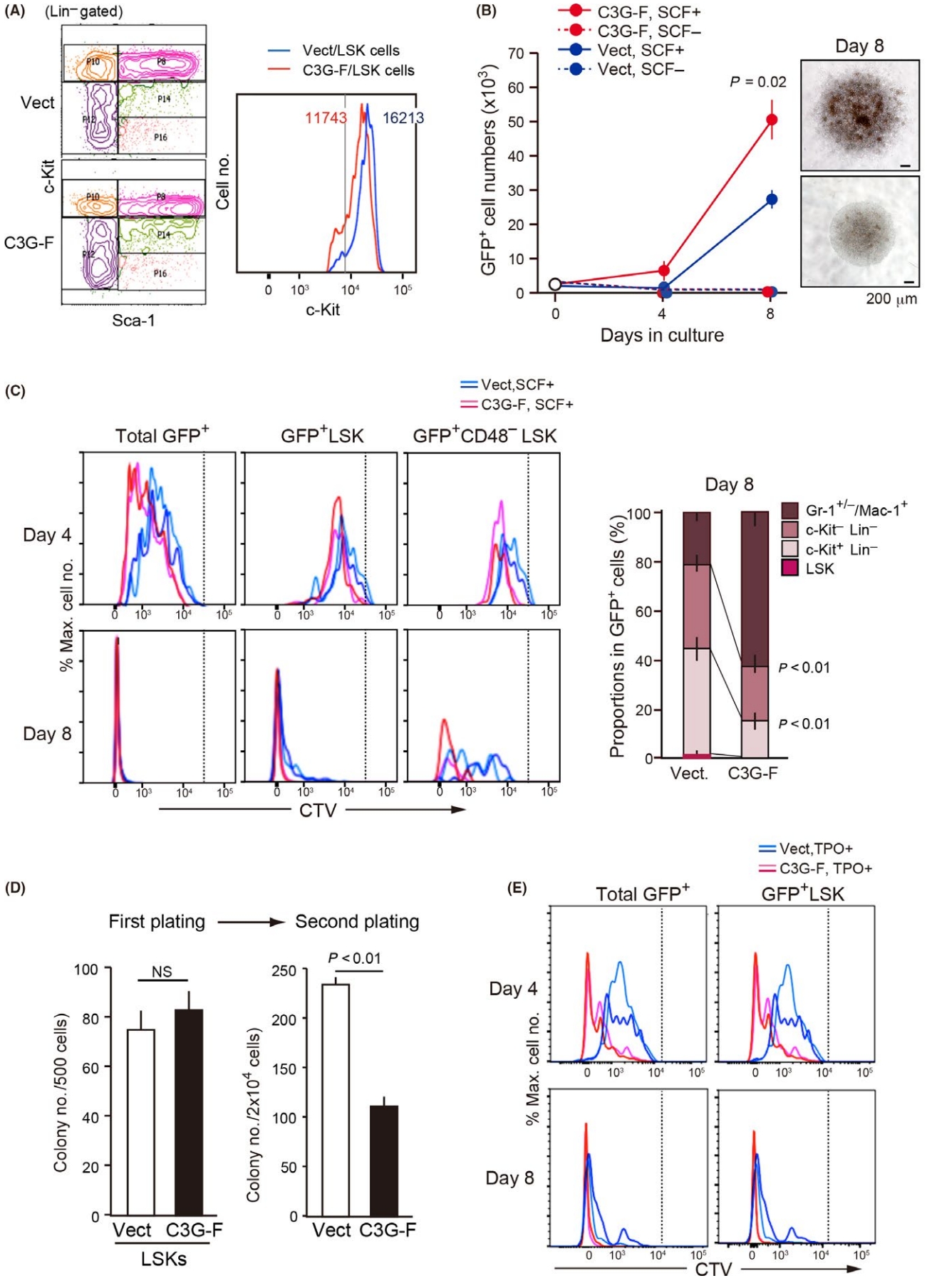
We then directly examined the effects of *C3G-F* expression on SCF-mediated c-Kit receptor signaling with the use of a Ba/F3 hematopoietic cell line transduced with *c-Kit* (c-Kit-Ba/F3). Stable transduction of *C3G-F* in the c-Kit-Ba/F3 cells caused a marked increase in basal Rap1 GTP as expected, without affecting the surface c-Kit expression levels at all (Figure 6A, Figure S7). *Vect*/c-Kit-Ba/F3 and *C3G-F*/c-Kit-Ba/F3 cells were starved for 4 hours and then cultured in the presence or absence of SCF. Both cells rapidly died out in the absence of SCF. In the presence of SCF, however, *C3G-F*/c-Kit-Ba/F3 cells showed significantly enhanced expansion compared to *Vect*/c-Kit-Ba/F3 cells (Figure 6B, left). Also, in a Boyden chamber assay, the *C3G-F*/c-Kit-Ba/F3 cells showed remarkably enhanced SCF-mediated migration compared to *Vect*/c-Kit-Ba/F3 cells (Figure 6B, right). We then examined tyrosine phosphorylation of c-Kit, which is crucial for c-Kit signaling.[25] *Vect*/c-Kit-Ba/F3 cells showed transient c-Kit phosphorylation at 2 minutes after SCF stimulation, which rapidly subsided after 5 minutes. In contrast, *C3G-F*/c-Kit-Ba/F3 cells showed quite prolonged phosphorylation of c-Kit lasting for at least 20 minutes after SCF stimulation, although peak magnitudes of the phosphorylation remained comparable (Figure 6C, Figure S7). Concordantly, activation of the major downstream signaling including ERK and AKT phosphorylation was also prolonged and enhanced in the *C3G-F*/c-Kit-Ba/F3 cells compared to *Vect*/c-Kit-Ba/F3 cells (Figure 6D, Figure S7). Of note, SCF induced no detectable activation of Rap1 in *Vect*/c-Kit-Ba/F3 cells, and also a markedly high level of Rap1GTP in *C3G-F*/c-Kit-Ba/F3 cells per se resulted in no detectable ERK/AKT activation in the absence of SCF (Figure 6D, Figure S7), making it unlikely that the enhanced ERK/AKT activation was mediated directly by the Rap1 signal bypassing c-Kit signaling. Rather, these results strongly suggested that the increased intracellular Rap1GTP levels caused the prolonged c-Kit receptor activation upon ligation with SCF.

4 | DISCUSSION

Maintenance of HSPC for life-long hematopoiesis in adults crucially depends on the specific hematopoietic microenvironment in BM niches, where a steady-state balance of HSPC quiescence, self-renewal, proliferation, and differentiation is regulated by cues from the niches.[3,4] Recent extensive investigations identified a variety of cellular components providing niche factors maintaining long-term adult hematopoiesis, among which SCF and CXCL12 play dominant roles.[4-6]

In the present study, we investigated the role of Rap1 in adult hematopoiesis. While Rap1 is activated at plasma and vesicular membranes by specific GEF recruited through signaling from diverse receptors,[13] we took advantage of C3G-F, a membrane-targeted form of C3G, that constitutively activates Rap1 bypassing the receptor coupling.[18] BMT with *C3G-F*/BMC resulted in remarkably compromised hematopoietic reconstitution of all lineages in the recipients. In BMT with normal BMC, the vast majority of donor HSPC are first retained in the spleen soon after BMT, but these cells are eventually repopulated exclusively in the BM for long-term hematopoiesis.[26] *C3G-F*/LSK showed essentially similar distribution in the spleen and BM at 1 week after BMT. However, afterwards, *C3G-F*/LSK progressively decreased with time in the BM until 5 months, whereas they were sustained in the spleen for much longer periods than *Vect*/LSK for over 2 months. Although splenic *C3G-F*/LSK apparently contributed inefficiently to overall hematopoiesis, these cells effectively initiated hematopoietic reconstitution when allowed to relocate in BM with secondary BMT, suggesting that splenic *C3G-F*/LSK retained progenitor potential. Conversely, BMT with *Sipa1*/BMC resulted in rather increased repopulation of *Sipa1*/LSK in BM compared to Vect/LSK,

FIGURE 4 Farnesylated form of C3G/Lin$^-$ Sca-1$^+$ c-Kit$^+$ cells (*C3G-F*/LSK) show enhanced proliferation and differentiation in response to stem cell factors (SCF) with accelerated progenitor cell exhaustion. A, Lin$^-$ bone marrow (BM) cells from 5-fluorouracil-treated B6 mice were infected with empty or *C3G-F*-retrovirus in the presence of mixed hematopoietic factors and multi-color analyzed for expression of indicated markers. Histograms for c-Kit expression at the GFP$^+$ *Vect*/LSK and *C3G-F*/LSK gates are also indicated with mean fluorescence intensities. B, C, At 2 days post-infection, GFP$^+$ LSK were sorted from *Vect* and *C3G-F* transfected bone marrow cells (BMC), labeled with Cell Trace Violet (CTV), starved for 1 hour, and cultured in the absence (dotted lines) or presence (solid lines) of SCF (100 ng/mL). Cells were harvested on day 4 and day 8, and viable cell numbers were counted with FACS (B). Means and SE of four samples from two independent experiments are indicated, and statistical analysis was done with two-tailed Student's *t* test. Representative pictures of the culture wells at day 8 are also shown. At days 4 and 8, CTV expression was analyzed with FACS in total GFP$^+$, GFP$^+$ LSK, and GFP$^+$ CD48$^-$ LSK cell gates of *Vect*/BMC and *C3G-F*/BMC (C, left). Data from two independent cultures were overlaid. At day 8, the cultured cells were harvested and analyzed for the expression of lineage-markers at GFP$^+$ cell gates (C, right). Mean proportions and SE of the indicated cell types in four samples from two independent experiments are shown, and statistical analysis was done with two-tailed Student's *t* test. D, The sorted GFP$^+$ LSK from *Vect*/BMC (open columns) and *C3G-F*/BMC (solid columns) were cultured in methylcellulose cultures containing mixtures of hematopoietic factors (stem cell factors, interleukin (IL)-3, IL-6, erythropoietin) at 500 cells/dish for 10 days, and the colony numbers were counted (left). The colonies were harvested and replated in the secondary methylcellulose cultures at 2×10^4 cells/dish for 12 days (right). Means and SE of triplicate cultures are indicated, and statistical analysis was done with two-tailed Student's *t* test. E, Sorted *Vect*/LSK and *C3G-F*/LSK were labeled with CTV and cultured in the absence or presence of 25 ng/mL thrombopoietin (TPO), and the expression of CTV was FACS-analyzed on days 4 and 8. The profiles of two independent cultures are overlaid. NS, not significant


(A)
(Lin⁻gated)
Vect
C3G-F
c-Kit
Sca-1
P10
P8
P14
P12
P16
Vect/LSK cells
C3G-F/LSK cells
11743
16213
Cell no.
c-Kit
(B)
C3G-F, SCF+
C3G-F, SCF–
Vect, SCF+
Vect, SCF–
P = 0.02
GFP⁺ cell numbers (x10³)
Days in culture
Day 8
200 μm
(C)
Vect,SCF+
C3G-F, SCF+
Total GFP⁺
GFP⁺LSK
GFP⁺CD48⁻ LSK
Day 4
Day 8
% Max. cell no.
CTV
Day 8
Gr-1⁺/⁻/Mac-1⁺
c-Kit⁻ Lin⁻
c-Kit⁺ Lin⁻
LSK
Proportions in GFP⁺ cells (%)
P < 0.01
P < 0.01
Vect.
C3G-F
(D)
First plating
Second plating
NS
P < 0.01
Colony no./500 cells)
Colony no./2x10⁴ cells)
Vect
C3G-F
LSKs
(E)
Vect,TPO+
C3G-F, TPO+
Total GFP⁺
GFP⁺LSK
Day 4
Day 8
% Max. cell no.
CTV

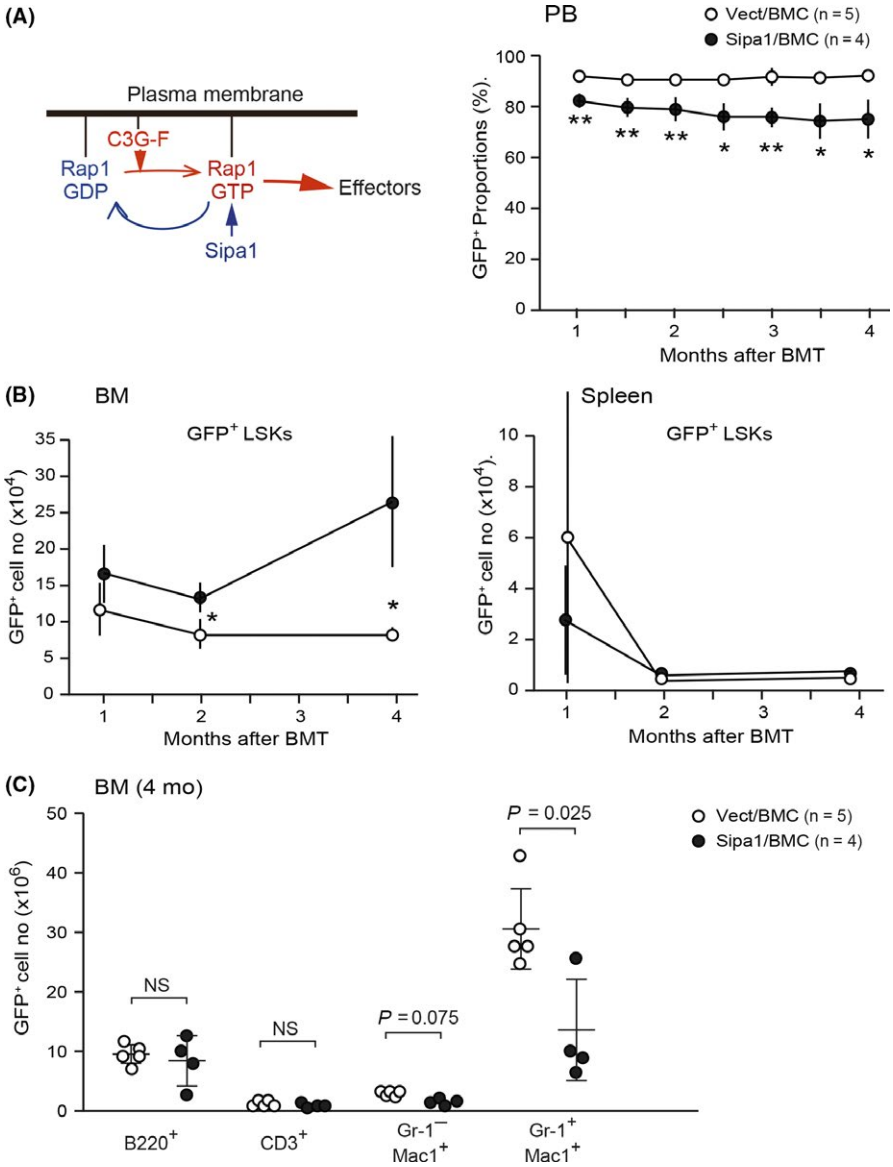


FIGURE 5 *Sipa1*/Lin$^-$ bone marrow cells (BMC) show increased hematopoietic stem/progenitor cells in BM with compromised hematopoietic reconstitution. A, Schematic representation of the function of the farnesylated form of C3G (C3G-F) and Sipa1 in Rap1 signaling (left). Lin$^-$ BM cells from 5-fluorouracil-treated B6 mice were retrovirally transduced with *Vect* (open circles) or *Sipa1* (closed circles), and the sorted GFP$^+$ cells were transplanted into 8.5 Gy-irradiated mice at 2 × 10^4 cells/mouse. Percentages of GFP$^+$ leukocytes in peripheral blood (PB) at varying periods after bone marrow transplantation (BMT) were determined with FACS (right). Means and SD of the indicated numbers of recipients are shown, and statistical analysis was done with Welch's *t* test. **P* < .05, ***P* < .01. B, BMC and spleen cells from the BMT recipients of *Vect*/BMC (open circles, five mice) and *Sipa1*/BMC (closed circles, four mice) were analyzed for the expression of GFP, Lin-markers, Sca-1, and c-Kit, and the numbers of GFP$^+$ Lin$^-$ Sca-1$^+$ c-Kit$^+$ cells (LSK) were determined at indicated periods after BMT. Means and SD of three to five mice at each time point are shown, and statistical analysis was done with Welch's *t* test. **P* < .05. C, BMC from the recipients of *Vect*/BMC (open circles, five mice) and *Sipa1*/BMC (closed circles, four mice) at 4 months were analyzed for the expression of GFP and linage markers, and the numbers of indicated GFP$^+$ cell types were determined. Means and SD are shown, and statistical analysis was done with Welch's *t* test. NS, not significant

with negligible retention in spleen. These results are consistent with the notion that endogenous Rap1 activation status in adult HSPC plays a crucial role in controlling their long-term sustenance in BM, although unexpected effects by overexpression of Rap1-modulators could not be eliminated.

It remains elusive as to why only BM exclusively provides the hematopoietic niche sites in adults, because the major niche factors such as SCF and CXCL12 are also expressed in other organs. Indeed, the spleen is a major homing site for mobilized HSPC into the circulation[27,28] and contains potential hematopoietic niches in the red pulp along with rare HSPC that are functionally comparable to those in the BM.[29,30] A variety of hematopoietic stresses mobilize HSPC from the BM to the spleen and may initiate extramedullary hematopoiesis (EMH), in which splenic endothelial/stroma cells producing SCF

and CXCL12 play essential roles.[29] EMH is commonly induced by pathological conditions that injure the normal hematopoietic niches in BM to compensate for the marrow hematopoiesis, although the efficiency may be much lower partly due to the paucity of other hematopoietic factors.[27,31] Whole-body irradiation in BMT strongly enhances niche factor production in hematopoietic tissues,[32] and intrinsic responsiveness of HSPC to the niche factors may be an important factor in regulating their stable repopulation in hematopoietic niches.

Despite the compromised hematopoietic reconstitution capacity in vivo, *C3G-F*/LSK showed significantly enhanced proliferation and differentiation in response to major niche factors SCF and TPO in vitro. Notably, at 8 days in culture with SCF or TPO, most of the progeny of *C3G-F*/LSK underwent more than eight cycles of cell division, whereas at least a proportion of *Vect*/LSK remained to have experienced only limited cell cycles of <4 times. Concordantly, *C3G-F*/LSK yielded much greater proportions of mature hematopoietic cells with fewer residual LSK than *Vect*/LSK. Consistently, the *C3G-F*/LSK-derived hematopoietic colony cells showed diminished replating efficiency compared to those from *Vect*/LSK. The differentiation profile of *C3G-F*/LSK into myeloid and lymphoid lineages was comparable to that of *Vect*/LSK, and no lineage bias was observed. These results suggested that *C3G-F*/HSPC showed an increased responsiveness to SCF and TPO with accelerated progenitor pool exhaustion. Of note, primitive HSC in fetal liver and neonatal BM, which show much more rapid cell-cycling rates with splenic preference than those in adult BM, are reported to show increased intrinsic responsiveness to SCF despite unchanged expression levels of c-Kit receptor.[33,34] Further, deficiency of *RapGEF2*, a Rap1-GEF, was reported to show selective defect of embryonic, but not adult, hematopoiesis.[35] It may be suggested that modulation of Rap1 activation states is also involved in the developmental changes of HSPC.

Stem cell factor-mediated activation of the c-Kit receptor occurs rapidly and transiently within minutes, which induces receptor dimerization and transphosphorylation of the cytoplasmic domains leading to the activation of downstream kinase pathways such as MAPK, PI3K, and Src family kinases.[25] The current study showed that *C3G-F*/c-Kit/Ba/F3 hematopoietic cells, which constitutively showed an increased basal Rap1GTP level, showed remarkably prolonged phosphorylation of c-Kit lasting up to as long as 20 minutes in response to SCF and accordingly prolonged and enhanced ERK and AKT activation. Importantly, SCF stimulation per se hardly induced Rap1 activation in control c-Kit/Ba/F3 cells, nor did *C3G-F*/c-Kit/Ba/F3 cells show activation of downstream signaling in the absence of SCF. Hence, it was suggested that enhanced and longer ERK/AKT activation was indeed due to prolonged SCF-mediated c-Kit activation under a high Rap1GTP level rather than direct effects through Rap1 signaling bypassing the c-Kit receptor. Rapid decrease of PTK receptor signaling is mediated by receptor internalization followed by degradation, in which Vav2, a Rho GEF, may play a role.[36] However, receptor internalization may play additional roles in PTK receptor signaling, such as endosomal signal transduction and receptor recycling for prolonged signaling.[37,38] It is reported that C3G-F affects endosomal function through activation of Rap1 associated with vesicular membranes.[20,39] It is an intriguing possibility that Rap1 signal may regulate intracellular trafficking of SCF-ligated c-Kit receptor, leading to prolonged c-Kit signaling by endosomal signaling or receptor recycling. A minor proportion of *C3G-F*/LSK also showed accelerated cell cycle rates in response to TPO, which induces the proliferation of megakaryocytic progenitors as well as the survival of HSC through Mpl.[40] It seems possible that the increased basal Rap1 activation status also causes increased activation of TPO-engaged Mpl, a PTK receptor, although the possibility remains to be verified.

Although *C3G-F*/LSK showed remarkably enhanced proliferation and differentiation in response to SCF and TPO in a short-term assay in vitro, the overall effects in longer-term steady state in vivo remain to be verified, particularly because the LSK population consists of cells at varying developmental stages. We previously reported that adult *Sipa1*$^{-/-}$ mice contained reduced numbers of LSK in BM at steady state, similar to the current *C3G-F*/BMC BMT recipients.[17] Notably, the *Sipa1*$^{-/-}$ LSK in BM showed rather reduced cell-cycling rates with markedly increased c-Myc activation and concordant p53 activation,[17] a major factor controlling HSPC quiescence.[11] The effects are consistent with the persistent activation of the ERK pathway.[41,42] Given that *C3G-F*/LSK show an even higher Rap1 activation state than *Sipa1*$^{-/-}$ LSK, it seems conceivable that the exaggerated c-Kit signaling under the high Rap1GTP state causes dual overall effects on HSPC; it induces accelerated proliferation and differentiation of forward-committed progenitors, whereas it may eventually cause the forced quiescence of more primitive progenitors by the c-Myc/p53 pathway. *Sipa1*$^{-/-}$ mice developed pancytopenia and often overt MPN only after long latency at over 1 year of age.[21] In the current study, the BMT recipients of *C3G-F*/HSPC showed a persistent hypo-hematopoietic state for 5 months and longer, being reminiscent of human MDS. Although *C3G-F*/LSK showed no consistent bias for the differentiation, the high variations in lineage-differentiation among individual *C3G-F*/BMC recipients may partly reflect the instability of *C3G-F*/HSPC under persistent c-Myc/p53 activation. Careful longer-term observation of *C3G-F*/BMC recipients is needed to examine their predisposition to overt leukemia.

In conclusion, our present results strongly suggest that basal Rap1 activation status in HSPC is an important determinant in maintaining HSPC at BM niche for long-term hematopoiesis in adults, through tuning their intrinsic c-Kit responsiveness to the crucial niche factor SCF. Thus, *C3G-F*/HSPC with a high basal Rap1GTP level show reduced repopulation in BM and impaired long-term hematopoietic reconstitution after BMT. The effects are attributable at least partly to the prolonged SCF-mediated c-Kit activation of *C3G-F*/HSPC, which causes an enhanced drive of HSPC for proliferation and differentiation with apparently accelerated exhaustion. In addition to pathological conditions affecting the hematopoietic environment, accumulating evidence indicates that intrinsic alterations in HSPC per se also play an important role in changes in adult hematopoiesis, for instance with age.[43] Current

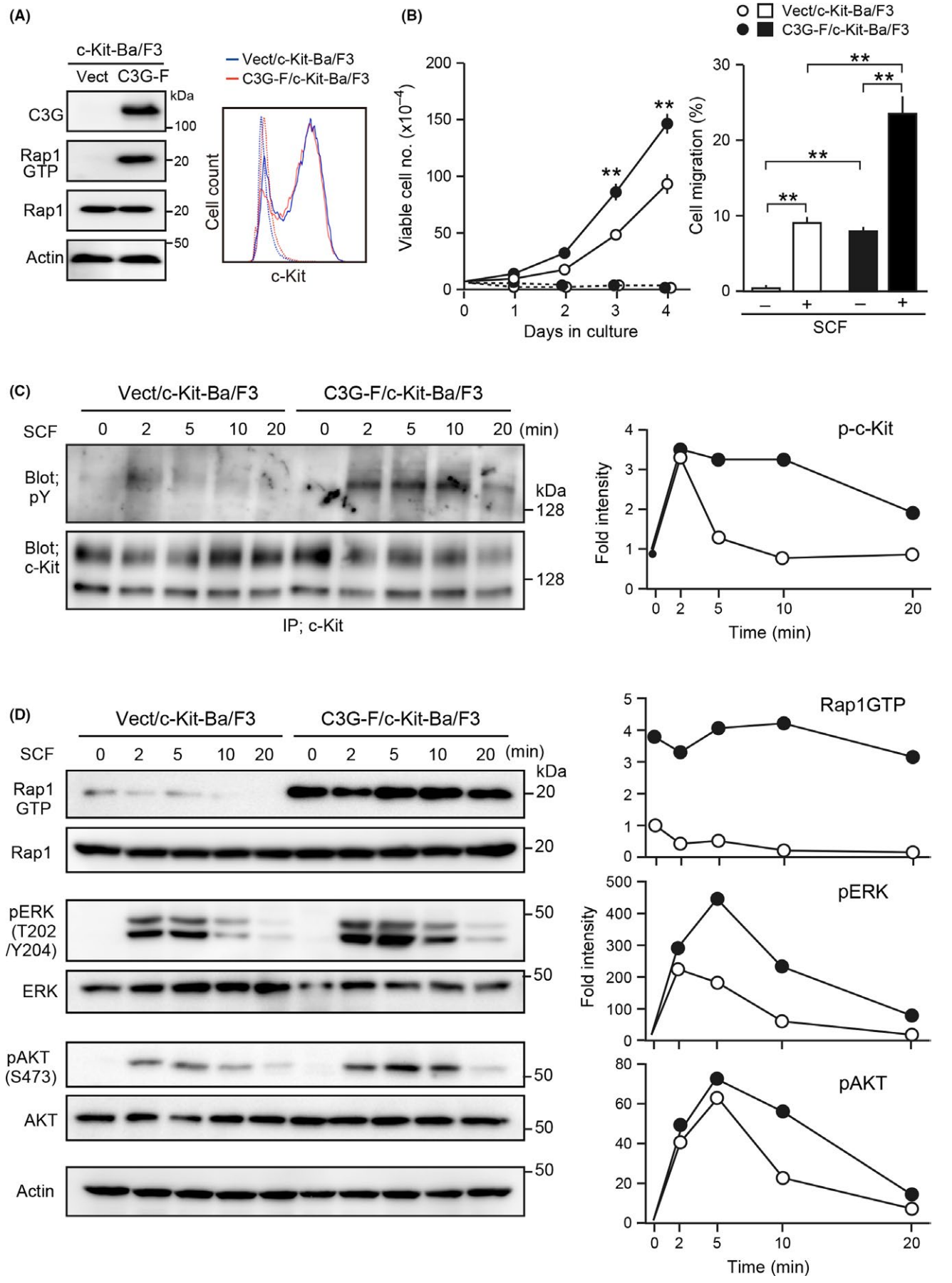

(A)
c-Kit-Ba/F3
Vect C3G-F
kDa
C3G
100
Rap1 GTP
20
Rap1
20
Actin
50
Vect/c-Kit-Ba/F3
C3G-F/c-Kit-Ba/F3
Cell count
c-Kit
(B)
Vect/c-Kit-Ba/F3
C3G-F/c-Kit-Ba/F3
Viable cell no. (x10⁻⁴)
Days in culture
Cell migration (%)
SCF
(C)
Vect/c-Kit-Ba/F3
C3G-F/c-Kit-Ba/F3
SCF 0 2 5 10 20 0 2 5 10 20 (min)
Blot; pY
Blot; c-Kit
kDa
128
IP; c-Kit
p-c-Kit
Fold intensity
Time (min)
(D)
Vect/c-Kit-Ba/F3
C3G-F/c-Kit-Ba/F3
SCF 0 2 5 10 20 0 2 5 10 20 (min)
Rap1 GTP
Rap1
pERK (T202 /Y204)
ERK
pAKT (S473)
AKT
Actin
20
50
Rap1GTP
pERK
pAKT
Fold intensity
Time (min)

FIGURE 6 Increased basal Rap1GTP level leads to prolonged stem cell factor (SCF)-mediated c-Kit receptor activation and downstream signaling. A, Ba/F3 cell line stably expressing *c-Kit* (c-Kit-Ba/F3) was retrovirally transduced with *Vect* or the farnesylated form of C3G (*C3G-F*). Cells were examined for the expression of indicated proteins with immunoblotting and pull-down assay (left). Cell surface expression of c-Kit was analyzed with FACS in *Vect*/c-Kit-Ba/F3 (blue line) and *C3G-F*/c-Kit-Ba/F3 (red line) cells (right). B, *Vect*/c-Kit-Ba/F3 (open symbols) and *C3G-F*/c-Kit-Ba/F3 (closed symbols) cells that had been maintained in WEHI3 conditioned medium were starved for 4 hours, cultured in the absence (dotted line) or presence (solid line) of 100 ng/mL SCF, and viable cell numbers were counted at indicated days (left). Aliquots of the cells were cultured in the upper wells of Boyden chambers in the presence or absence of SCF (100 ng/mL) in the lower wells, and the migrated cell numbers were counted with FACS (right). Means and SE of triplicate cultures are indicated, and statistical analysis was done with two-tailed Student's *t* test. $^{**}P < .01$. C, *Vect*/c-Kit-Ba/F3 (open circles) and *C3G-F*/c-Kit-Ba/F3 (solid circles) cells were stimulated with SCF (100 ng/mL) for varying times, and the cell lysates were immunoprecipitated (IP) with anti-c-Kit antibody followed by immunoblotting with anti-p-Y and anti-c-Kit antibodies (left). Relative signal intensities of p-c-Kit were analyzed with densitometry (right). D, *Vect*/c-Kit-Ba/F3 and *C3G-F*/c-Kit-Ba/F3 cells stimulated with SCF (100 ng/mL) for varying times were lysed and immunoblotted with the indicated antibodies (left). Relative signal intensities were analyzed with densitometry (right). Uncropped digital images are provided in Figure S7

results may provide a clue for understanding altered hematopoiesis in hematopoietic disorders and aging.

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CONFLICTS OF INTEREST

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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