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The role of Fas-associated phosphatase 1 in leukemia stem cell persistence during tyrosine kinase inhibitor treatment of chronic myeloid leukemia

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Abstract

Chronic myeloid leukemia (CML) is characterized by expression of Bcr-abl, a tyrosine kinase oncogene. Clinical outcomes in CML were revolutionized by development of Bcr-abl-targeted tyrosine kinase inhibitors (TKIs), but CML is not cured by these agents. CML leukemia stem cells (LSCs) are relatively TKI insensitive and persist even in remission. LSC persistence results in relapse upon TKI discontinuation, or drug resistance or blast crisis (BC) during prolonged treatment. We hypothesize that increased expression of Fas-associated phosphatase 1 (Fap1) in CML contributes to LSC persistence and BC. As Fap1 substrates include Fas and glycogen synthase kinase-3β (Gsk3β), increased Fap1 activity in CML is anticipated to induce Fas resistance and stabilization of β-catenin protein. Resistance to Fas-induced apoptosis may contribute to CML LSC persistence, and β-catenin activity increases during BC. In the current study, we directly tested the role of Fap1 in CML LSC persistence using in an *in vivo* murine model. In TKI-treated mice, we found that inhibiting Fap1, using a tripeptide or small molecule, prevented TKI resistance, BC and relapse after TKI discontinuation; all events observed with TKI alone. In addition, Fap1 inhibition increased Fas sensitivity and decreased β-catenin activity in $CD34^+$ bone marrow cells from human subjects with CML. Therapeutic Fap1 inhibition may permit TKI discontinuation and delay in progression in CML.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

INTRODUCTION

In chronic myeloid leukemia (CML), t(9;22) results in expression of the Bcr-abl oncogene.[1] Bcr-abl-selective tyrosine kinase inhibitors (TKIs) revolutionized CML therapeutics, but do not cure most patients.[2–5] Even in optimal responders, CML leukemia stem cells (LSCs) persist in the bone marrow, as demonstrated by studies attempting TKI discontinuation.[6–8] Time to remission upon retreatment of these subjects was longer than initial remission induction, suggesting LSC expansion during treatment.[6] Not all CML patients are optimal responders and 50% develop imatinib (IM) resistance or intolerance by 5 years.[9] These patients may respond to later-generation agents, but persisting CML LSCs provide a reservoir for disease progression.

Nonproliferating CML LSCs are relatively TKI insensitive.[10–12] One hypothesis for LSC persistence during TKI treatment is resistance to Fas-induced apoptosis. Fas resistance in CML is not due to decreased Fas or Fas ligand, but our studies suggested a role for Fas inhibition by Fas-associated phosphatase 1 (Fap1).[13–16] During progression to blast crisis (BC), β-catenin activity increases, expanding the LSC pool.[14,17] Increased β-catenin activity in CML is not due to altered Wnt or Wnt receptors, but our studies implicated glycogen synthase kinase-3β (Gsk3β) inhibition by Fap1.[13,18] Therefore, we hypothesize increased Fap1 contributes to TKI resistance and BC in CML.

PTPN13 (encoding Fap1) is repressed by the interferon consensus sequence binding protein (Icsbp).[14–16] Icsbp expression is decreased relative to normal in chronic phase (CP) CML bone marrow, rises with remission and is lowest in BC.[13,19] Icsbp is a leukemia suppressor in murine models and Fap1 inversely correlates with Icsbp in human CML.[13–16,20–22] Fas and Gsk3β are Fap1 substrates and we found Fap1-dependent Fas resistance, and Fap1/Apc/Gsk3β-dependent β-catenin stabilization in Bcr-abltransduced murine myeloid progenitors.[14–16]

Fap1 interacts with the C-termini of Fas or Apc via a PDZ domain (PDZ2).[23,24] A tripeptide representing the Fas-C terminus (SLV) blocks this domain, increasing apoptosis sensitivity and decreasing β-catenin *in vitro*.[14,15,24–26] In the current studies, we investigated effects of blocking Fap1-PDZ2 on CML progression and LSC persistence *in vivo* in mice. The goal was determining contributions of Fap1 to TKI resistance, BC and relapse after TKI discontinuation.

MATERIALS AND METHODS

Plasmids

p210-Bcr-abl in MiGR1 was obtained from Dr Ravi Bhatia (University of Alabama, Birmingham, Birmingham, AL, USA) and Fap1-PDZ2 cDNA from Addgene (Cambridge, MA, USA).

Flow cytometry

Bone marrow or peripheral blood was analyzed for green fluorescent protein (GFP) expression on a Becton-Dickinson FACScan (Cambridge, MA, USA). For apoptosis, cells

were incubated for 12 h with SLV or VLS peptide (20 mM) or Quinobene (20 μM), 24 h with anti-Fas antibody (5 μg/ml CH11; BD Bioscience Inc., San Jose, CA, USA), labeled with phycoerythrin-conjugated CD34 antibody and analyzed by Annexin V-Apoptosis Detection Kit I (eBioscience, San Diego, CA, USA).

Quantitative PCR

RNA was isolated with Triazol reagent (Invitrogen, Carlsbad, CA, USA) and tested for integrity by electrophoresis. Primers were designed with Applied Biosystems software (Grand Island, NY, USA) and PCR performed by SYBR green method. Result were normalized to 18S.

Western blots

Cells were lysed in SDS sample buffer, separated by SDS–polyacrylamide gel electrophoresis, transferred to nitrocellulose and serially probed with antibodies. Other lysate proteins were immunoprecipitated under nondenaturing conditions with Fap1-antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and SPA Sepharose before analyzing as above. Experiments were repeated 3 × with different lysates.

Confocal microscopy

Cells were paraformaldehyde fixed, methanol permeabilized, incubated with β-catenin antibody+Cy3-secondary antibody and slides mounted with anti-fade reagent+4',6-diamidino-2-phenylindole (DAPI). Signals were acquired by Zeiss laser scanning microscope (Chesterfield, VA, USA).

Murine bone marrow transduction

Mononuclear cells from femurs of C57/BL6 mice were cultured in Dulbecco's modified Eagle media, 10% fetal calf serum, 1% penicillin–streptomycin with 10 ng/ml granulocyte-macrophage colony-stimulating factor, 10 ng/ml interleukin-3, 100 ng/ml stem cell factor followed by CD34 separation (‘myeloid progenitor conditions’), or interleukin-3, 10 ng/ml interleukin-6, stem cell factor followed by Sca1 separation (‘hematopoietic stem cell conditions’) (R&D Systems Inc., Minneapolis, MN, USA). $CD34^+$ or $Sca1^+$ cells were isolated by magnetic bead affinity technique (Miltenyi Biotechnology, Auburn, CA, USA). $CD34^+$ cells represent the LSC population in murine CP CML.[27]

Retrovirus was prepared by transfecting 293T cells with Bcr-abl-MiGRI and Ecopack plasmids.[28] Supernatants collected 48 h post transfection were titered in NIH3T3 cells. Murine bone marrow cells were transduced by incubation with retroviral supernatant (~10^7 PFU/ml) supplemented with polybrene (6 μg/ml).[29,30] Transgene expression was confirmed by PCR and $\%GFP^+$.

Murine bone marrow transplantation

Primary donors were treated with 150 mg/kg of 5-flurouracil by intraperitoneal injection and bone marrow harvested after 4 days.[28] Cells were transduced with Bcr-abl-MiGR1 under hematopoietic stem cell conditions. 5×10^5 viable cells were transplanted into lethally irradiated, syngeneic mice. Recipients were killed when peripheral white blood cells

(WBCs) were > 30 000 with > 50% polymorphonuclear neutrophils (PMNs, granulocytes) but < 5% blasts. Bone marrow from primary recipients was transplanted into sublethally irradiated secondary recipients (2×10^6 cells). After 4 weeks, recipients were treated with IM (100 mg/kg/day), SLV or VLS peptide (5 mg/day), Quinobene (50 μg/day), or IM +peptide or Quinobene by intraperitoneal injection (10/group). Each cohort included recipients from four different donors. Mice were killed for WBCs > 60 000/mm^3, hemoglobin < 6.0 g/dl, platelets < 1 00 000/mm^3 or organomegaly. Bone marrow harvested from secondary recipients at 32 weeks was transplanted (2×10^6 cells) into sublethally irradiated tertiary recipients.

Analysis of murine peripheral blood and bone marrow

Blood was obtained from tail veins for automated blood counts. Blast counts were determined on May-Grunwald-Giemsa-stained peripheral smears (300 cells/slide). Sternal bone marrow was stained with hematoxylin– eosin by the Cancer Center Pathology Core (Chicago, IL, USA).

IM resistance assay

Transduced murine bone marrow was cultured 4 weeks under myeloid progenitor conditions. VLS or SLV peptide (10 mM) or Quinobene (20 μM) was added ± IM at 0.2 μg/ml, increasing to 2.0 μg/ml over 6 weeks.[16,29]

Fluorescent polarization

Recombinant Fap1-PDZ2-GST was generated as previously described.[31] The 3–15 amino acid Fas C-terminal peptides were synthesized by GenScript (Piscataway, NJ, USA). The 10 amino acid peptide (NFRNEIQSLV) bound Fap1-PDZ2-GST with greatest affinity. Screening was performed with 5680 compounds from MicroSource Spectrum (Gaylordsville, CT, USA), NIH Clinical Collection (Bethesda, MD, USA) and NCI Discovery (Bethesda, MD, USA) sets. Fap1-PDZ2-GST+FITC-NFRNEIQSLV or probe alone were controls. Z factor = 0.78.

Fluorescence thermal shift

Fap1-PDZ2 was incubated with a dose titration of candidate molecule and fluorescence measured across a temperature gradient. T_m shift was determined by Boltzmann sigmoidal function.

AlphaScreen

Biotin-labeled NFRNEIQSLV peptide was bound to streptavidin donor beads and Fap1-PDZ2-GST to GST antibody acceptor beads (Perkin Elmer, Waltham, MA, USA). Assays were started with candidate molecule and signals detected on a multi-function plate reader.

Statistical analysis

Significance was determined by two-tailed Student's t-test or analysis of variance (for ⩾3 samples) using SigmaStat (Systat Software Inc., San Jose, CA, USA). Data are reported as average ± s.e.m. and $P < 0.02$ considered significant. Differences in survival/relapse were

analyzed by Mann–Whitney rank-sum test. Differences in blood counts in treatment cohorts at treatment start were analyzed by Kruskal-Wallis one-way analysis of variance on ranks.

Human and murine studies

This study was approved by the institutional review board or animal care and use committee of Northwestern University and Jesse Brown VA Medical Center.

RESULTS

Inhibiting Fap1 increased Fas sensitivity and decreased β-catenin activity in human CML

Fap1 influences apoptosis and β-catenin in Bcr-abl-expressing murine bone marrow cells.[14–16] To investigate Fap1 in the more molecularly complex setting of human CML, we determined expression in $CD34^+$ bone marrow cells from subjects presenting in CP CML versus control subjects. We also determined expression of Gas2; another Icsbp target gene implicated in CML by murine studies.[29] Fap1 and Gas2 mRNA (Figure 1a) and protein (Figure 1b) were significantly greater in CML versus control ($P < 0.001$, $n = 6$), consistent with publically available databases.[13] We next determined effects of Fap1 inhibition on apoptosis using SLV peptide (versus VLS control). $CD34^+$ CML cells were relatively resistant to Fas-induced apoptosis compared with control cells ($P < 0.001$, $n = 3$; Figure 1c). SLV peptide increased Fas sensitivity in CML cells ($P < 0.001$, $n = 3$) but not control ($P = 0.28$, $n = 3$).

To investigate the effects of Fap1 on β-catenin, we determined β-catenin target gene expression (c-myc, survivin). Target gene mRNA was increased in $CD34^+$ CML cells versus control ($P < 0.001$, $n = 6$; Figure 2a). β-Catenin protein (Figure 2b), but not mRNA ($P = 0.2$, $n = 6$; Figure 2a), was increased in CML cells.[14] Treatment of CML cells with SLV peptide abrogated the increase in β-catenin, c-myc and survivin protein (Figure 2b), c-myc and survivin mRNA and nuclear β-catenin (Supplementary Figure S1). β-Catenin influences proliferation, and granulocyte-macrophage colony-stimulating factor-induced proliferation of CML cells was greater than control, but inhibited by SLV ($P < 0.001$, $n = 3$; Figure 2c).

Secondary recipients of Bcr-abl-transduced bone marrow develop CP CML

As Fap1 affected the physiology of human CML, we next investigated its influence on drug resistance and LSC persistence in murine double-transplant model.[28] This model generates homogeneous cohorts of animals for testing. We transplanted primary recipient mice with Bcr-abl-transduced bone marrow (in a vector coexpressing GFP) or control vector. We used p210-Bcr-abl as it is the most common form in adult CML.[32] Transduction efficiency was 30.6 ± 2.5% with Bcr-abl vector. An average of $2.96 \pm 0.34 \times 10^4$ $Sca1^+GFP^+$ cells were transplanted per recipient and peripheral blood counts determined every 2–4 weeks.

Recipients of $Bcr\text{-}abl^+$ bone marrow developed CP CML an average of 16.8 ± 1.2 weeks post transplant ($n = 4$); defined as WBC ⩾30 000/mm^3, ⩾50% PMNs (68.2 ± 5.9%, $n = 4$) and < 5% blasts (1.4 ± 0.7%, $n = 4$; Figure 3a). Hemoglobin and platelets were maintained

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(10.8 ± 0.5 g/dl and 757.8 ± 103.0 platelets/mm^3, respectively). Blood counts in control bone marrow recipients were not different than in nontransplanted mice.

Four primary recipients of Bcr-abl^+ bone marrow, from independent transductions, were donors for secondary recipients. At the time of killing, total bone marrow cells in primary Bcr-abl^+ recipients were significantly greater than control ($6.5 \pm 0.5 \times 10^7$ versus $2.2 \pm 0.3 \times 10^7$ cells, $P < 0.0001$, $n = 4$). Livers were ~ 2 × and spleens ~ 7 × enlarged, and tissues infiltrated with PMNs (Figure 3b). Increased bone marrow PMNs and progenitors in Bcr-abl^+ recipients were confirmed by flow cytometry (Figure 3c).

Bone marrow from each primary recipient was transplanted into 10–12 secondary recipients (2×10^6 cells; 27.8 ± 2.2% GFP^+, $n = 4$). After 4 weeks, treatment was initiated with combinations of Fap1 inhibitor and IM. At this point, blood counts demonstrated recovery of hematopoiesis (hemoglobin 12.1 ± 0.8, WBC $4.5 \pm 0.9 \times 10^3/mm^3$, platelets $658 \pm 98 \times 10^3/mm^3$). Circulating WBCs were predominantly lymphocytes (57.8 ± 2.9%), but PMNs infiltrated bone marrow and tissues, consistent with PMN retention in murine bone marrow (Figure 3b).[33]

Fap1 inhibition prevented IM resistance or BC in a murine CP CML model

We used SLV peptide as one approach to inhibiting Fap1. As a proof of principle, we used a fluorescence polarization screen to identify a Fap1-blocking small molecule to also study (Supplementary Figure S2A).[34] We identified four compounds that disrupted Faspeptide probe/recombinant Fap1-PDZ2 interaction and confirmed binding by fluorescence thermal shift and AlphaScreen assays.[35,36] Two of these, Quinobene and NSC-34931, were structurally similar (Supplementary Figure S2B). We used Quinobene in our studies, as its pharmacokinetics in mice were known.[37,38] Quinobene bound Fap1-PDZ2 in a dose-dependent manner in all three assays (Supplementary Figures S2A, C, D).

Quinobene blocked co-immunoprecipitation of Fas or Apc with Fap1 in Bcr-abl^+ murine bone marrow cells, and decreased β-catenin and survivin in a manner augmented by IM, but did not alter Fap1-independent Bcr-abl targets (Supplementary Figures S3A and B). In an *in vitro* resistance assay,[16] either SLV peptide or Quinobene impaired Bcr-abl^+ cell outgrowth in IM ($P < 0.001$, $n = 3$; Supplementary Figure S3C).

We treated cohorts of secondary Bcr-abl^+ bone marrow recipients with SLV peptide, Quinobene or VLS control peptide ± IM (10/group). Average PMN and hemoglobin in the 6 cohorts were not different at treatment initiation ($P > 0.7$, $n = 10$). After 8 weeks of control peptide treatment, circulating PMNs were > 7000 and hemoglobin was falling (Supplementary Figures S4A and B). Of these mice, 60% developed stable CP and 40% BC (> 10% circulating myeloid blasts) with half initially in CP (Figures 4a and b). The 50% survival was 11.3 ± 0.5 weeks (Figure 4c). Splenomegaly and hepatomegaly were present at death (0.70 ± 0.06 and 2.04 ± 0.07 g, respectively) and bone marrow was infiltrated with granulocytes or myeloid blasts (Figure 5a). Results were not significantly different in a cohort of buffer-treated mice (not shown).

In contrast, a subset of IM+VLS-treated recipients maintained normal blood counts through the treatment period (Supplementary Figures S4A and B). A rise in PMNs at ~ 18 weeks represented those with progression. Survival was twice that with VLS alone ($P < 0.0001$, $n = 10$) and 30% survived 32 weeks (Figure 4c). In addition, 50% developed CP and 30% BC with half initially in CP ($P < 0.001$, $n = 10$ compared with VLS; Figures 4a and b). Animals expiring during treatment had enlarged spleens and livers (0.68 ± 0.40 and 1.8 ± 0.5 g) and bone marrow infiltrated with PMNs or blasts. Mice surviving 32 weeks were in hematologic remission (HR) per peripheral blood counts and bone marrow histology.

Most Fap1 inhibitor-treated secondary recipients maintained normal blood counts for > 30 weeks (Supplementary Figures S4A and B). The PMN rise at 20–22 weeks represented 10% (SLV) or 20% (Quinobene) developing CP CML and 5% of these progressed to BC (Figures 4a and b). Of the Fap1 inhibitor-treated mice, 80% survived the entire treatment period in HR (Figure 4c). The 50% survival was not reached in Fap1 inhibitor-treated mice, and survival, CP and BC rates were significantly different than VLS ($P < 0.001$, $n = 10$). Spleens and livers were enlarged in mice with progression (0.45 ± 0.03 and 1.59 ± 0.40 mg, respectively). The addition of Fap1 inhibitor to IM altered the disease course. No mice treated with either combination relapsed during treatment ($P < 0.01$, $n = 10$ compared with Fap1 inhibitor or VLS+IM; Figures 4a and b). Thus, 100% survived 32 weeks in HR; significantly longer than VLS+IM ($P < 0.001$, $n = 10$; Figure 4c).

Fap1 inhibition during TKI treatment decreased LSC persistence Except for the control peptide group, all cohorts of secondary Bcr-abl$^+$ bone marrow recipients had normal blood counts and bone marrow morphology after 8 weeks of treatment (Figure 5b). Bcr-abl transcripts in bone marrow were 3 logs greater in control peptide-treated mice versus SLV, Quinobene or IM+control peptide ($P < 0.0001$, $n = 3$). Transcripts decreased an additional 2 logs with IM+Fap1 inhibitor ($P < 0.0001$, $n = 3$; Figure 5c) and were stable with sustained HR.

We performed tertiary transplants to investigate relapse after treatment discontinuation. 1×10^6 bone marrow cells from secondary Bcr-abl$^+$ recipients in HR at 32 weeks were transplanted into tertiary recipients (2 donors/5 recipients/condition; average % GFP$^+$: IM = 4.3%, Fap1 inhibitor = 1.8%, IM+Fap1 inhibitor = 0.7%). Mice received no additional treatment. Recipients from IM+Fap1 inhibitor-treated donors maintained normal peripheral blood counts over 24 weeks (Supplementary Figures S4C and D). In contrast, recipients from IM+control peptide-treated donors began CP relapse at 10 weeks and BC progression at 14 weeks (70%; $P < 0.001$, $n = 10$ compared with IM+Fap1 inhibitor; Figure 6a). Recipients from Fap1 inhibitor-treated donors fared better with CP relapse in 30% from SLV-treated and 15% Quinobene-treated donors ($P < 0.001$, $n = 10$; Figure 6a).

The 50% survival was 16 weeks for recipients from IM+VLS-treated donors but was not reached in recipients from IM+Fap1 inhibitor-treated donors ($P < 0.01$, $n = 10$; Figure 6b). After 24 weeks, bone marrow Bcr-abl transcripts were significantly greater in CP or BC versus HR ($P < 0.001$, $n = 3$; Figure 6c). Bcr-abl transcripts were significantly less in recipients from IM+Fap1 inhibitor-treated donors versus other groups ($P < 0.001$, $n = 3$).

DISCUSSION

Our study addressed the role of Fap1 in CML LSC persistence during TKI treatment. This is discrete from acquired TKI resistance because of *BCRABL* point mutation or duplication.[39] We explored the hypothesis that Fas resistance contributes to intrinsic CML LSC TKI insensitivity. The corollary to this hypothesis is that blocking Fap1 delays TKI resistance and prevents relapse after treatment discontinuation. Gsk3β is a Fap1 substrate and Fap1 inhibition decreased β-catenin activity.[14] As increased β-catenin in CML LSCs is associated with BC, inhibiting Fap1 may have multiple benefits.[40]

Consistent with our hypotheses, a Fap1-blocking tripeptide increased sensitivity to Fas-induced apoptosis and decreased β-catenin activity in human $CD34^+$ CML cells. In a CML murine model, Fap1 inhibition decreased *in vivo* LSC persistence during TKI treatment and delayed relapse. No mice treated with IM+Fap1 inhibitor developed BC, although BC occurred with the treatments individually. In addition, no recipients of bone marrow from combined treatment cohorts in remission relapsed upon treatment discontinuation. Our results suggested inhibiting Bcr-abl+Fap1 improved disease control *in vivo*.

As a proof of principle, we studied Quinobene, a Fap1-blocking small molecule that replicated SLV functions. Quinobene is known to protect cells from HIV infection through undefined mechanisms. Additional studies will be required to optimize compounds for oral administration. It is possible these molecules influence other Fap1 substrates, or other PDZ proteins. This is a focus of our ongoing studies.

Secondary recipients in our studies were a relatively homogeneous group, facilitating therapeutic comparison. Long lead times to overwhelming CML in this model is advantageous as it permits evaluation of animals not succumbing to disease. As most human CML patients achieve TKI-induced remission, we focused on sustaining remission. We initiated treatment when CP CML was established, but disease was not overwhelming. In mice achieving HR, Bcr-abl transcripts in the bone marrow were stable from 8 to 32 weeks at encouraging levels. Mice treated with IM+Fap1 inhibitor had ~ 2 transcripts/100 cells, whereas mice treated with IM alone had ~ 800–1000 transcripts and Fap1 inhibitor alone ~ 300–500 transcripts (~6 00 000 in VLS-treated mice).

Tertiary transplant studies also indicated favorable results with combination treatment. No additional treatment was given, and hence relapse reflected viable leukemia-initiating cells. We found relapse in bone marrow recipients from IM+control peptide-treated donors was similar to the 60% rate in human discontinuation studies. Relapse was infrequent in recipients from Fap1 inhibitor-treated donors, and recipients from TKI+Fap1 inhibitor-treated donors did not relapse. This suggested Fap1 inhibition disfavors LSC persistence during TKI treatment. The combination also prevented BC, possibly by destabilizing β-catenin.

Our current study indicates inhibiting pathways downstream from Bcr-abl enhances the impact of TKIs on LSCs. This may be because some events, such as Fap1 expression, involve stable proteins that persist in LSCs without ongoing TK activity. Additional work will clarify this.

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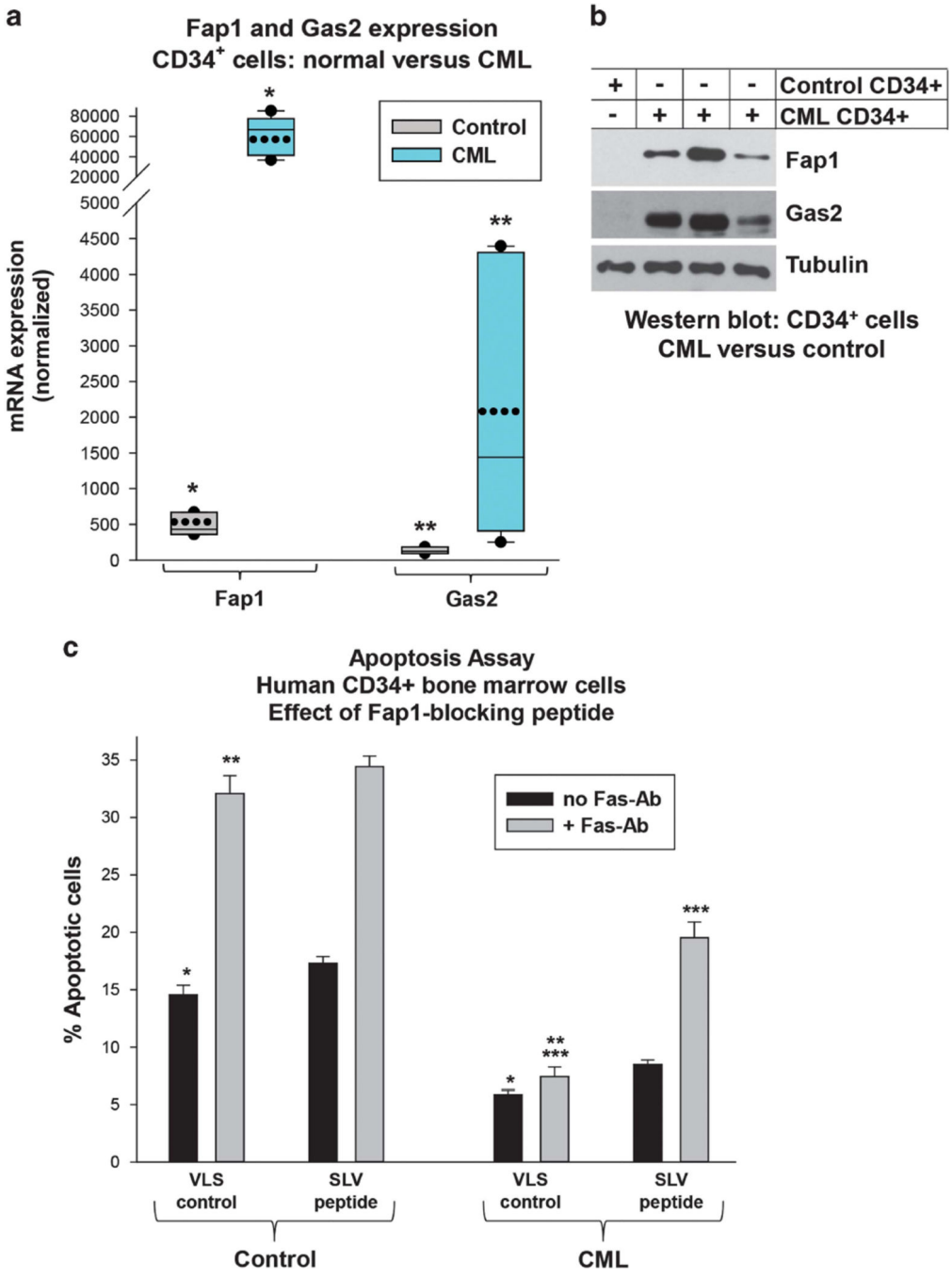

Figure 1.

Fap1 inhibition increased Fas sensitivity in human CD34$^+$ CML cells. CD34$^+$ bone marrow cells from CML or control subjects were studied ($n = 6$). (**a**) Fap1 and Gas2 mRNA were increased in CML versus control. Line = median, dotted line = mean, • = 95% confidence. (**b**) Fap1 and Gas2 proteins were increased in CML versus control. Western blots were probed as indicated. (**c**) Fap1 inhibition increased Fas-induced apoptosis in CML. Cells were treated with Fap1 blocking SLV peptide or VLS control and apoptosis determined. Significant differences are indicated by *, ** or *** ($P < 0.01$).

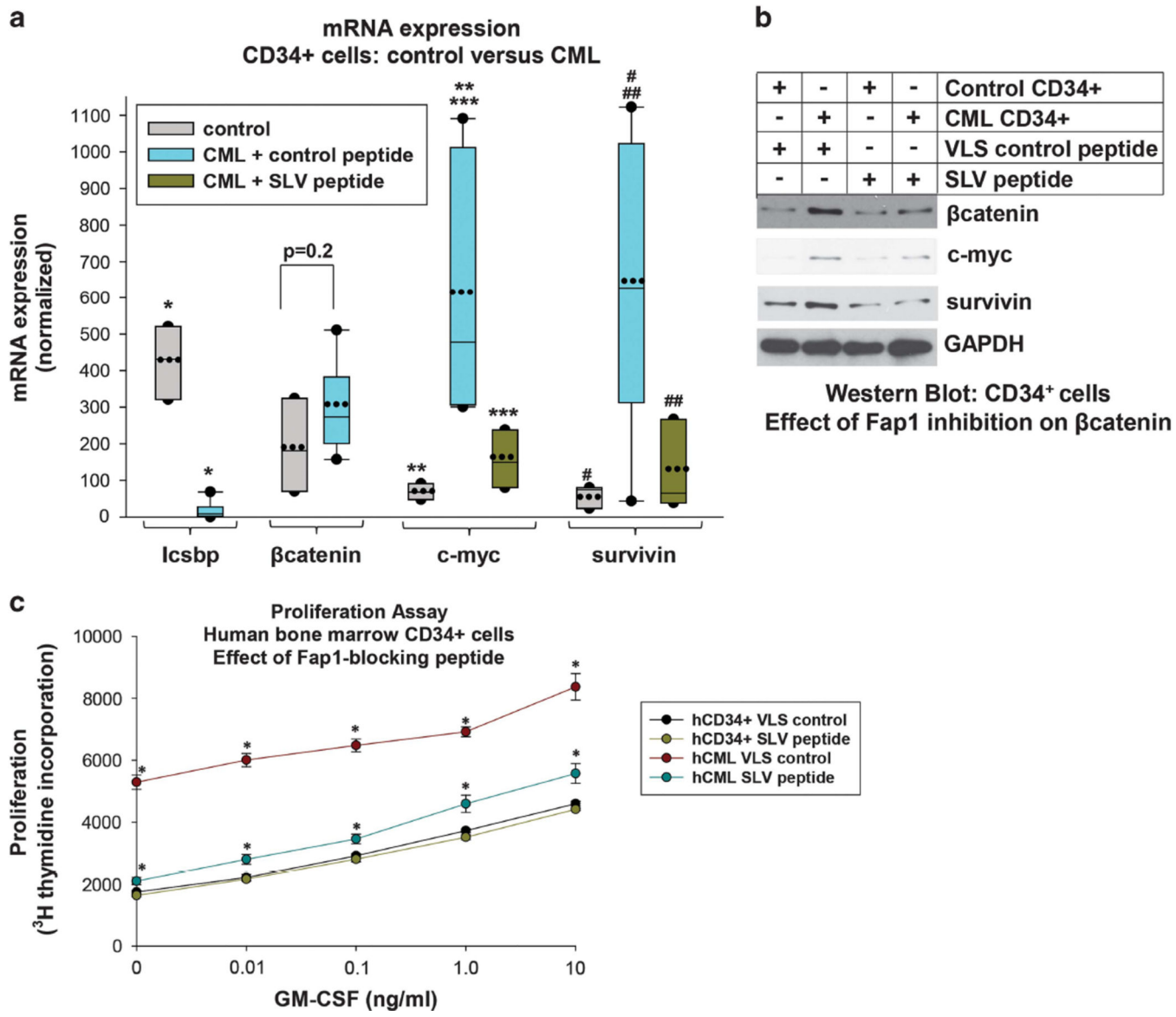

Figure 2.

Fap1 inhibition decreased β-catenin activity in human CD34$^+$ CML cells. Cells were treated with SLV peptide or VLS control ($n = 6$). (**a**) Fap1 inhibition decreased β-catenin target gene expression in CML. β-Catenin, Icsbp and β-catenin target gene (c-myc, survivin) mRNA was quantified. Line = median, dotted line = mean, • = 95% confidence. (**b**) Fap1 inhibition decreased β-catenin protein in CML. Cell lysates were analyzed by western blot. (**c**) Fap1 inhibition reversed cytokine hypersensitivity of CML cells. Cell proliferation was determined at various granulocyte-macrophage colony-stimulating factor (GM-CSF) doses. Significant differences indicated by *, **, ***, $^{\#, \#\#}$ or $^{\#\#\#}$ ($P < 0.01$).

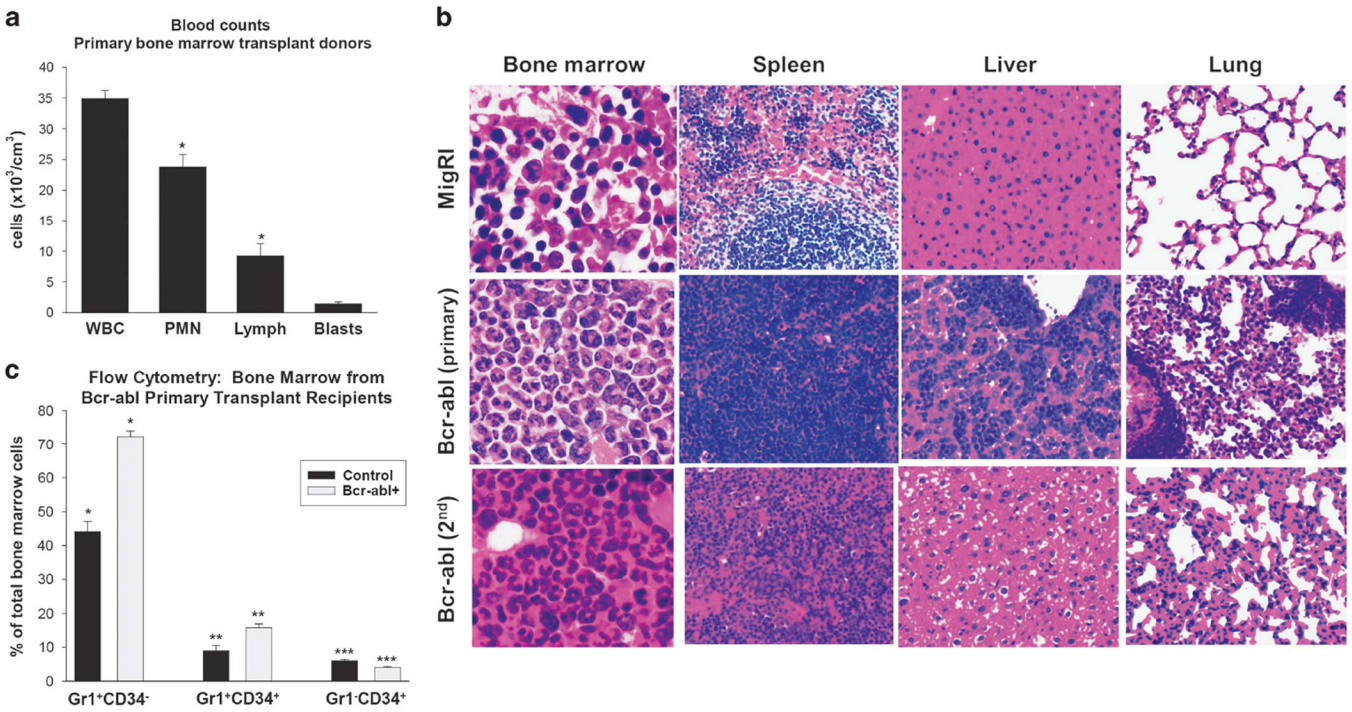

Figure 3.

Recipients of Bcr-abl-transduced bone marrow developed CP CML. (**a**) Circulating PMNs predominated in primary Bcr-abl-transduced bone marrow recipients. (**b**) Mature PMNs infiltrated bone marrow, liver, spleen and lungs of Bcr-abl$^+$ bone marrow recipients. Primary recipients of Bcr-abl or control vector-transduced bone marrow or secondary Bcr-abl$^+$ recipients 4 weeks post transplant are shown (×40 or × 20 magnification). (**c**) CD34$^+$ and Gr1$^+$ populations are expanded in primary Bcr-abl$^+$ bone marrow recipients. Significant differences indicated by *, ** or *** ($P < 0.01$; $n = 4$).

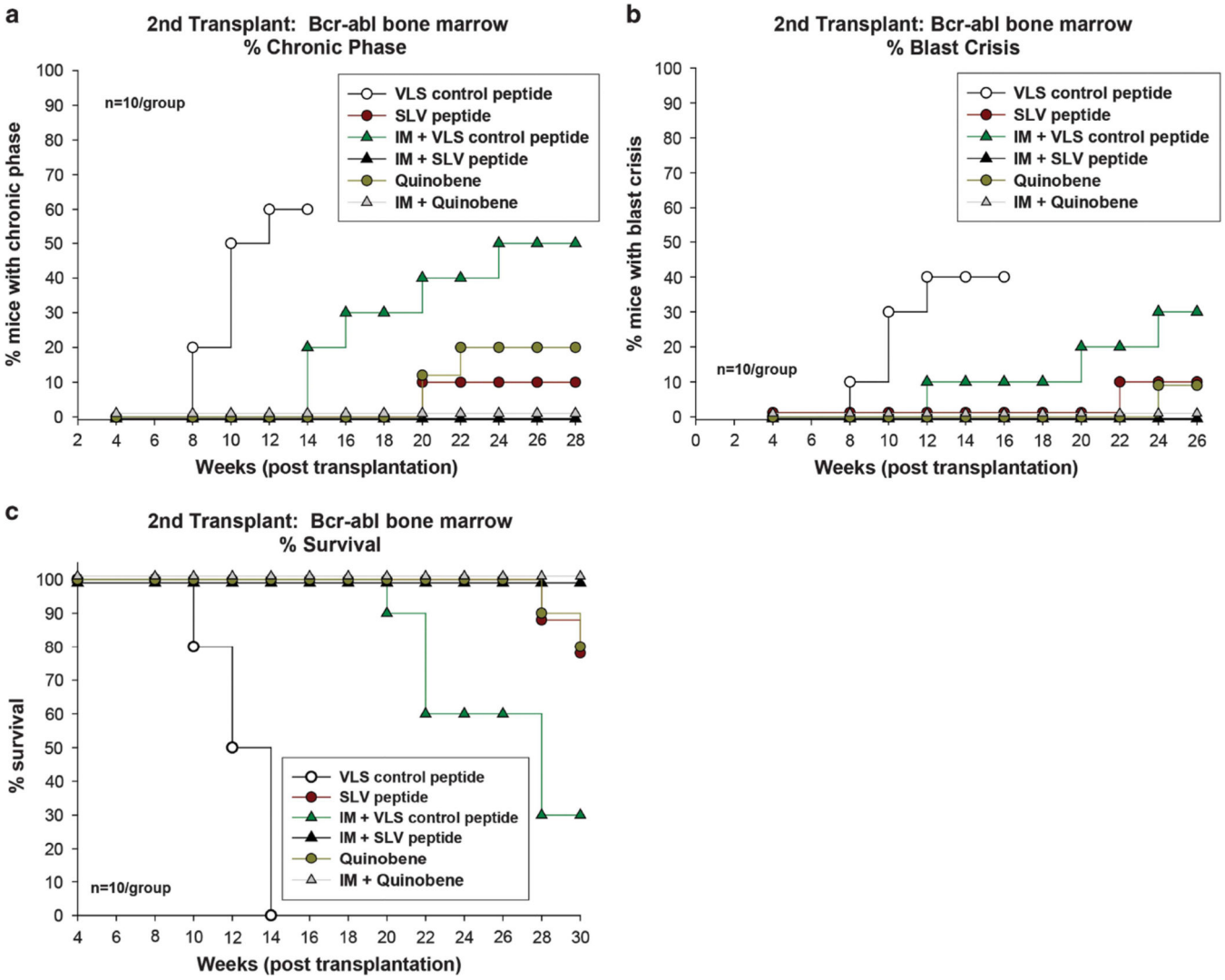

Figure 4.

Fap1 inhibitor delayed relapse and prevented BC in IM-treated CP CML mice. Secondary recipients of Bcr-abl^{+} bone marrow were treated with SLV, VLS (control) peptide or Quinobene ± IM (n = 10/group). (**a**) Fap1 inhibitor or IM delayed CP relapse compared with control. Mice treated with IM+Fap1 inhibitor did not relapse. (**b**) Fap1 inhibitor or IM delayed BC versus control. No mice treated with IM+Fap1 inhibitor developed BC. (**c**) Mice treated with IM+Fap1 inhibitor survived significantly longer than Fap1 inhibitor alone or IM +VLS.

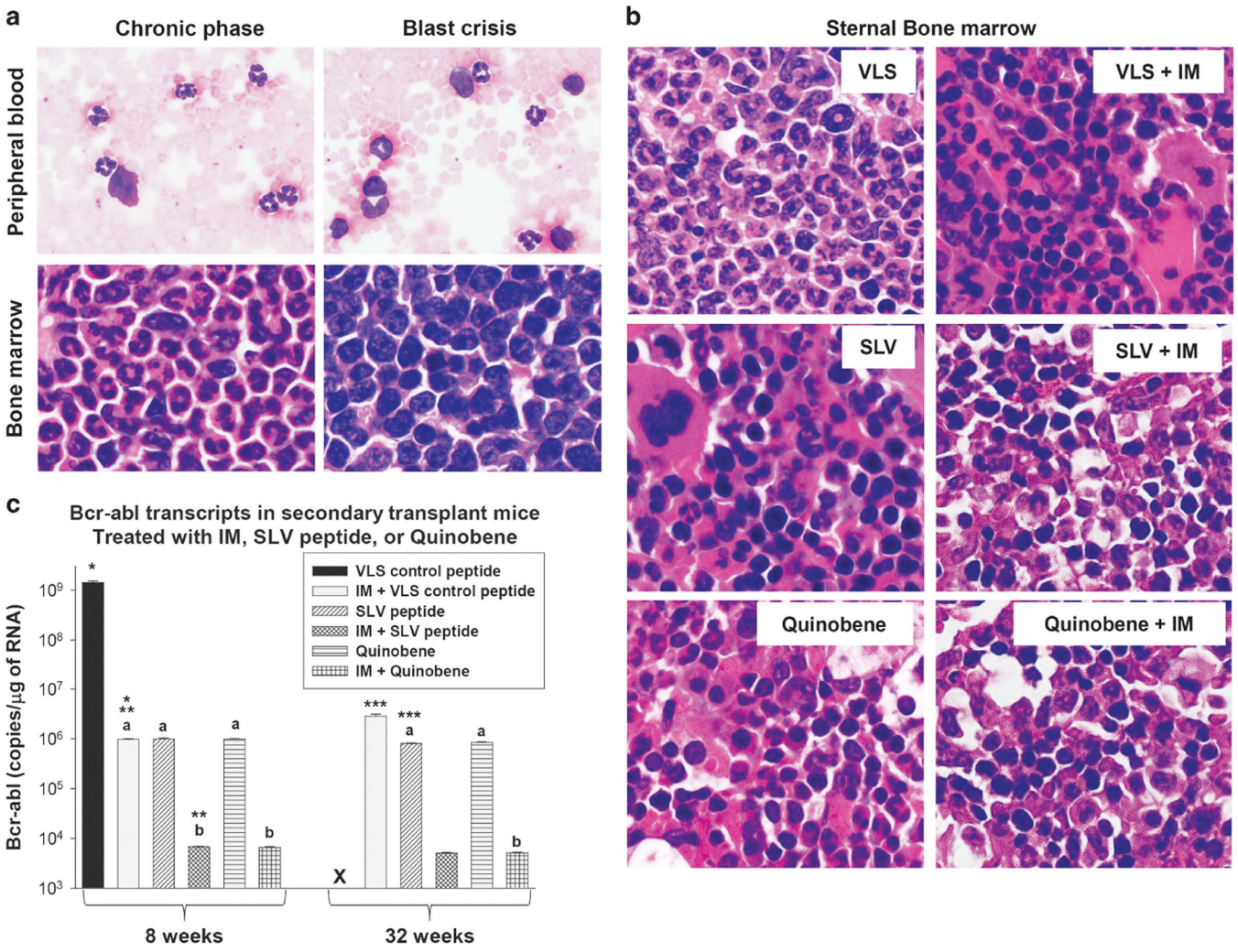

Figure 5.

Remission in secondary Bcr-abl^{+} bone marrow recipients correlated with transcript abundance. (**a**) Circulating and bone marrow myeloid blasts were correlated. Sterna were obtained from mice with peripheral blood evidence of CP or BC and Wright-Giemsa-stained sections examined at × 40. (**b**) Sternal bone marrow histology documented HR in mice after 8 weeks of Fap1 inhibitor or IM ± Fap1 inhibitor. Sections examined at × 40. (**c**) Treatment with IM ± Fap1 inhibitor decreased Bcr-abl transcripts versus any of the three alone. Significant differences indicated by *, **, *** ($P < 0.01$; $n = 8$). Not significantly different values are indicated by ‘a’ or ‘b’.

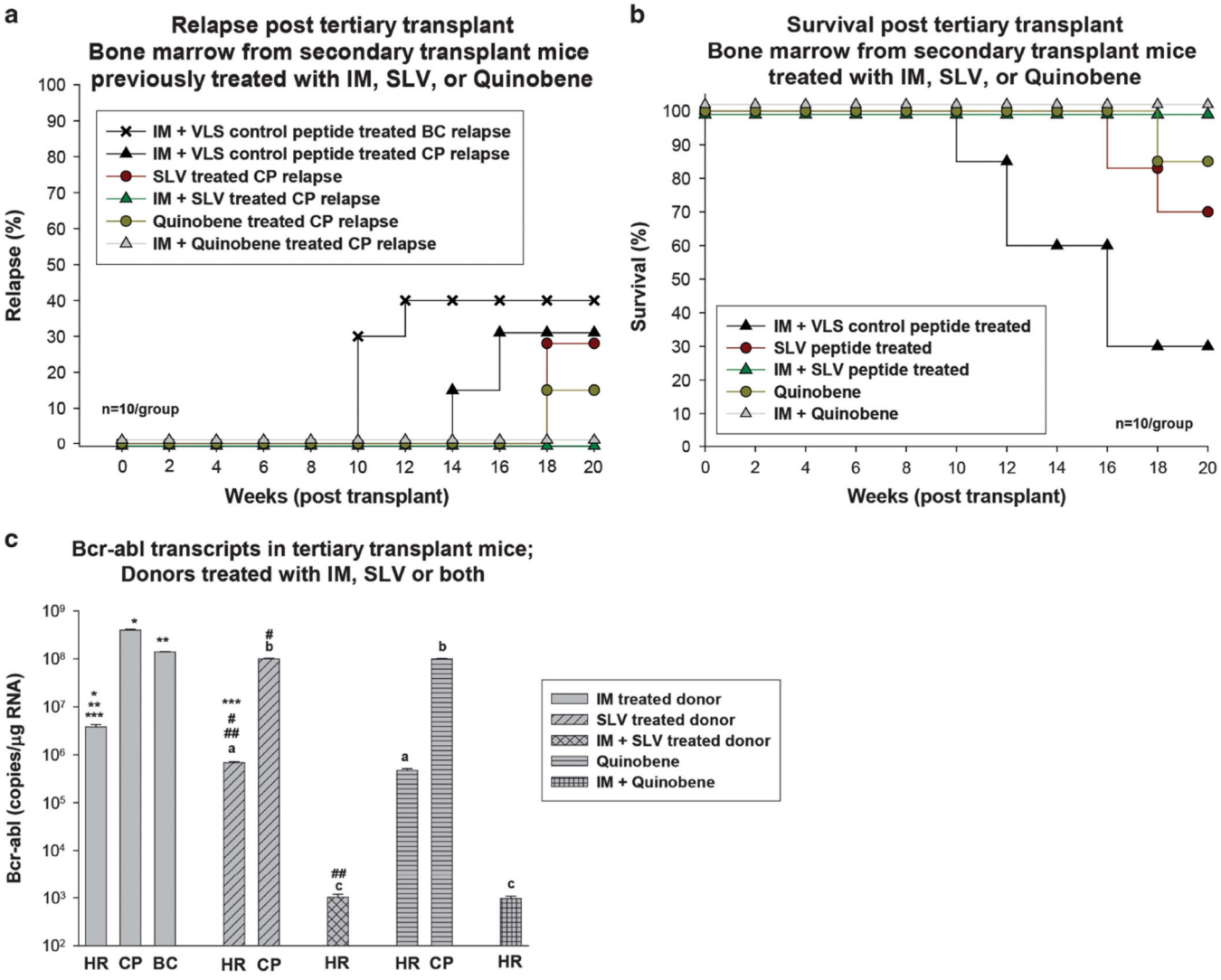

Figure 6.

Fap1 inhibition decreased LSC persistence during IM treatment of Bcr-abl^{+} bone marrow recipients. Bone marrow from secondary recipients in HR after 32 weeks of IM+control peptide or Fap1 inhibitor ± IM was transplanted into tertiary recipients (n = 10/group). No further treatment was given. (**a**) Relapse rates and type differed in tertiary recipient cohorts. Recipients from donors treated with IM+control developed CP or BC, with SLV or Quinobene CP, but with IM+Fap1 inhibitor did not relapse. (**b**) Survival in recipients from IM+Fap1 inhibitor-treated donors was significantly longer than other cohorts. (**c**) Bcr-abl mRNA in bone marrow was significantly less in recipients from IM+Fap1 inhibitor-treated donors versus other cohorts. Significant difference indicated by *, **, ***, $^{\#}$ or $^{\#\#}$ ($P < 0.01$). Not significantly different values are indicated by ‘a’, ‘b’ or ‘c’.